University of Novi Sad
INSTITUTE OF FOOD TECHNOLOGY IN NOVI SAD

FINS

tech congress

16-18 October, 2024
Novi Sad, Serbia

5th INTERNATIONAL CONGRESS FOOD TECHNOLOGY, QUALITY AND SAFETY

e-PROCEEDINGS

ISBN 978-86-7994-064-3

V INTERNATIONAL CONGRESS "FOOD TECHNOLOGY, QUALITY AND SAFETY", 16-18 OCTOBER 2024, NOVI SAD, REPUBLIC OF SERBIA

Publisher

University of Novi Sad
Institute of Food Technology
Bulevar cara Lazara 1
21000 Novi Sad
Republic of Serbia

Main editor
Dr Nemanja Teslić

Editor
Dr Jelena Miljanić
Dr Danka Dragojlović
Dr Aleksandra Bajić
Dr Dragana Ubiparip Samek

Paper Review
All papers are peer-reviewed and supervised by the International Scientific Committee

Technical editor
Milana Matić
Olja Todorić

Cover
Boris Bartula, BIS, Novi Sad, Serbia

Organization of Congress:

- INSTITUTE OF FOOD TECHNOLOGY IN NOVI SAD, University of Novi Sad, Republic of Serbia

Co-Organizers of Congress:

- SHANDONG AGRICULTURAL UNIVERSITY, People's Republic of China
- INSTITUTE OF MEAT HYGIENE AND TECHNOLOGY, University of Belgrade, Republic of Serbia

Congress is supported by:

- Ministry of Science, Technological Development and Innovation, Belgrade, Republic of Serbia
- Provincial Secretariat for Higher Education and Scientific Research, Autonomus Province of Vojvodina, Republic of Serbia
- Provincial Secretariat for Agriculture, Water Management and Forestry, Autonomus Province of Vojvodina, Republic of Serbia
- Provincial Secretariat for Regional Development, Interregional Cooperation and Local Self-Government, Autonomus Province of Vojvodina, Republic of Serbia
- Food and Feed Research, Journal of the Institute of Food Technology in Novi Sad, University of Novi Sad, Republic of Serbia

Congress President:

Ljubiša Šarić, Institute of Food Technology in Novi Sad, University of Novi Sad, Republic of Serbia

General sponsor: Analysis d.o.o. – Novi Beograd, Republic of Serbia

INTERNATIONAL SCIENTIFIC COMMITTEE

President:

Tamara Dapčević Hadnađev, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Members:

Aleksandar Marić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Aleksandra Jovanović Galović, Faculty of Pharmacy Novi Sad, University Business Academy, Serbia

Aleksandra Mišan, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Aleksandra Nikolić, Faculty of Agriculture and Food Science, University of Sarajevo, Bosnia and Herzegovina

Aleksandra Novaković, Faculty of Education in Bijeljina, University of East Sarajevo, Bosnia and Herzegovina

Alexandrina Sirbu, FMMAE Râmnicu Vâlcea, Constantin Brâncoveanu University of Pitesti, Romania

Alena Stupar, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Anamarija Mandić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Anita Klaus, Faculty of Agriculture, University of Belgrade, Serbia

Antonio Moretti, Institute of Sciences of Food Production, National Council of Research CNR-ISPA, Italy

Biljana Pajin, Faculty of Technology Novi Sad, University of Novi Sad, Serbia

Biljana Stojanovska Dimzoska, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University in Skopje, North Macedonia

Bojan Butinar, Institute for Olive culture, Science and Research Centre Koper, ZRS Koper, Slovenia

Bojana Kokić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Bojana Šarić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Branislav Šojić, Faculty of Technology Novi Sad, University of Novi Sad, Serbia

Breda Jakovac Strajn, Veterinary Faculty, University of Ljubljana, Slovenia

Brijesh Tiwari, Teagasc - Agriculture and Food Development Authority, Ireland

Cristina M. Rosell, Department of Food and Human Nutritional Sciences, University of Manitoba, Canada

Dapeng Li, College of Food Science and Engineering, Shandong Agricultural University, China

Dejan Dragan Miladinović, Center for Feed Technology, Norwegian University of Life Sciences, Norway

Diego A. Moreno-Fernández, CEBAS-CSIC, Spanish National Research Council (CSIC), Spain

Dragomir Catalin, National Institute for Research and Development in Animal Biology and Nutrition, Romania

Dragan Milić, Faculty of Agriculture, University of Novi Sad, Serbia

Dragana Plavšić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Dragana Ubiparip Samek, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Dubravka Škrobot, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Elena Velickova Nikova, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, North Macedonia

Fatih Ozogul, Faculty of Fisheries, Cukurova University, Turkey

Feng Li, College of Food Science and Engineering, Shandong Agricultural University, China

Mia Eeckhout, Department of Food Technology, Safety and Health, Ghent University, Belgium

Mian N. Riaz, Dept. of Food Science and Technology, Texas A&M University, USA

Mihaela Jurdana, Institute for Kinesiology Research, Science and Research Centre Koper, ZRS Koper, Slovenia

Milan Ilić, Faculty of Pharmacy Novi Sad, University Business Academy, Serbia

Milan Vraneš, Faculty of Sciences, University of Novi Sad, Serbia

Milena Stranska, Faculty of Food and Biochemical Technology, University of Chemistry and Technology Prague, Czech Republic

Milica Pojić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Milivoj Radojčin, Faculty of Agriculture, University of Novi Sad, Serbia

Miloš Pelić, Scientific Veterinary Institute Novi Sad, Serbia

Miona Belović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Miroljub Barać, Faculty of Agriculture, University of Belgrade, Serbia

Miroslav Hadnađev, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Mladen Brnčić, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia

Mladenka Pestorić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Nataša Ćurčić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Nataša Jovanović Lješković, Faculty of Pharmacy Novi Sad, University Business Academy, Serbia

Nebojša Ilić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Nemanja Teslić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Nikola Tomić, Faculty of Agriculture, University of Belgrade, Serbia

Nikolina Čukelj Mustač, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia

Olivera Đuragić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Omer Said Toker, Chemical and Metallurgical Engineering Faculty, Food Engineering Department, Yildiz Technical University Istanbul, Turkey

Patrik Drid, Faculty of Sport and Physical Education, University of Novi Sad, Serbia

Pavle Jovanov, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Pedro Silva, Instituto de Medicina Molecular, University of Lisbon, Portugal

Peter Raspor, Biotechnical Faculty, University of Ljubljana, Slovenia

Predrag Ikonić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Qingguo Wang, College of Food Science and Engineering, Shandong Agricultural University, China

Rada Jevtić Mučibabić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Relja Suručić, Faculty of Medicine, University of Banja Luka, Bosnia and Herzegovina

Renata Kovač, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Ružica Tomičić, Faculty of Technology, University of Novi Sad, Serbia

Sandra Zavadlav, Department of Food Technology, Karlovac University of Applied Sciences, Croatia

Sanja Belić, Faculty of Sciences, University of Novi Sad, Serbia

Saša Despotović, Faculty of Agriculture, University of Belgrade, Serbia

Slađana Rakita, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Slobodan Gadžurić, Faculty of Sciences, University of Novi Sad, Serbia

Spiros Paramithiotis, Department of Biological Applications and Technologies, University of Ioannina, Greece

Szabo Balazs, Cereal Research Nonprofit Ltd., Hungary

Szabolcs Halazsi, Faculty of Pedagogy, Gal Ferenc University, Hungary

Tatjana Peulić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Verica Dragović-Uzelac, Faculty of Food Technology and Biotechnology, Croatia

Vesna Đorđević, Institute of Meat Hygiene and Technology, University of Belgrade, Serbia

Viktor Nedović, Faculty of Agriculture, University of Belgrade, Serbia

Wei Chen, College of Food Science and Engineering, Shandong Agricultural University, China

Xiaoyong Sun, College of Information Science and Engineering, Shandong Agricultural University, China

Xuguang Qiao, College of Food Science and Engineering, Shandong Agricultural University, China

Yilun Chen, College of Food Science and Engineering, Shandong Agricultural University, China

Yimin Zhang, College of Food Science and Engineering, Shandong Agricultural University, China

Žarko Kevrešan, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Zhixiang Xu, College of Food Science and Engineering, Shandong Agricultural University, China

Zlata Kralik, Faculty of Agrobiotechnical Sciences, Josip Juraj Strossmayer University of Osijek, Croatia

Zorica Stojanović, Faculty of Technology, University of Novi Sad, Serbia

Zorica Tomičić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Zsuzsanna Jakabné Sándor, Research Centre for Aquaculture and Fisheries (HAKI), Hungarian University of Agriculture and Life Sciences, Hungary

EXECUTIVE COMMITTEE

Bojana Kokić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Bojana Šarić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Jovana Kos, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Ljubiša Šarić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Pavle Jovanov, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Predrag Ikonić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Tamara Dapčević Hadnađev, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

ORGANIZING COMMITTEE

President:

Pavle Jovanov, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Members:

Aleksandar Marić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Aleksandra Bajić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Alena Stupar, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Biljana Cvetković, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Bojana Radić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia

Branislava Đermanović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Danka Dragojlović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Dragana Tomanić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Dragana Plavšić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Dragana Ubiparip, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Ivana Čabarkapa, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Jelena Miljanić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Jelena Tomić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Jovana Delić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Jovana Kojić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Lidija Perović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Milana Matić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Milica Vidosavljević, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Miloš Županjac, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Miona Belović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Miroslav Hadnađev, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Nataša Đerić Ilić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Nataša Ćurčić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Nedeljka Spasevski, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Nemanja Teslić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Nikola Maravić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Olivera Šimurina, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Olja Todorić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Petar Ilić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Radmila Radović, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Renata Kovač, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Strahinja Vidosavljević, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Tatjana Peulić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Viktor Stojkov, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Vojislav Banjac, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Zorica Tomičić, Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia
Fan Liu, College of Food Science and Engineering, Shandong Agricultural University, China
Feng Li, College of Food Science and Engineering, Shandong Agricultural University, China
Kang Xu, College of Food Science and Engineering, Shandong Agricultural University, China
Qingqing Li, College of Food Science and Engineering, Shandong Agricultural University, China
Yannan Chen, College of Food Science and Engineering, Shandong Agricultural University, China

EVALUATION OF THE ADDITION OF QUINCE SEED OIL CAKE ON THE TEXTURE AND SENSORY PROPERTIES OF TOAST BREAD DURING STORAGE

Aleksandra Ćirković*, Milica Stevanović, Biljana Rabrenović, Jovanka Laličić-Petronijević, Dragana Paunović, Mirjana Demin

Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Zemun, Serbia

*Corresponding author:
E-mail address: cirkovicco@gmail.com

ABSTRACT

Bread enriched with fruit seed oil cake is an innovation in the food industry, which is increasingly focusing on the development of functional foods. The use of oil cake as a by-product in food production is a multifunctional approach that contributes to food safety, sustainability and public health. By using these nutrient resources, the composition and quality of food can be significantly influenced, waste reduced and healthier eating habits promoted. The aim of this study was to analyze the chemical composition, technological and functional properties of quince seed oil cake flour and the effects of its addition on the freshness and sensory properties of standard toast bread. The oil was extracted from the quince seeds by cold pressing, and the resulting cake was then ground into flour. Before being added to the dough, the quince seed oil cake flour was hydrothermally processed. 5 % and 10 % quince seed oil cake flour were added to the standard recipe for toast bread. The quince seed oil cake affected the chemical composition of the toast by increasing the protein, lipid, mineral and moisture content and decreasing the carbohydrate content. The addition of quince seed oil cake influenced the textural property of hardness and had a positive effect on the freshness of the final product. The change in freshness was lowest in the Q10 sample with 10 % quince seed oil cake flour (22.0 %) and highest in the control sample (63.0 %). The greatest negative change during storage was observed in the crumbliness, while the odor and taste characteristics showed the least negative changes. After seven days of storage, mold appeared in all samples.

Keywords*: quince, oil cake, toast, technological and functional properties, sensory evaluation*

INTRODUCTION

Quince seed oil cake is a secondary product after cold pressing that can be used as flour, protein isolate and protein concentrate, potential source of antioxidants and dietary fiber, etc. in the food industry and bakery production (Bárta et al., 2021). Bakery products offer immense variety and adaptability, so they can be easily modified depending on the ingredients available and the desired characteristics. Recently, the food industry has focused on increasing the health benefits of bread through the addition of bioactive substances. This approach is not only in line with sustainable food production practices, but also improves the nutritional quality of the bread, although it poses technological challenges due to the potential impact on the dough properties and texture of the final product when new ingredients are added (Guiné, 2022). Texture is the sensory experience of a food's structure and how it responds to applied forces, combining mechanical, geometric, and surface properties perceived by the senses (de Paula & Conti-Silva, 2013). When developing food products, it's crucial to understand customer needs and effectively communicate the product's value. Therefore, sensory evaluation is an important tool in the new product development process. Sensory analysis helps to evaluate product quality and assess consumer expectations and reactions throughout the development process (Świąder & Marczewska, 2021).

In this study supplementation of wheat flour with 5 % and 10 % quince seed oil cake flour and it's impact on chemical composition, hardness as texture property, freshness of toast breads and sensory evaluation were investigated.

MATERIAL AND METHODS

Material

Wheat flour T 500 (Čeković mill) was used as the main ingredient for the production of toast bread and contained 13.74 % moisture, 0.51 % ash, 9.80 % protein, 1.39 % lipid and 74.57 % carbohydrates. Quince seed samples were obtained from a private plantation (Trampling Ćirković), cold pressed to remove the fats and stored in a freezer. The resulting oil cake contained 8.07 % moisture, 4.21 % ash, 32.64 % protein, 10.12 % lipid and 44.96 % carbohydrates. It was coarsely crushed and finely ground with a high-speed grinder (Delimano, China, MC 343, 200 W) for 30-60 seconds, with at least 50 % of the obtained particles pass through a 335 μm sieve. Other ingredients were yeast (Alfa, Lesaffre), salt (Solana Tuzla), Soft'r Premium (Puratos) for dough stability, Propi San (Puratos) as a preservative for longer freshness and water from the municipal supply.

Toast bread preparation

The finely crushed quince seed flour was mixed with hot water (50℃) in a ratio of 1:2, the water was absorbed for 30 minutes and made into a sticky, compact cake. This absorbed water was included in the total amount of water required for kneading. For the standard dough 58 % water was used, for the Q5 dough 65 % and for the Q10 dough 76 %, which reflects the water absorption capacity of the quince seeds. Water was added in amount to produce dough with acceptable handling characteristics. The dough consistency and stickiness were subjectively estimated by an experienced baker.Other ingredients were added in an amount of 3,5 % yeast, 2 % salt, 1 % improver and 1 % preservative, calculated in relation to the total amount of flour. The mixture was prepared in a spiral mixer (Bongard, France) for 15 minutes in two phases. Up to 90 % of the total amount of water was added in the first phase and the rest in the second phase, with the flour temperature at 23.4 °C and the water temperature fluctuating slightly between 21.6 °C and 23.6 °C. During kneading, the mixer heated the ingredients so that test pieces with temperatures between 29.1 °C and 29.7 °C were formed. The dough was then formed into balls. After 10 minutes of proofing, the dough for the toasts was divided into 600 g pieces, which were left to proof for a further 10 minutes.

The dough was placed in the molds for the toasts. It was then left to ferment in a proofing chamber (Bongard, France) at 35 °C and 80 % humidity for 45 to 50 minutes. Baking took place at 250-260 °C for 30 minutes. After baking, the bread was cooled, sliced and packed in polypropylene bags. It was then stored at room temperature for 7 days to observe the changes in textural and sensory properties at 0, 3, 5 and 7 days.

Chemical composition

The moisture, ash, protein and lipid content of the toast bread samples were determined according to AOAC (AOAC, 2006). The carbohydrate content was determined according to the formula given in Millar et al. (2019): 100 – sum of moisture, ash, protein and fat.

Texture determination

The textural properties of the toast breads were determined by double compression texture profile analysis (TPA) using the TA1 texture analyzer (Ametek Test & Calibration Instruments, Texture Analyzer Machine TA1 Series, LLOYD TA 500). A cylinder with a diameter of 25 mm and a load cell of 50 N were used as part of the analyzer. The toast was cut into 16 equal pieces of 15 mm thickness using a slicing machine (Graef, Bradford Great Britain, ABS 07), of which the 4th, 8th, 9th and 13th pieces were analysed in terms of hardness. Freshness was expressed as a percentage increase in hardness during storage.

Sensory evaluation

The sensory characteristics of the produced breads enriched with different proportions of quince seed oilcakes were determined on the 0th, 3rd, 5th and 7th day after baking by a

panel of 10 assessors – experts – using the relevant ISO standards (2023). The evaluation procedure was carried out according to the instructions in Stikić et al. (2012).

Statistical analyses

Statistical analysis was performed using STATISTICA 12 software, with results expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was used to assess differences between group means. Tukey's HSD test (honestly significant difference) with a confidence level of 95% ($p < 0.05$) was performed to determine statistically significant differences between samples.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the toast is shown in Figure 1. The determination of the moisture content was necessary during the baking and cooling process in order to maintain the desired quality and extend the shelf life (Rico et al., 2020).

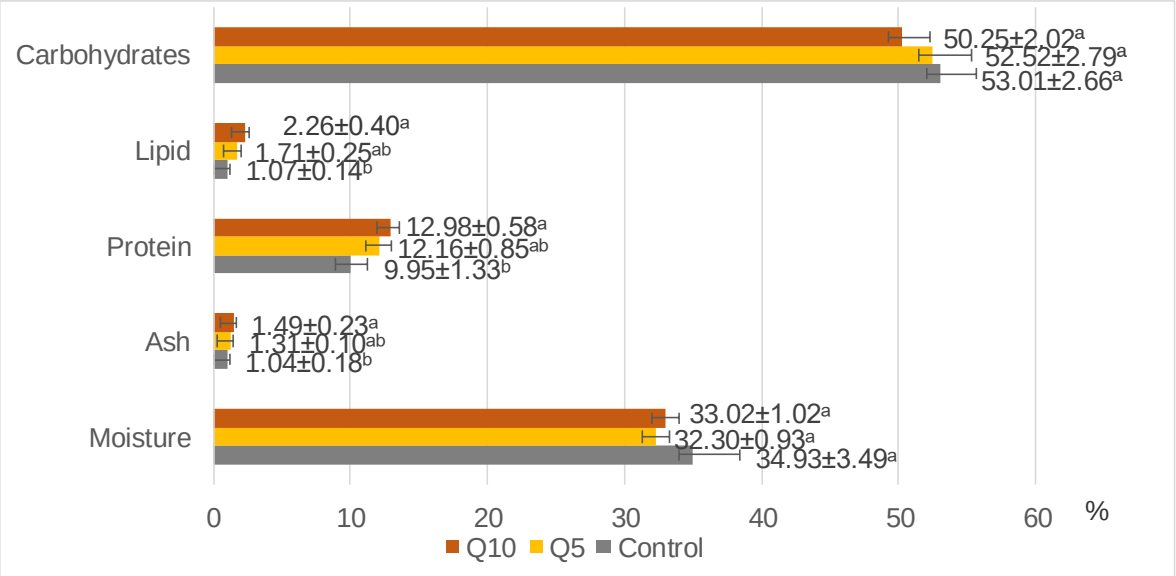


Figure 1. The chemical composition of control and toast bread with 5 % (Q5) and 10 % (Q10) quince seed oil cake flour. Similar letters indicate no significant difference at p ≤ 0.05.

The moisture content of the quince seed-enriched bread samples corresponded to the moisture content of the control sample: the control sample had a moisture content of 34.93±3.49 %, Q5 32.30±0.93 % and Q10 33.02±1.02 % (Figure 1). According to Hassan and Ali (2014), the moisture content of toast bread enriched with different percentages of defatted grape fruit albedo flour ranged from 22.65 to 33.50 %, which was within the range of the results of this study.

The partial replacement of wheat flour with oilseed quince cake flour had a noticeable effect on the ash, protein and fat content. With the increase in the proportion of quince oil cake flour, the content of the macronutrients mentioned also increased compared to the control (Figure 1). The ash content of control, Q5 and Q10 was 1.04±0.18 %, 1.31±0.10 % and 1.49±0.23 %, respectively. Enriching the wheat flour with 10 % quince oil cake flour had the greatest influence on the ash content.

Baiano et al. (2009) found that toast made from two different types of durum wheat flour contained 1.56 and 2.54 % ash. Ash content can be considered an indicator of the quality of the bread as it influences the purity of the flour, the milling process, the color as well as the texture and taste of the final product (Ismail, 2017). The protein content increased with higher enrichment of wheat flour. The control had 9.95±1.33 %, Q5 had 12.16±0.85 % and

Q10 had 12.98±0.58 % proteins (Figure 1). Proteins are essential for the production of toast as they affect nutritional and structural properties (del Mercado et al., 2021). Zidan (2021) published that wheat flour toast bread had a protein content of 14.83 %, which was higher than the results of the present study. Addition of 10 % quince seed oil cake flour had the greatest positive effect on protein content compared to the control sample.
Although the lipids are only present in small quantities, they play an important role in bread quality: they stabilize the gas cells during baking and influence the volume, crumb structure and shelf life (Pareyt et al., 2011). As the proportion of added oil cake increased, the proportion of lipids increased compared to the control. The lipid content for the control, Q5 and Q10 was 1.07±0.14 %, 1.71±0.25 % and 2.26±0.40 %, respectively. Sadeghzadeh Benam et al. (2021) reported that toast bread made from wheat flour had a lipid content of 2.03 %, which was higher than the lipid content of the toast bread in this study. The differences in the results could be explained by different wheat varieties, recipe and toast production methods. A slight decrease in carbohydrate content was found in toasted breads. Millar et al. (2019) published carbohydrate values in white wheat bread 54.3 %, which were close to the results obtained in this research for the same group of compounds. The carbohydrate content determined in the control was 53.01±2.66 %, Q5 52.52±2.79 % and Q10 50.25±2.02 % (Figure 1). Reducing the carbohydrate content of bread and enriching it with bioactive substances (protein, fiber, antioxidants, phenols) is very popular among consumers (Mohamed et al., 2006). The differences between the results can be explained by different types of wheat flour, storage, recipe and bread making condition. Small differences in the content of this macronutrient could be due to the low proportion of wheat flour that was replaced by quince seed oil cake.

Texture

For bread, hardness is most commonly determined as the main texture parameter of the final product, as there is a correlation between the crumb hardness and the consumer's perception of the freshness of the bread (Carr et al., 2006). The results of toast crumb hardness during storage on day 0, 3, 5 and 7 measured with the Texture Analyzer and the freshness, expressed as a percentage increase in hardness, after 7 days are shown in Table 1.

Table 1. The hardness and freshness of toast bread during storage

TOAST BREAD (N)					
Samples	**0 day**	**3rd day**	**5th day**	**7th day**	**Freshness change during storage after 7 days (%)**
Control	2,65±0,16^{c}	4,19±0,48^{b}	4,26±0,07^{b}	4,32±0,21^{c}	63,02
Q5	3,23±0,21^{b}	4,62±0,21^{ab}	4,73±0,39^{ab}	5,05±0,23^{b}	56,35
Q10	5,02±0,19^{a}	5,51±0,63^{a}	5,56±0,69^{a}	6,12±0,41^{a}	21,91

Q5 – toast bread with 5 % quince seed oil cake flour; Q10 – toast bread with 10 % quince seed oil cake flour. Similar letters indicate no significant difference at $p \leq 0.05$.

The hardness of the fresh samples was statistically significantly different (Table 1). This quality parameter was influenced by the amount of oil cake added, so that the hardness increased with the increasing cake proportion and storage time. The same trend was observed in the samples after the 3rd, 5th and 7th day of storage. In the fresh samples, the lowest hardness was found in the control (2.65±0.16 N), while it was 1.9 times higher in the Q10. In the stored samples, the difference in hardness between the samples with the same storage period is lower. The highest hardness was measured on the seventh day of storage: 4.32±0.21 N, 5.05±0.23 N, 6.12±0.41 N for the control, Q5 and Q10 respectively. Addition of 10 % quince oil cake showed the lowest change in freshness after the 7-day storage (21.91 %), while the control sample showed the highest change in freshness (63.02 %). The higher quantity of oilseed cake means less change in the freshness of the toasted bread. The control and Q5 show the greatest change in freshness after the third day of storage (36.75 % and 30,09 %, respectively), while Q10 shows the greatest change in freshness between the

fifth and seventh day of storage (9.15 %). According to Nasir et al. (2020), the differences in firmness correspond to the different degrees of wheat flour substitution, with harder bread requiring higher force to compresse due to the lower moisture content and the interactions between gluten and fibre materials. In the study by Nasir et al. (2020), the hardness of wheat bread was 2.10 N (for fresh samples), which is consistent with the results published in this study.

Sensory evaluation

The sensory evaluation results of the toast breads tested is shown in Figure 2. After cooling and stabilization on day 0, the assessors found that the control toast bread had a pleasant smell and taste, standard appearance and a good chewiness (Figure 2. a). The sample Q5 had a mild odor and taste (score 3.75), high scores for appearance (score 4.23) and texture, low crumbliness and good chewiness. The sample Q10 had a strong marzipan flavor and a denser texture, but the increased seed content caused irritation in the palate and throat. This sample also required more time to chew and swallow.

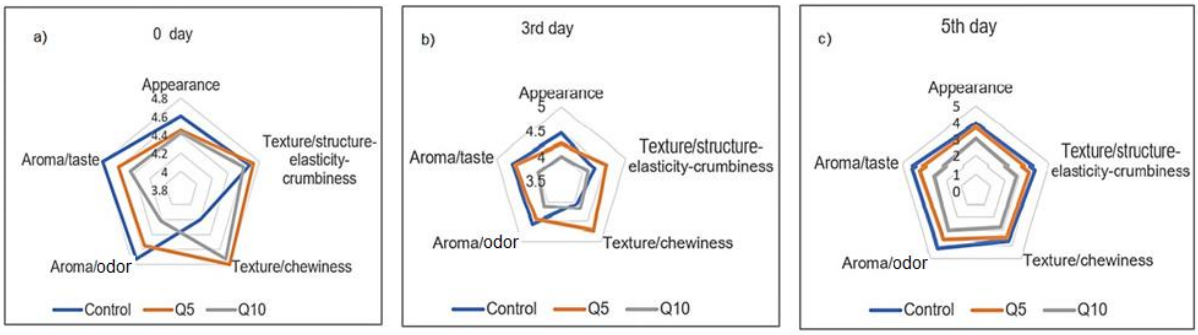


Figure 2. Sensory evaluation of control and toast bread with 5 % (Q5) and 10 % (Q10) quince seed oil cake flour: a) fresh samples; b) after 3rd day, and c) after 5th day of storage.

After the 3rd day of storage at room temperature in polypropylene bags, the toast bread samples showed reduced freshness. The sample Q5 retained a similar taste and odor to the control sample and received the highest ratings for appearance and odor (3.75 and 4.23, respectively). In particular, the sample Q10 showed increased crumbliness, making the bread harder and more difficult to chew, but with a more pronounced taste. After the 5th day of storage, the greatest change was observed in the sensory parameters that indicate bread freshness. The control sample retained the greatest freshness, while the quince seed-enriched bread samples dried out visibly and showed a lower volume and a sinking in the middle, which led to lower ratings. The sample Q5 exhibiting an almost imperceptible odor, but received the highest score for appearance (4.23). The sample Q10 showed a noticeable reduction in aroma, became particularly crumbly and dry in the middle, difficult to chew and approached the shelf life limit. After the 7th day at room temperature, the samples became unusable due to mold growth and excessive dryness. Although the textual properties were evaluated, the samples were not subjected to further testing or sensory evaluation.

CONCLUSIONS

The replacing part of the wheat flour with quince seed oil cake flour affected the chemical composition, texture and sensory properties of toasted breads. The bread with 10 % of this ingredient had better chemical composition, while the sample with 5 % performed better in terms of texture and sensory properties. The incorporation of quince seed oil cake flour in toast bread production contributes to the reduction of by-products after quince processing and to the expansion of the range of bakery products. On the other hand, quinces are not grown in large quantities in Serbia, so a good long-term plan for the supply of this raw material is necessary for this product to reach the market and be sustainable.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministry of Education, Science and Technological Development, Republic of Serbia, Contract number 451-03-65/2024-03/200116 (University of Belgrade, Faculty of Agriculture).

REFERENCES

AOAC (2006). Official methods of analysis (17thed.). Gaithersburg MD: Association of Official Analytical Chemists.

Baiano, A., Romaniello, R., Lamacchia, C., La Notte, E. (2009). Physical and mechanical properties of bread loaves produced by incorporation of two types of toasted durum wheat flour. *Journal of Food Engineering,* 95(1):99–207.

Bárta, J., Bártová, V., Jarošová, M., Švajner, J., Smetana, P., Kadlec, J., Filip, V., Kyselka, J., Berčíková, M., Zdráhal, Z., Bjelková, M., Kozak, M. (2021). Oilseed Cake Flour Composition, Functional Properties and Antioxidant Potential as Effects of Sieving and Species Differences. *Foods*, 10(11):2766.

Carr, G. L., Rodas, A. B. M., Della Torre, C. M. J., Tadini, C. C. (2006). Physical, textural and sensory characteristics of 7-day frozen part-baked French bread. *LWT - Food Science and Technology*, 39(5):540-547.

de Paula, A. M., Conti-Silva, A. C. (2013). Texture profile and correlation between sensory and instrumental analyses on extruded snacks. *Journal of Food Engineering*, 121:9-14.

del Mercado, P. V., Mojica, L., Morales-Hernández, N. (2022). Protein Ingredients in Bread: Technological, Textural and Health Implications. *Foods*, 11(16):2399.

Guiné, P. F. R. (2022). Textural Properties of Bakery Products: A Review of Instrumental and Sensory Evaluation Studies. *Applied Sciences*, 12(17):8628.

Hassan, A. M. M., Ali, M. H. (2014). Physico-Chemical Properties and Sensory Evaluation of Toast Bread Fortified with Different Levels of White Grapefruit (Citrus paradise L.) Albedo Layer Flour. *World Journal of Dairy & Food Sciences*, 9(2): 228-234.

Ismail, B. P. (2017). Ash Content Determination. *Food Analysis Laboratory Manual*. Food Science Text Series: 117-119.

ISO 8586:2023. (2023). Sensory Analysis – Selection and training of sensory assessors. Edition 2.

Millar, K. A., Barry-Ryan, C., Burke, R., McCarthy, S., Gallagher, E. (2019). Dough properties and baking characteristics of white bread, as affected by addition of raw, germinated and toasted pea flour. *Innovative Food Science & Emerging Technologies*, 56:102189.

Mohamed, A. A., Rayas-Duarte, P., Shogren, L. R., Sessa, J. D. (2006). Low carbohydrates bread: Formulation, processing and sensory quality. *Food Chemistry*, 99(4):686-692.

Nasir, S., Mehraj Allai, F., Gani, M., Ganaie, S., Gul, K., Jabeen, A., Majeed, D. (2020). Physical, Textural, Rheological, and Sensory Characteristics of Amaranth-Based Wheat Flour Bread. *International Journal of Food Science*.

Pareyt, B., Finnie, M. S., Putseys, A. J., Delcour, A. J. (2011). Review Lipids in bread making: Sources, interactions, and impact on bread quality. *Journal of Cereal Science,* 54(3): 266-279.

Rico, D., González-Paramás, M. A., Brezmes, C., Martín-Diana, B. A. (2020). Baking Optimization as a Strategy to Extend Shelf-Life through the Enhanced Quality and Bioactive Properties of Pulse-Based Snacks. *Molecules*, 25(16):3716.

Sadeghzadeh Benam, N., Goli, M., Seyedain Ardebili, M. S., Vaezshoushtari, N. (2021). The quality characteristics of dough and toast bread prepared with wheat flour containing different levels of Portulaca oleracea leaf powder. *Food Science and Technology (Campinas)*, 42(6).

Stikić, R., Glamočlija, Dj., Demin, M., Vucelic-Radović, B., Jovanović, Z., Milojković-Opsenica, Z., Jacobsen, S. E., Milovanović, M. (2012). Agronomical and nutritional evaluation of quinoa seeds (Chenopodium quinoa Willd.) as an ingredient in bread formulations. *Journal of Cereal Science*, 55(2): 132-134.

Świąder, K., Marczewsk, M. (2021). Trends of Using Sensory Evaluation in New Product Development in the Food Industry in Countries That Belong to the EIT Regional Innovation Scheme. *Foods*, 10(2):446.

Zidan, S. N. (2021). Utilization Of Millet (pennisetum glaucum) flour To produce gluten-free toast bread. *Egyptian Journal of Nutrition*, 36(3):1-21.

ALTERNATIVES FOR NITRITES FROM NATURAL RESOURCES IN COOKED MEAT PRODUCTS

Branislav Šojić[1]*, Branimir Pavlić[1], Predrag Ikonić[2], Nataša Živković Šojić[3], Danijela Jojkić Pavkov[3], Milo Mujović[4], Saša Novaković[5], Milivoj Radojčin[6]

[1]Faculty of Technology Novi Sad, University of Novi Sad, Bul. cara Lazara 1, 21000 Novi Sad, Serbia
[2]Institute of Food Technology in Novi Sad, University of Novi Sad, Bul. cara Lazara 1, 21000 Novi Sad, Serbia
[3]Faculty of Medicine, Institute for Child and Youth Health Care Vojvodina, University of Novi Sad, Hajduk Veljkova 10, 21000 Novi Sad, Serbia
[4]Gombit d.o.o., Kralja Petra I 194c, 22320 Inđija, Serbia
[5]Teagasc Food Research Centre Ashtown, D15 DY05 Dublin, Ireland
[6]Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: sojic@tf.uns.ac.rs

ABSTRACT

Nitrites are the most essential synthetic additives and preservatives in the meat processing industry. They are related to the control of different pathogenic bacteria (e.g., *Clostridium botulinum*, *Listeria monocytogenes*), the development of reddish-pink color and cured flavor, and the delaying of oxidative reactions (lipid and protein oxidation) in cured meat products. However, these preservatives have been linked to several health risks, leading to recent regulatory changes. Also, according to "clean label" trends, customer demand for natural meat products has increased. Hence, scientists and meat industry processors focus on developing nitrite alternatives. This paper reviews nitrate-containing vegetable and plant extracts as potential replacements (used entirely or partially) for synthetic nitrites in manufacturing of cooked meat products.
***Keywords**: nitrites, cured meat products, nitrosamines, plant extracts*

INTRODUCTION

Meat curing is one of the most used methods in the modern meat industry to enhance the quality and prolong the shelf-life of highly perishable foodstuffs. This process involves adding salts, nitrites, nitrates, and other ingredients such as sugar, hydrocolloids, phosphates, spices, and spice extracts (Alahakoon et al., 2015; de Carvalho et al., 2024; Honikel, 2008).
Nitrites (sodium - E250 and potassium nitrite - E249) are registered as allowable food preservatives in cooked meat products (e.g., sausages and ham). According to EU legislation: Regulation no. 1129/2011, the addition of nitrites in cooked meat products is limited to 150 mg/kg (European Commission, 2011).
Also, it should be noticed that the residual nitrites in the final products are lower than the initially added amount. The reduction of nitrites during processing and storage is probably the result of NO-myoglobin, microorganisms and enzyme activity, oxidation to nitrate, etc. (Honikel, 2008; Lotfi & Bryan, 2024; Premi et al., 2024; Šojić et al., 2019). These preservatives are predominantly used to ensure microbiological safety, focusing on inhibiting *C. botulinum* and *L. monocytogenes* growth and delaying oxidative reactions; moreover, they play a vital role in the formation of a typical cured flavor and typical reddish-pink color for cured meat products (Melios et al., 2024; Premi et al., 2024).
Regardless, nitrites are noticeably harmful due to the possibility of forming carcinogenic N-nitroso compounds (NOCs) such as N-nitrosoamines. The finding of NOCs raised significant safety concerns about using nitrites in cured meat processing (Alahakoon et al., 2015; Honikel, 2008; Jin et al., 2018; Premi et al., 2024; Zhang et al., 2023).
Nitrosamines can form in the mouth or stomach when foods containing nitrosamine precursors, such as nitrites and nitrates, are consumed. This reaction occurs through the interaction of nitrites with secondary amines, which can lead to the formation of nitrosamines within the digestive system. Additionally, nitrosamines can develop during the processing of

foods, especially in conditions involving high temperatures and acidic environments, further contributing to their presence in the final products. Consequently, reducing the residual nitrite level in meat products can decrease the potential risk of NOCs formation (Zhang et al., 2023).
According to the numerous investigations, the International Agency for Research on Cancer (IARC) marked curing meat products as carcinogenic to the humans - Group 1 (Flores & Toldrá, 2021). Concerning these issues, an acceptable daily intake (ADI) of 0.07 mg nitrite per kg of body weight was set by the Joint Expert Committee of the Food and Agriculture Organization (JECFA) and the World Health Organization (WHO) that appears to be safe for healthy newborns, children, and adults (Shakil et al., 2022).
Considering the impact of nitrites on human health, it is essential to note that consuming vegetables allows for approx. 80% of nitrite sources, while processed meat products contain only 5%. Also, van Breda et al. (2019) reported that nitrate from drinking water provides NOCs formation independently of processed meat consumption. Consequently, the classification report should consider drinking water nitrate levels (Flores & Toldrá, 2021; van Breda et al., 2019). Moreover, some studies have suggested that nitrites have benefit for the human cardiovascular system. Nitrogen-monoxide (NO), formed from nitrate or nitrite, is accepted to be one of the chief signaling molecules in the body. Its continuous generation is essential for the integrity of the cardiovascular system. Recently, an interest in the therapeutic effect of inorganic NO intake has been enlarged (Bryan & Ivy, 2015; Lotfi & Bryan, 2024).
The “clean labeling“ trend, a significant innovation in the food sector, is direct response to the growing consumer consciousness about the ingredients in their food (de Carvalho et al., 2024). This trend, which prioritizes the creation of healthier and minimally processed food, has gained traction by excluding synthetic chemicals (de Carvalho et al., 2024; Zhang et al., 2023).
As consumer demand for "clean label" products continues to grow, it is vital for researchers to explore alternatives to synthetic nitrites in cooked meat products (Zhang et al., 2023). This review article addresses possible replacement of synthetic nitrites by plant based ingredients including spices, medical herbs, fruits and vegetables.

Alternatives for nitrites in cooked meat products

Several methods have been assessed to find alternatives for synthetic preservatives (nitrates/nitrites) and enhance the quality and shelf-life of cooked meat products. Usage of nitrate-containing plant materials is the most dominant method to produce “natural” meat products without synthetic preservatives (Melios et al., 2024). Celery, spinach, Swiss chard, radish parsley, and nettle can be natural nitrate sources. Celery is the most widely studied plant among these plants and has been used commercially because it does not have negative impact on the sensory properties of meat products (Jin et al., 2018).
During the processing of cooked meat products, the addition of nitrate-containing plant materials can be approached in two ways. The first method involves the direct addition of all ingredients (e.g., salts, plants/plant extracts, starter cultures) in the brine or sausage batters. The second method utilizes the “pre-fermented” or “pre-converted” nitrate-containing plant material, which has already been treated with a starter culture (e.g., *Staphylococcus carnosus*). Adding the starter cultures during processing facilitates the conversion of nitrate into nitrite. Therefore, optimizing processing parameters is crucial for the successful use of natural nitrate sources in meat products (Flores & Toldrá, 2021; Melios et al., 2024; Ozaki et al., 2021).
In different formulations of meat batters, addition of nitrate/nitrite from plant material can also provide the formation of NOCs. However, different plants, besides significant concentrations of nitrates, could be reservoirs of phytochemicals, which have strong inhibiting potential during nitrosation. Consequently, the addition of nitrate-containing plant materials in cured meats, in some cases, can reduce the endogenous formation of NOCs (Melios et al., 2024).

Several authors examined the application of nitrate-containing plant materials as alternatives for synthetic preservatives in cooked meat products (Choi et al., 2017; Hwang et al., 2018; Jin et al., 2018; Riel et al., 2017; Sindelar et al., 2007).

Sindelar et al. (2007) added powder of celery juice in combination with starter culture in cooked sausages. As a result of this study, sausages treated with celery juice powder exhibited similar physico-chemical and microbiological quality to those made with sodium nitrite. In another study, Jin et al. (2018) determined a similar preservative potential of celery powder (0.8%) in cured meat product. Choi et al. (2017) determined that “pre-converted” nitrite from red beets (10%) and ascorbic acid, applied in the emulsified sausages, possess similar antioxidant and antimicrobial potential as synthetic sodium nitrite. Riel et al. (2017) reported that the parsley extract powder (nitrate > 60 ppm) showed similar antimicrobial potential against *L. monocytogenes* as synthetic sodium nitrite, in mortadella-type sausages. The impact of “pre-converted” nitrites from spinach, lettuce, celery, and red beet (3%) on the quality and shelf-life of raw and cooked sausages were also determined. Among the obtained plant materials, spinach exhibited the most power as an alternative to synthetic sodium nitrite, maintaining the color and delaying oxidative reactions in cooked pork sausage (Hwang et al., 2018). Kim et al. (2019) reported that “pre-fermented” Swiss chard retarded lipid oxidation and enhanced the redness of cooked loin ham.

Besides nitrate-containing plants, many natural extracts (e.g., essential oils, oleoresins, water, and ethanolic extracts) have an antioxidative and antimicrobial potential; hence, they could be used as potential substitutes for nitrites in cured meat products (Melios et al., 2024). The level of bioactive compounds (polyphenols, flavonoids, and terpenoids) in plant extracts is the most important for their preservative potential in the food sector. Principally, plant essential oils (EOs) attract interest as potential food additives since they are generally recognized as safe (GRAS) and have broad consumer acceptance (Burt, 2004; Šojić et al., 2020).

Also, it should be noted that the effectiveness of plant extracts depends on many factors (e.g., climate conditions during plant harvest, physicochemical properties of meat batters, and interactions among other constituents of meat batters). Moreover, some extracts possess an intense aroma and inadequate biopotential for further application in the food industry. Hence, selecting and optimizing extraction technologies are essential for recovering high-quality plant extracts (Melios et al., 2024).

Several authors determined the application of plant extracts in cooked meat products (Table 1).

Sweet paprika (*Capsicum annuum* L.) is one of the most widely used spice in the meat industry. It is added to enhance meat products' color, aroma, and flavor. Due to its high content of carotenoids and other bioactive compounds, sweet paprika exhibits some antioxidant potential. Sweet paprika oleoresins (SPO) are often used to improve the color of meat products because they show higher thermal stability than ground paprika (Kim & Chin, 2021). Given these benefits, Kim and Chin (2021) explored the possibility of partially substituting nitrites with SPO to produce cooked sausages. In this study, SPOs were dissolved in sunflower oil at 1% and 5%. The resulting extracts were dosed at a concentration of 0.1% as partial substitutes for nitrites in cooked sausages. The results of this study indicate a significant antioxidant and antimicrobial effect of the added SPOs, as well as their positive impact on the color of cooked sausages produced with reduced nitrite content (75 mg/kg). The interaction of nitrite (75 mg/kg) and 0.1% added SPO affected the final product's shelf-life as in the control sausages, which were produced with an addition of 150 mg/kg sodium nitrite (Kim and Chin, 2021).

Given the well-known protective properties of lipophilic rosemary extracts in meat products, Abbasi et al. (2023) investigated the potential for partial and complete substitution of nitrites with rosemary essential oil (REO) in cooked sausages. This study found that REO at a concentration of 120 ppm reduced lipid oxidation (TBARs test) and the growth of aerobic mesophilic bacteria in cooked sausages produced without added nitrites.

Additionally, it was found that a combination of REO and nitrites (60:60 ppm) achieved a similar antioxidant effect as the addition of nitrites alone (120 ppm) in these meat products.

On the other hand, it is essential to note that the complete substitution of nitrites with 120 ppm REO needs to achieve adequate color formation. Therefore, the authors emphasize that partially substituting nitrites with this EO is the optimal solution for meeting all quality criteria for cooked sausages (Abbasi et al., 2023).

The encapsulation process has been increasingly used in recent years to enhance the functional properties of EOs. Vafania et al. (2019) applied the nanoencapsulation technique to thyme essential oil (TEO) to improve its antioxidant and antimicrobial potential and to use it as a partial substitute for nitrites in cooked sausages. Before industrial application, the encapsulation process was optimized. It was determined that the encapsulate with optimal physicochemical properties contained nearly 40% of EO and a carrier in a combination of chitosan and gelatin (1:6). The encapsulate with optimal properties (500 ppm), as well as the free TEO (1000 ppm), was added to cooked sausages with reduced nitrite content (20 ppm), while the control sample contained 120 ppm nitrites. The results of this study showed that TEO, both in its free form and in the encapsulated form combined with reduced nitrite content (20 ppm), effectively inhibited the growth of *C. botulinum*, showing similar antimicrobial potential to the 120 ppm nitrite addition in the control sausages. Additionally, it is essential to note that adding the encapsulate improved the sensory quality of the final product compared to the free-form EO (Vafania et al., 2019).

Table 1. Plant extracts as alternatives for nitrites in cooked meat products

Plant material	Type of extract	Dose	Storage condition	Effect	Reference
Sweet paprika	Oleoresin	0.1%	4 °C, 35 days	DLO, RMG	(Kim & Chin, 2021)
Rosemary	EO	60 ppm	4 °C, 35 days	DLO, RMG	(Abbasi et al., 2023)
Thyme	EO	500 ppm	4 °C, 20 days	DLO, RMG	(Vafania et al., 2019)
Coriander	EO	0.075-0.150 µl/g	4 °C, 60 days	DLO, RMG	(Šojić et al., 2019)
Peppermint	EO	20-60 ppm	4 °C, 30 days	DLO	(Ghabraie et al., 2016)
Peppermint + tomato pomace	EO + SFE	0.150 µl/g	4 °C, 60 days	DLO, RMG	(Šojić et al., 2020)
Satureja montana L.	EO	7.80; 15;60; 31.25 µl/g	25 °C, 30 days	DLO	(Coutinho de Oliveira et al., 2012)

EO – essential oil; SFE – supercritical fluid extracts; RMG – reducing microbial growth; DLO – decreasing lipid oxidation

Coriander is an aromatic spice plant with extensive application in the food industry. It is characterized by a high content of EOs rich in oxygenated monoterpenes, with linalool being a prominent component (Šojić et al., 2019b). To find an alternative to nitrites in cooked sausages, Šojić et al. (2019) used coriander EO (CEO) at various concentrations (0.075 - 0.150 µl/g). The authors determined that CEO cannot wholly replace nitrites in meat products, primarily concerning color formation. However, the combination of nitrites

(50 mg/kg) and CEO led to a significant reduction in lipid oxidation and the growth of aerobic mesophilic bacteria. A stable pink-red color was also achieved, which can be attributed to the synergistic effect of the bioactive compounds from the EO and nitrites with the myoglobin pigment (Šojić et al., 2019b). In this study, using an artificial neural network, it was predicted that extending the shelf-life of these meat products from 45 to 52 days would be achievable in sausages with reduced nitrite content (60 mg/kg) to which CEO was added at a concentration of 0.12 µl/g. The limiting criteria for the sustainability of cooked sausages were set as: TBARs value (< 0.12 mg MDA/kg), total number of aerobic mesophilic bacteria (< 2.5 log CFU/g), and color (CIEa* > 11) (Šojić et al., 2019b). In addition to its application in traditional medicine, as well as tea and other beverages, peppermint is increasingly used as a functional ingredient in the food industry (Ghabraie et al., 2016; Šojić et al., 2020). This plant is rich in EO with potent antioxidant and antimicrobial potential (Ghabraie et al., 2016). For these reasons, Ghabraie et al. (2016) investigated the potential use of peppermint EO (PEO) as a partial substitute for nitrites in cooked sausages. The PEO demonstrated antimicrobial potential against *E. coli* and *L. monocytogenes*. These researchers partially substituted nitrites (120 ppm) with 20, 40, and 60 ppm of PEO. The results of this study indicated that partial substitution of nitrites with 60 ppm of this EO effectively reduced lipid oxidation (TBARs test) while improving the final product's color (Ghabraie et al., 2016). To enhance the bioactive potential of PEO, its synergistic effect with supercritical tomato extract, a by-product of vegetable processing, was also investigated (Šojić et al., 2020).
A high content of lycopene characterizes the lipophilic tomato extract. This pigment could reduce oxidation levels and improve the color of meat products with reduced nitrite content (Šojić et al., 2020). The results of this study indicate that the synergistic effect of PEO (0.075 µl/g) and tomato pomace extract (0.075 µl/g) obtained by supercritical fluid extraction (SFE) contributes to reduced oxidation levels and improved red color formation in cooked sausages with reduced nitrite content (50 mg/kg) (Šojić et al., 2020).
Due to its high content of thymol and carvacrol, the EO isolated from *Satureja montana* L. shows significant potential for application in the food industry (de Oliveira et al., 2012). Sausages produced with 0, 50, and 100 ppm nitrites were inoculated with *C. perfringens* type A. These authors found that *Satureja montana* L. EO reduced the growth of *C. perfringens*, both in conventionally nitrite-added sausages (100 ppm) and sausages with reduced nitrite content (0 and 50 ppm). The results suggested that this natural extract could be used as an alternative for nitrites in cooked meat processing.

CONCLUSIONS

Following the IARC classification of nitrite-cured meat as a Group 1 carcinogen, many investigators have tried discovering alternative ingredients to replace nitrite or nitrate. Using nitrate-containing plants in combination with starter cultures could be an alternative to synthetic nitrites in cooked meat products. There are some appropriate advantages, such as the removal of E numbers and the opportunity to obtain “clean-label” meat products. However, nitrites, even at trim levels, pose the risk of generating N-nitrosamines.
Hence, adding plant extracts (e.g., EOs, oleoresins, water extracts) could be another alternative for nitrites. In this way, the N-nitrosamines are restricted, but, unfortunately, these natural compounds did not fulfill the preservative effect. Up-to-day investigations have allowed the partial replacement of synthetic nitrites with different plant materials. The development of hurdle processing technologies for obtaining natural extracts and their encapsulation could allow the industrial production of high-quality material for further application in the meat industry. It is imperative that governments and meat processors invest in this transition for the future. By taking proactive steps and investing in research and development, we can collectively steer the meat industry towards a safer and more sustainable future.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Education, Science and Technological Development: Project 451-03-65/2024-03/200134.

REFERENCES

Abbasi, M. A., Ghazanfari, S., Sharifi, S. D., & Ahmadi Gavlighi, H. (2023). Effect of rosemary essential oil as nitrite substitute on quality of sausage produced using chicken fed by thymus essential oil and rapeseed oil. *Journal of Food Science and Technology*, *60*(3), 856–867. https://doi.org/10.1007/s13197-020-04786-8

Alahakoon, A. U., Jayasena, D. D., Ramachandra, S., & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science & Technology*, *45*(1), 37–49. https://doi.org/10.1016/J.TIFS.2015.05.008

Bryan, N. S., & Ivy, J. L. (2015). Inorganic nitrite and nitrate: evidence to support consideration as dietary nutrients. *Nutrition Research*, *35*(8), 643–654.

Coutinho de Oliveira, T. L., Malfitano de Carvalho, S., de Araújo Soares, R., Andrade, M. A., Cardoso, M. das G., Ramos, E. M., & Piccoli, R. H. (2012). Antioxidant effects of Satureja montana L. essential oil on TBARS and color of mortadella-type sausages formulated with different levels of sodium nitrite. *LWT - Food Science and Technology*, *45*(2), 204–212. https://doi.org/10.1016/J.LWT.2011.09.006

de Carvalho, T. B., Oliveira, M., Gomes, A. M., Monteiro, M. J., Pintado, M., Komora, N., Durães, T., Nunes, F. M., Cosme, F., & Patarata, L. (2024). Clean labelling sodium nitrite at pilot scale: In-situ reduction of nitrate from plant sources and its effects on the overall quality and safety of restructured cooked ham. *Meat Science*, 109572.

European Commission. (2011). Regulation (EC) no 1129/2011 of the European Parliament and of the council of 12 November 2011 amending annex II to regulation (EC) no 1333/2008 of the European Parliament and of the council by establishing a union list of food additives. Official Journal of the European Union, L295, 1.

Flores, M., & Toldrá, F. (2021). Chemistry, safety, and regulatory considerations in the use of nitrite and nitrate from natural origin in meat products - Invited review. *Meat Science*, *171*, 108272. https://doi.org/10.1016/J.MEATSCI.2020.108272

Ghabraie, M., Vu, K. D., Huq, T., Khan, A., & Lacroix, M. (2016). Antilisterial effects of antibacterial formulations containing essential oils, nisin, nitrite and organic acid salts in a sausage model. *Journal of Food Science and Technology*, *53*(6), 2625–2633. https://doi.org/10.1007/s13197-016-2232-x

Honikel, K.-O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, *78*(1–2), 68–76. https://doi.org/10.1016/j.meatsci.2007.05.030

Jin, S.-K., Choi, J. S., Yang, H.-S., Park, T.-S., & Yim, D.-G. (2018). Natural curing agents as nitrite alternatives and their effects on the physicochemical, microbiological properties and sensory evaluation of sausages during storage. *Meat Science*, *146*, 34–40. https://doi.org/10.1016/j.meatsci.2018.07.032

Kim, G. H., & Chin, K. B. (2021). Evaluation of quality characteristics of low-nitrite pork sausages with paprika oleoresin solution during refrigerated storage. *Food Science of Animal Resources*, *41*(3), 428–439. https://doi.org/10.5851/KOSFA.2021.E8

Lotfi, A., & Bryan, N. S. (2024). Role of sodium nitrite curing of meat in curing human disease. *Encyclopedia of Meat Sciences*, 453–460. https://doi.org/10.1016/B978-0-323-85125-1.00006-5

Melios, S., Grasso, S., Bolton, D., & Crofton, E. (2024). Sensory quality and consumer perception of reduced/free-from nitrates/nitrites cured meats. *Current Opinion in Food Science*, *58*, 101183. https://doi.org/10.1016/J.COFS.2024.101183

Premi, L., Rocchetti, G., Lucini, L., Morelli, L., & Rebecchi, A. (2024). Replacement of nitrates and nitrites in meat-derived foods through the utilization of coagulase-negative staphylococci: A review. *Current Research in Food Science*, *8*, 100731. https://doi.org/10.1016/J.CRFS.2024.100731

Shakil, M. H., Trisha, A. T., Rahman, M., Talukdar, S., Kobun, R., Huda, N., & Zzaman, W. (2022). Nitrites in cured meats, health risk issues, alternatives to nitrites: A review. *Foods*, *11*(21), 3355.

Šojić, B., Pavlić, B., Ikonić, P., Tomović, V., Ikonić, B., Zeković, Z., Kocić-Tanackov, S., Jokanović, M., Škaljac, S., & Ivić, M. (2019). Coriander essential oil as natural food additive improves quality and safety of cooked pork sausages with different nitrite levels. *Meat Science*, *157*. https://doi.org/10.1016/j.meatsci.2019.107879

Šojić, B., Pavlić, B., Tomović, V., Kocić-Tanackov, S., Đurović, S., Zeković, Z., Belović, M., Torbica, A., Jokanović, M., Urumović, N., Ivić, M., & Škaljac, S. (2020). Tomato pomace extract and organic peppermint essential oil as effective sodium nitrite replacement in cooked pork sausages. *Food Chemistry*, *330*. https://doi.org/10.1016/j.foodchem.2020.127202

Vafania, B., Fathi, M., & Soleimanian-Zad, S. (2019). Nanoencapsulation of thyme essential oil in chitosan-gelatin nanofibers by nozzle-less electrospinning and their application to reduce nitrite in sausages. *Food and Bioproducts Processing*, *116*, 240–248. https://doi.org/10.1016/J.FBP.2019.06.001

van Breda, S. G., Mathijs, K., Sági-Kiss, V., Kuhnle, G. G., Van der Veer, B., Jones, R. R., Sinha, R., Ward, M. H., & de Kok, T. M. (2019). Impact of high drinking water nitrate levels on the endogenous formation of apparent N-nitroso compounds in combination with meat intake in healthy volunteers. *Environmental*

Health, *18*, 1–12.
Zhang, Y., Zhang, Y., Jia, J., Peng, H., Qian, Q., Pan, Z., & Liu, D. (2023). Nitrite and nitrate in meat processing: Functions and alternatives. *Current Research in Food Science*, *6*, 100470. https://doi.org/10.1016/J.CRFS.2023.100470

DETERMINATION OF OPTIMAL QUANTITY OF TOMATO BY-PRODUCT IN FERMENTED CRACKERS USING MATLAB – A SUSTAINABLE APPROACH TO AGRICULTURAL WASTE VALORIZATION

Gjore Nakov[1]*, Zlatin Zlatev[2], Marko Jukić[3], Ivan Dimov[1], Jasmina Lukinac[3]

[1]College of Sliven, Technical University of Sofia, 59 Burgasko Shose Blvd, Sliven, Bulgaria
[2]Faculty of Technics and Technologies, Trakia University, 8 Graf Ignatiev Street, 8602, Yambol, Bulgaria
[3]Faculty of Food Technology Osijek, Josip Juraj Strossmayer University of Osijek, 20 Franje Kuhača Street, 1000 Osijek, Croatia
[4]Vocational School of Tourism "Prof. Dr. Asen Zlatarov", 53, Stefan Stambolov Blvd, Burgas, Bulgaria

*Corresponding author:
E-mail address: gnakov@tu-sofia.bg

ABSTRACT

The use of certain waste products in the production of crackers can lead to improved properties and a higher nutrient content. However, the optimal amount of additives to replace basic ingredients is still uncertain. The present work aims to determine the optimal amount of tomato pomace in crackers. The crackers were developed using waste from tomato processing and their physical, chemical, and sensory properties were evaluated. The study used a selection method to select relevant characteristics for regression analysis and a second-order polynomial model. A linear programming algorithm was used to determine the optimal amount of the additive. The results of the study show that the addition of 9.27% tomato pomace to crackers improves their properties and remains acceptable to consumers. The optimal amount determined in the study is higher than the amount indicated in the literature, which allows the addition of 8% tomato pomace in crackers. The higher optimal amount identified in this work can help to replace more of the main raw materials in the production of crackers.
Keywords*: Food waste, Sustainability, Statistical analysis, Optimal quantity, Tomato pomace*

INTRODUCTION

The crackers, together with salted bread, grits, pretzels, etc., belong to the group of dry bakery products with a moisture content of 4 to 9% (Chonova & Karadzhov, 2014). According to Manley, 2011), there are two types of crackers: unsweetened (fermented and unfermented) and lightly sweetened (modified with enzymes).
The crackers are characterized by their dry texture, crunchiness and perforated surface, which makes them a pleasant snack. A major disadvantage of crackers is their relatively high energy value and low nutritional value. Some potentially harmful compounds are present in these products, including acrylamide, which is formed at high temperatures and low humidity during baking in the presence of reducing sugars and some amino acids such as asparagine. In recent years, there has been an increased interest in adding functional ingredients to various types of bakery products such as crackers, cookies and bread. Active ingredients contained in functional foods that are expected to have a positive effect on health should be consumed in an amount that align with daily food intake standards (Salem et al., 2020). In addition, functional foods should have the same form as everyday/normal foods, i.e. they should not be in tablet or capsule form (Siró et al., 2008). The food industry generates millions of tons of waste worldwide, which can be used as a source of important components such as proteins, dietary fibers, polysaccharides, aromatic compounds or various other phytochemicals. These biologically active substances can be a functional components in food, pharmaceutical, cosmetic or other products (Lauková et al., 2016; Quiles et al., 2018). The use of waste products from food production in the production of crackers leads to the reduction of waste and the creation of new products enriched with bioactive compounds.
According to Roy et al. (2023), of the total amount of waste products from food production worldwide, those from fruits are the largest compared to the other waste raw materials. Research on their use in bakery products is less affected by this.

According to FAOSTAT (2017), the annual production of fresh tomatoes is about 242 million tons (Campos et al., 2020). After processing fresh tomatoes into tomato juice, tomato paste or other tomato products, the waste, commonly referred to as pomace, contains skins/husks, seeds and a smaller amount of connective tissue (Zuorro et al., 2011).
Tomato pomace is used in various bakery products such as crackers (Ahmad Bhat & Ahsan, 2016; Akubor & Owuse, 2020; Isik & Topkaya, 2016; Tomic et al., 2016) and bread (Nour et al., 2018). According to the results reported in the cited literature sources, the addition of tomato pomace at 8% in crackers is acceptable to consumers. The content of protein, ash, fiber, minerals, total phenols, antioxidant capacity and the color of a and b values of the Lab color model are increased. The use of tomato pomace in biscuits up to 15% leads to changes in expansion factor, hardness and color. The investigated influence of 5 to 30% tomato pomace in bread shows that higher levels of the additive (above 10%) have a negative influence on the sensory properties, increase the mass and hardness of the bread and reduce the specific volume. Silva et al. (2023) in their review on the use of tomato pomace in bakery products pointed out that the addition of different amounts of tomato pomace in these products has a significant impact on their properties. However, it is still necessary to determine the optimal amount of the additive to minimize the impact on the properties of bakery products while ensuring the positive effects of using this waste raw material. These positive effects include enriching bakery products with fiber and improving their antioxidant properties (Ahmad Bhat and Ahsan, 2016).
Similarly, Akubor and Owuse (2020) found that the use of tomato pomace can lower the overall calorie content of the product without compromising its texture and taste. Isik and Topkaya (2016) recommended an addition of 8% tomato pomace as optimal to maintain consumer acceptance, while Tomic et al. (2016) pointed out that higher additions (up to 15%) could negatively affect the sensory properties of crackers.
However, most of these studies focused on relatively low addition levels and there is only a limited amount of research investigating the potential benefits and challenges associated with higher levels of tomato pomace. In their review, Silva et al. (2023) emphasize the need for further research to determine the optimal amount of addition that balances nutritional benefits with maintaining sensory acceptability.
Given the global increase in fresh tomato production and the generation of significant amounts of waste, it is important to find efficient ways to utilize it. This study aims to fill the gap in the existing literature by determining the optimal amount of tomato pomace in crackers that maximizes nutritional value without compromising product quality.

MATERIAL AND METHODS

Materials

Source data for tomato pomace crackers and the mixtures used for their production are available in Nakov et al. (2022). The formulation, technology and methods of product analysis are available in the indicated publication.

Methods

The technological characteristics of the products were used in this development.
Stylized images were created using Inkscape ver. 1.0.1 (https://inkscape.org, accessed 12.07.2024), which is an Open Source Scalable Vector Graphics Editor. Cracker images are created in vector SVG file format and colored with the averaged color for the respective sample. For preview in this work, they are converted to bitmap PNG file format.
The methodology for determining the optimum amount of tomato pomace to add to crackers consists of the following steps:
The color digital images of the crackers were used. They were obtained using a video sensor of a mobile phone model LG L70 (LG Electronics, Inc., Seoul, Korea). The video sensor is a VB6955CM (STMicroelectronics International N.V., Geneva, Switzerland). Resolution: 2600x1952 pixels. Pixel size: 1,4x1,4 μm. The images were taken from a height of 20 cm. Homogeneous lighting is provided by a white dome-shaped part with installed white LEDs,

with the greatest intensity of emitted light at 450 nm. Thus, the images of the crackers were obtained under the same conditions and are comparable.
Color digital images were obtained in the RGB color model, which were converted to the Lab color model, according to CIE 1976. Color component conversion functions were used at observer 2^{o} and illuminance D65.
For the color analysis of the crackers with added tomato pomace, a total of 16 color index values (L, a, b, C, h, YI, WI, BI, SI, CIRG, COL, CI, ECB, FCI, WL, PACI) were calculated according to the method of Pathare et al. (2012). These indices provide a detailed insight into the changes in the visual properties of the product, which are decisive for consumer acceptance.
The color indices were used according to the color changes they reflect. YI measures the degree of yellowness. WI quantifies how close an object's color is to white. BI measures object browning. SI represents the saturation or intensity of the color. CIRG shows changes in green color. COL reflects illuminance or brightness level. CI quantifies the chromaticity of an object, specifically the ratio of green-red (a) to blue-yellow (b). The ECB evaluates the balance between chrominance and brightness. FCI reflects the degree of fading or loss of color. WL evaluates the level of whiteness. PACI quantifies changes in the green color of an object.
A data set containing a total of 40 features describing the change in the characteristics of crackers at different amounts of added tomato pomace was compiled. Table 1 lists these features.
Data collection: a vector was compiled comprising the physicochemical properties, antioxidant activity, organoleptic data and color characteristics of crackers with different amounts of tomato pomace.
Selection of characteristics: The RReliefF method was applied to select the traits that best described the changes in cracker properties at different addition levels. Traits with a weighting coefficient above 0.6 were selected to reduce the dimensionality of the data and focus the analysis on the most important parameters (Vasilev et al., 2021).
Dimensionality reduction: Principal component analysis (PCA) was used to reduce the number of variables in the feature vector to two principal components that explain over 95% of the variance in the data. This facilitates the creation of more accurate regression models.
Development of a regression model: A second-order model (polynomial regression) was developed to describe the relationship between the reduced data set and the amount of tomato pomace (Georgieva et al., 2020). The model was evaluated using the coefficient of determination (R^2), the sum of squared errors (SSE) and the root mean square error (RMSE) to assess its accuracy.
Determining the optimal amount to add: A linear programming algorithm implemented in Matlab using the "linprog" function was used to determine the optimal amount of tomato pomace addition that maximizes positive product attributes while maintaining sensory acceptability (Yang & Yamashita, 2018).

Table 1. Features of Crackers with Tomato Pomace and the Mixtures Used in Their Production

F1	Peak Viscosity	F11	Snapping Force	F21	Aroma	F31	WI
F2	Breakdown	F12	Moisture	F22	Taste	F32	BI
F3	Final viscosity	F13	Lipids	F23	Odor	F33	SI
F4	Pasting Temperature	F14	Proteins	F24	OA	F34	CIRG
F5	Width	F15	Ash	F25	L	F35	COL
F6	Thickness	F16	Water Activity	F26	a	F36	CI
F7	Spread factor	F17	Total fibre	F27	b	F37	ECB
F8	Volume	F18	ABTS	F28	C	F38	FCI
F9	Specific volume	F19	Appearance	F29	h	F39	WL
F10	Hardness	F20	Texture	F30	YI	F40	PACI

The data obtained were processed using the Matlab program system (The Mathworks Inc., Natick, MA, USA.). All data were processed with a significance level of α=0.05.

RESULTS AND DISCUSSION

Figure 1 shows in a general stylized form, by their averaged colors, the crackers with the addition of tomato pomace. As the amount of additive increases, the color of the products changes from light to darker.

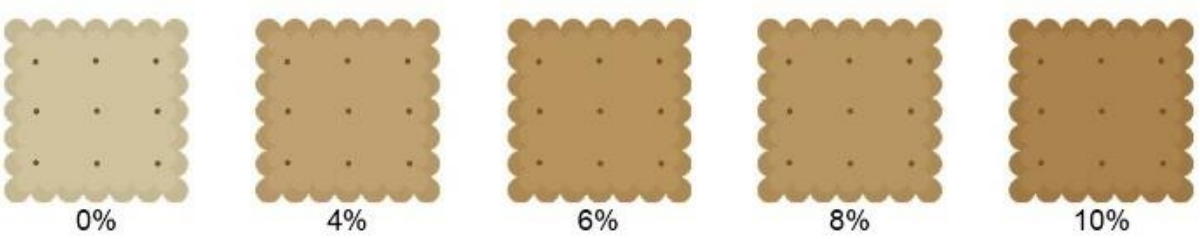


Figure 1. Crackers with tomato pomace – general view

Figure 2 shows the distribution of the component values from the laboratory model for crackers with tomato presses. The control sample differed significantly from the samples with the addition of tomato pomace. The 4% sample also stands out from the others. Increasing the amount of tomato pomace leads to an overlap of the experimental data.

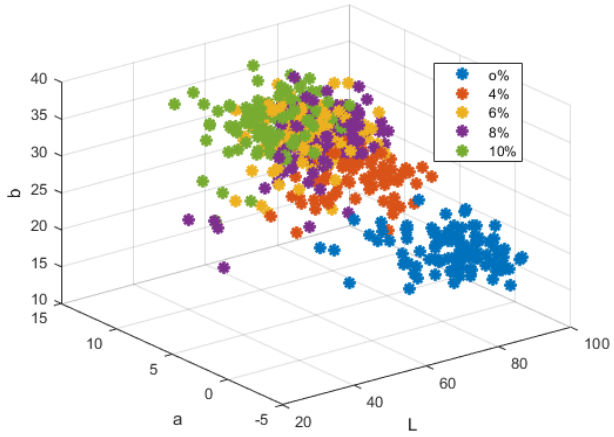


Figure 2. Distribution of Lab values for crackers with tomato pomace

Table 2 shows the color index values for pressed tomato crackers. Components L and h decrease as the amount of tomato pomace increases. Components a, b and C increase their values. Of the color indices, the values for SI, CIRG, COL, CI, ECB and PACI increase. The values for WI, BI, FCI and WL decrease.

Table 2. Color indices of crackers with tomato pomace (TP). The statistical significant difference is at p<0,05

TP / Index	0%	4%	6%	8%	10%
L	78,83±8,88	68,15±6,27	63,01±6,67	63,99±8,68	57,39±6,78
a	1,21±0,66	4,55±1,59	6,82±1,78	5,98±1,36	8,19±1,76
b	20,47±2,46	28,83±2,25	32,93±2,62	32,52±3,21	34,19±2,52
C	4,4±0,34	5,77±0,3	6,3±0,3	6,2±0,31	6,5±0,26
h	1,51±0,04	1,42±0,05	1,37±0,05	1,39±0,04	1,34±0,05
YI	37,85±7,97	60,86±6,46	75,17±6,96	73,35±7,4	85,78±7,34
WI	70±7,35	56,52±4,71	49,67±4,49	50,56±5,66	44,45±4,46
BI	190,83±55,17	74,41±16,3	56,6±11,22	61,01±11,91	46,66±8,62
SI	20,52±2,44	29,23±2,37	33,67±2,73	33,09±3,21	35,19±2,57
CIRG	2,2±0,25	2,43±0,22	2,6±0,27	2,6±0,44	2,83±0,33
COL	7,06±3,84	23,39±8,36	34,86±9,94	31,17±10,55	44,7±11,83
CI	0,06±0,04	0,16±0,05	0,21±0,05	0,18±0,04	0,24±0,05

ECB	0,08±0,04	0,22±0,08	0,32±0,08	0,28±0,07	0,39±0,09
FCI	58,36±10,1	39,32±6,15	30,08±5,72	31,48±6,66	23,21±5,33
WL	3,93±0,76	2,37±0,26	1,92±0,18	1,97±0,19	1,68±0,14
PACI	15,4±7,9	66,87±25,78	107,96±33	94,19±29,83	142,28±39,26

A selection of meaningful characteristics was made to describe the changes in the crackers when different amounts of tomato pomace were added. Figure 3 graphically shows the result of this selection. Those characteristics with weight coefficients above 0.6 are taken into account. 9 technological characteristics were selected, all components from the color models Lab and LCh as well as 9 color indices.

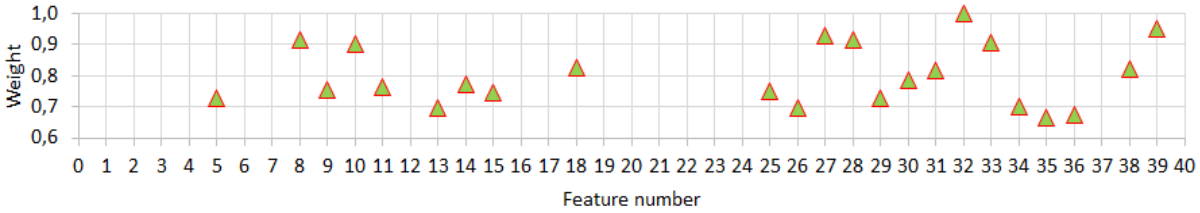


Figure 3. Results from feature selection

The following vector of 23 features was selected using the RReliefF method (Eq. 1):

$$FV = [F5 \cdot F8 \cdot F9 \cdot F10 \cdot F11 \cdot F13 \cdot F14 \cdot F15 \cdot F18 \cdot F25 \cdot F26 \cdot F27 \cdot F28 \cdot F29 \cdot F30 \cdot F31 \cdot F32 \cdot F33 \cdot F34 \cdot F35 \cdot F36 \cdot F38 \cdot F39] \quad (1)$$

The data of the feature vector are reduced to two principal components. They describe over 95% of the variance in the selected vector data.
The following regression model was determined (Eq. 2):

$$TP = 5.21 + 0.77PC_2 + 0.001PC^2{}_2 - 0.05PC^2{}_2 \quad (2)$$

The evaluation of the obtained model shows that the coefficient of determination is R^2=0.84. The sum of squared errors is SSE=0.19. The mean squared error is 0.44. This shows that the obtained model describes the experimental data with sufficient accuracy.
Figure 4 shows the resulting model in graphical form. The optimum percentage for the addition of tomato pomace in crackers was determined. From the calculations and analysis using the Linpprog method, it was found that 9.27% tomato pomace in crackers was acceptable to consumers and did not significantly change the physicochemical, geometric and color properties of the product.

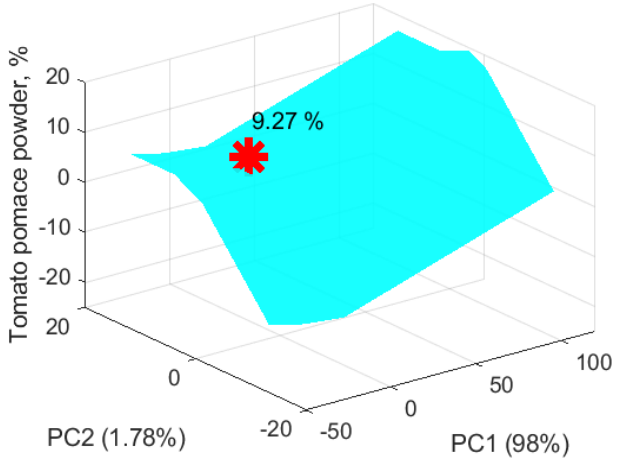


Figure 4. Determining the appropriate amount of tomato pomace in crackers

Statistical data analysis methods such as principal component analysis and linear programming algorithm were used in determining the optimal amount of tomato pomace in crackers.

The results of this study indicate that the optimal amount of tomato pomace addition in crackers is 9.27%, which is significantly higher than the 8% previously recommended by Nakov et al. (2022) and Isik & Topkaya (2016). This difference may be due to different methods, raw material compositions or sensory criteria used in our study. The increased addition amount resulted in improved nutritional properties of the product, including an increase in fiber content, proteins, and antioxidant compounds, which is consistent with the findings of Ahmad Bhat and Ahsan (2016).

However, it is important to note that higher levels of additives may affect the sensory acceptability of the product. While our results showed that an addition of 9.27% remains acceptable to consumers, there is a risk of reduction in texture and color changes that may affect the market success of the product. Silva et al. (2023) highlighted the importance of balancing nutritional benefits with maintaining sensory properties, a point that is further reinforced by our findings.

The improved optimal addition rate enables producers to increase the proportion of tomato pomace in production, thereby reducing raw material costs and contributing to sustainable production. In addition, crackers with an enriched nutritional profile can be used as a healthy snack by all age groups.

CONCLUSIONS

In this study, additional research was conducted to determine the optimal amount of tomato pomace in crackers. To achieve this goal, appropriate statistical methods were used to analyze the data. The analysis revealed the ideal amount of tomato pomace and showed that the addition of 9.27% improved the key properties of the crackers while maintaining consumer acceptance. A total of 40 properties were investigated, covering the physicochemical, sensory and color changes of crackers with added tomato pomace. Of these, 23 were considered sufficiently informative, including 9 technological characteristics, 6 color components and 9 color indices. The results of this study provide a useful method to explore how waste products from food production can be reused to promote sustainability and ensure food health.

ACKNOWLEDGEMENTS

This work was financially supported by the Bulgarian Ministry of Education and Science, National Research Fund under contract number: КП-06-КОСТ/20.

REFERENCES

Ahmad Bhat, M., & Ahsan, H. (2016). Physico-Chemical Characteristics of Cookies Prepared with Tomato Pomace Powder. *Journal of Food Processing & Technology*, 07(01).

Akubor, P. I., & Owuse, A. U. (2020). Chemical Composition, functional and biscuit making properties of tomato peel flour. *South Asian Journal of Food Technology and Environment,* 06(01), 874–884.

Campos, D. A., Gómez-García, R., Vilas-Boas, A. A., Madureira, A. R., & Pintado, M. M. (2020). Management of Fruit Industrial By-Products—A Case Study on Circular Economy Approach. *Molecules*, Vol. 25, Page 320, 25(2), 320.

Chonova V., & Karadzhov G. (2014). Technology of bakery and confectionery products. *Academic publishing house of UHT - Plovdiv*.

Georgieva, T., Mihaylova, A., & Daskalov, P. (2020). Research of the possibilities for determination of some basic soil properties using image processing. 2020 In: *7th International Conference on Energy Efficiency and Agricultural Engineering (EE&AE)*, 1–4. 12"-14" of November, Ruse, Bulgaria.

Isik, F., & Topkaya, C. (2016). Effects of tomato pomace supplementation. Italian *Journal of Food Science*, 28(3), 525–535.

Lauková, M., Kohajdová, Z., & Karovičová, J. (2016). Effect of hydrated apple powder on dough rheology and cookies quality. *Potravinarstvo Slovak Journal of Food Sciences*, 10(1), 506–511.
Manley, D. (2011). Technology of Biscuits, Crackers and Cookies.
Nakov, G., Brandolini, A., Estivi, L., Bertuglia, K., Ivanova, N., Jukić, M., Komlenić, D. K., Lukinac, J., & Hidalgo, A. (2022). Effect of Tomato Pomace Addition on Chemical, Technological, Nutritional, and Sensorial Properties of Cream Crackers. *Antioxidants*, 11(11), 2087.
Nour, P., Ropota, T. & Trandafir C.. (2018). Nutritional and bioactive compounds in dried tomato processing waste. *CyTA - Journal of Food*, 16(1), 222–229.
Pathare, P. B., Opara, U. L., & Al-Said, F. A. J. (2012). Colour Measurement and Analysis in Fresh and Processed Foods: A Review. *Food and Bioprocess Technology* 6:1, 6(1), 36–60.
Quiles, A., Campbell, G. M., Struck, S., Rohm, H., & Hernando, I. (2018). Fiber from fruit pomace: A review of applications in cereal-based products. *Food Reviews International*, 34(2), 162–181.
Roy, P., Mohanty, A. K., Dick, P., & Misra, M. (2023). A Review on the Challenges and Choices for Food Waste Valorization: Environmental and Economic Impacts. *ACS Environmental Au*, 3(2), 58–75.
Salem, B. R., El-Sahy, K. M., Sulieman, A. M., & Gouda, M. R. (2020). Use of tomato pomace, mango seeds kernel and pomegranate peels powders for the production of functional biscuits. *Zagazig Journal of Agricultural Research*, 47(4), 1011–1023.
Silva, F. D., Garcia, V. A. dos S., Vanin, F. M., Yoshida, C. M. P., & Carvalho, R. A. de. (2023). Application of Tomato Byproduct in Food Products-A Review. *Food Science and Engineering*, 103–115.
Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance--a review. *Appetite*, 51(3), 456–467.
Tomic, J., Belovic, M., Torbica, A., Pajin, B., Loncarevic, I., Petrovic, J., & Fistes, A. (2016). The influence of addition of dried tomato pomace on the physical and sensory properties of whole grain rye flour cookies. *Food and Feed Research*, 43(2), 145–152.
Vasilev, M. D., Shivacheva, G. I., & Krastev, K. I. (2021). Predicting the day of storage of dairy products by data combination. *IOP Conference Series: Materials Science and Engineering*, 1031(1), 012056.
Yang, Y., & Yamashita, M. (2018). An arc-search O(nL) infeasible-interior-point algorithm for linear programming. *Optimization Letters*, 12(4), 781–798.
Zuorro, A., Fidaleo, M., & Lavecchia, R. (2011). Enzyme-assisted extraction of lycopene from tomato processing waste. *Enzyme and Microbial Technology*, 49(6–7), 567–573.

NUTRITIVE PROFILE EVALUATION OF THE CELERY DEHYDRATED BY DIFFERENT METHODS

Milenko Košutić[1], Jelena Filipović[1], Milica Nićetin[2], Vladimir Filipović[2], Ivica Đalović[3], Dragan Živančev[3]
[1]Institute of Food Technology in Novi Sad, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[3]Institute of Field and Vegetable Crops, National Institute of the Republic of Serbia, Maksima Gorkog 30, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: milenko.kosutic@fins.uns.ac.rs

ABSTRACT

Celery (*Apium graveolens* L.) is an aromatic vegetable, a member of the Apiaceae family. Celery is a globally cultivated vegetable. Celery root is rich in dietary fibre, mineral elements, vitamins and essential oils and, as such, has health benefits. Celery root has few calories, it can be consumed raw or treated.There are different dehydration methods used to preserve plant material, such as convective, lyophilisation and combined dehydration methods. Convective dehydration is characterized by a high temperature which influences the sensory and nutritional characteristics of the product. The lyophilisation method preserves the nutritional and sensory characteristics of products, but it involves increased process costs and longer dehydration times. The combined dehydration method consists of osmotic dehydration in molasses and lyophilisation as a successive dehydration phase. This study aims to investigate the effect of different dehydration methods on the chemical and mineral composition of the celery root. The significant differences between different methods of draying of celery root are confirmed by the application of post-hoc Tukey's HSD test at a 95% confidence limit. The calculation of Z-Score Analysis, based on chemical and mineral parameters, points out the best score of celery root dehydration by the combined method, obtaining 91.67%, while celery lyophilisation and convective dehydration obtained only 15.67% and 7.60% of maximal score values, respectively.
Keywords*: dehydration methods, chemical composition, mineral composition, celery root*

INTRODUCTION

The *Apiaceae* family, also known as the *Umbelliferae* family, is a significant and diverse family of flowering plants. It encompasses approximately 3780 species distributed across 434 genera. This family is globally widespread, with members commonly found in tropical high-altitude regions and the northern temperate zones. Onions, celery, and kale are rich sources of various health-promoting compounds. Scientific research has highlighted several bioactive compounds in these vegetables, contributing to their health benefits, particularly their antioxidant properties. A good deal of scientific articles have found that onions, celery, and kale are good sources of health-promoting compounds, i.e. apiin, apigenin, rutin, oleic acid, pantothenic acid, α-linolenic acid, succinate, vitamin E (Liu et al, 2020) glycosides of quercetin, kaempferol, coumaric acid, ferulic acid, sinapic acid, coffee acid (Olsen, Aaby & Borge, 2009), and have high antioxidant capacity (Liu et al., 2020, Kręcisz et. al 2023).
Celery (*Apium graveolens* L.) is an aromatic vegetable, a member of the family Apiaceae. Celery is a globally cultivated vegetable with three botanical varieties: *var. Rapaceum*, known as 'celeriac' with a large root tuber (popular in Europe); *var dulce*, forming a crisp stalk (popular in the USA and Western Europe); and *var. secalinum* (Asia) (Bruznican, De Clercq, Eeckhaut, Van Huylenbroeck, & Geelen, 2020). Celery root which has low calories can be consumed either raw or cooked (Kian-Pour 2023). In recent times, there is a growing emphasis on enhancing the utilization of secondary products from the food industry, such as sugar beet molasses. These by-products are valuable sources of natural antioxidants, minerals, and other functional ingredients. They can be effectively used to enrich food

products, thereby increasing their nutritional value and contributing to more sustainable food production practices.
Besides changes in the attractiveness and quality of food after dehydration, another major disadvantage of dehydration in the food industry is its high cost (Baysan et al. 2024).
In every technological process, it is very important to accurately define the production parameters of the process to achieve a good and consistent product quality while minimizing the loss of nutritional and functional properties of the raw materials (Košutić 2016, Košutić et all 2016), where combined dehydration method of peach was characterized by upgraded overall dehydration effectiveness, reduced time and energy consumption, and enhanced chemical and mineral matter content of dehydrated peach samples (Filipović et al., 2022a), indicating the research path for the celery root dehydration.
This study aims to investigate the effect of different dehydration methods on the chemical and mineral composition of the celery root. The findings are expected to guide the food processing industry in selecting the best dehydration technique to produce high-quality dried celery root with maximum health benefits and desirable physical properties.

MATERIAL AND METHODS

Material

Fresh celery root (Apium graveolens L. var. rapaceum, Alabaster variety) was sourced from a local greengrocery in Novi Sad, Serbia. The celery root had an average dry matter content of 9.05%. Sugar beet molasses, used as an osmotic solution in the osmotic pre-treatment process, was obtained from a sugar factory in Crvenka, Serbia, and had an average dry matter content of 86.04%.

Convective Dehydration

Diced celery samples were subjected to convective dehydration using the following procedure: **Dehydration Process**: The samples were dried to a constant mass in a dryer (Instrumentaria, Zagreb, Croatia) set at 50°C (Kręcisz et al., 2023; Marić et al., 2020).
Grinding: Once dehydrated, the samples were pulverized using a universal laboratory mill, type: WZ-1 (solem, ZBPP, Bydgoszcz, Poland), to obtain convectively dried celery root powder.

Lyophilisation

Fresh samples of diced celery were processed using the following lyophilisation procedure:
Freezing: The diced celery samples were frozen for at least 24 hours at -30°C.
Lyophilisation: The frozen samples were placed on metal trays in a freeze-dryer (Christ ALPHA1-2 LDPLUS, Osterode am Harz, Germany). The lyophilisation parameters were set as follows: absolute pressure: 1.6 Pa, condenser temperature: -57°C, duration: 48 hours. Duration of the lyophilization process was determined on the basis of previous tests, where the chosen process duration produced dehydrated products without water content.
Grinding: After lyophilisation, the samples were ground using a universal laboratory milltype: WZ-1 (solem, ZBPP, Bydgoszcz, Poland).

Combined Method of Dehydration

The combined method of dehydrating celery root was executed in two stages: osmotic dehydration followed by lyophilisation. Here are the detailed steps:
Preparation of Celery Root: fresh celery root was washed with running tap water, dried with paper towels, peeled, and cut into cubes approximately 1 cm x 1 cm x 1 cm.
Osmotic Dehydration: the celery cubes were immersed in vessels filled with molasses (molasses content as described in Lončar et al., (2021)) at a ratio of 1:5 (dehydrating material to osmotic solution) to prevent excessive dilution of the molasses and slow down the process kinetics. This process took place over 5 hours at atmospheric pressure in a

thermostatic chamber (Memmert IN160, Schwabach, Germany), with the temperature set and maintained at 20°C.
Post-Osmotic Treatment: after 5 hours, the osmotically treated celery samples were separated from the molasses and washed with running water to remove excess solution from the surface of the cubes. The samples were then blotted with paper towels to remove excessive water.
Freezing: the osmotically dehydrated celery samples were frozen and stored at -30°C for 24 hours.
Lyophilisation: the frozen samples were subjected to lyophilisation using the device Christ ALPHA1-2 LDPLUS (Osterode am Harz, Germany). The lyophilisation parameters were set to a absolute pressure of 1.6 Pa, a condenser temperature of -57°C, and process duration of 24 hours.
Post-Lyophilisation Processing: after lyophilisation, the dehydrated samples were finely ground into a powder of uniform particle size using a universal laboratory mill, type: WZ-1 (solem, ZBPP, Bydgoszcz, Poland).

Chemical analysis
Proximate chemical composition of cookie samples was conducted according to AOAC (Horowitz, 2019) standard methods: protein content (method No. 950.36), starch content (method No. 996.11), total sugars content (method No. 2020.07), cellulose content (method No. 973.18), lipid content (method No. 935.38) and ash content (method No. 930.22). Each measurement was performed in three replications.

Minerals analysis
The mineral content of potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn) and copper (Cu) of the cookies was determined in accordance to the standard methods of AOAC (Horowitz, 2019). Minerals were determined by atomic absorption spectrophotometry (method No. 984.27) on a Varian Spectra AA 10 (Varian Techtron Pty Ltd., Mulgvare Victoria, Australia). Each measurement was performed in three repetitions.

Methods of Statistical Analysis
Analysis of Variance
Analysis of variance (ANOVA) was applied in order of determinating the variations statistical significance on the set of all cookies' samples tested quality responses. ANOVA analysis was performed by using STATISTICA 12.0 software (2013), (StatSoft Europe, Hamburg, Germany).

Z-Score Analysis
In the Z-Score analysis min-max normalization is used for samples' different response values. They are recalculated and presented in a new dimensionless unit system, with the effort of comparisons and further mathematical calculations of different cookie samples' quality responses (Szarek et al., 2024).
The maximal obtained value of total Z-score values indicates on the optimum value of all segment Z-scores mathematically combined in defined manner, pointing at the optimal combination of all tested quality parameters.
The calculation of individual segment Z-scores is as following:
Cookie samples' technological quality segment Z-score:

$$S_{1i} = \frac{\sum_{k=1}^{12}\left(\frac{x_{ki}-x_{kmin}}{x_{kmax}-x_{kmin}}\right)}{12} \quad (1)$$

where x_k are: Proteins, Starch, Total sugars, Celullose, Lipids, Ash, Zn, Cu, Mg, Ca, Fe and K.
max $[S_i] \rightarrow$ optimum (2)

Z-score values were calculated using Microsoft Excel ver. 2016. (Microsoft Corporation, Redmond, WA, USA).

RESULTS AND DISCUSSION

Tables 1 and 2 present the results of the nutritive composition of celery dehydrated by different methods: convective dehydration, lyophilization, and a combined method (osmotic dehydration and lyophilization).
From the results presented in Table 1, it can be seen that celery dried by the combined method (O.D.+L.) has a statistically significantly higher content of proteins, total sugars, and ash, but a lower content of cellulose and lipids compared to celery samples dried by the convective method (C.D.) and lyophilization (L.).

Table 1. Dehydrated celery root chemical composition

Sample:	Proteins (% d.m.)	Starch (% d.m.)	Total sugars (% d.m.)	Cellulose (% d.m.)	Lipids (% d.m.)	Ash (% d.m.)
C.D.*	1.04 ± 0.04^{a}	0.85 ± 0.08^{a}	5.25 ± 0.15^{a}	2.64 ± 0.09^{b}	0.30 ± 0.01^{a}	0.81 ± 0.03^{a}
L.**	1.31 ± 0.05^{b}	1.01 ± 0.05^{b}	5.31 ± 0.09^{a}	2.71 ± 0.05^{b}	0.30 ± 0.01^{a}	0.79 ± 0.02^{a}
O.D.+L.***	4.36 ± 0.19^{c}	1.04 ± 0.03^{b}	24.99 ± 0.99^{b}	1.57 ± 0.04^{a}	0.26 ± 0.00^{a}	4.97 ± 0.49^{b}

* Convectively dehydrated, pulverized, celery root
** Lyophilizated, pulverized, celery root
*** Osmotically dehydrated and lyophilizated, pulverized, celery root
Results are shown as average value ± standard deviation of six replications
$^{a–c}$ Different letters in superscript of the same table column indicate the statistically significant difference between values at a level of significance of $p < 0.05$ (based on post-hoc Tukey HSD test)

The mineral composition of celery dehydrated by different methods is presented in Table 2. Celery dehydrated by the combined method (O.D.+L.) has a statistically significantly higher mineral content in terms of K, Mg, Ca, Fe, Zn, and Cu compared to celery dehydrated by the convective method and lyophilization. During osmotic dehydration stage of the combined method, secondary mass transfer – uptake of the solid from the osmotic solution (sugar beet molasses) in the dehydrating material (celery root) occurs, affecting the change of chemical and mineral content of celery root dry matter (Filipović et al., 2022b). Obtained results in this research also shows the increase of mineral matter content due to dry matter increase from the mineral-rich molasses during the osmotic dehydration stage of the process.

Table 2. Dehydrated celery root mineral content

Sample:	K (mg/100g d.m.)	Mg (mg/100 g d.m.)	Ca (mg/100 g d.m.)	Fe (mg/100 g d.m.)	Zn (mg/100 g d.m.)	Cu (mg/100 g d.m.)
C.D.	308.43 ± 0.94^{a}	23.13 ± 0.11^{a}	73.08 ± 0.21^{a}	0.78 ± 0.03^{a}	0.71 ± 0.02^{a}	0.51 ± 0.02^{a}
L.	309.75 ± 1.05^{a}	23.94 ± 0.24^{a}	73.94 ± 0.34^{a}	0.79 ± 0.02^{a}	0.74 ± 0.04^{ab}	0.52 ± 0.00^{a}
O.D.+L.	1825.74 ± 20.16^{b}	42.74 ± 1.75^{b}	120.49 ± 1.55^{b}	5.39 ± 0.75^{b}	1.35 ± 0.15^{b}	0.84 ± 0.03^{b}

Results are shown as average value ± standard deviation of six replications
$^{a–c}$ Different letters in superscript of the same table column indicate the statistically significant difference between values at a level of significance of $p < 0.05$ (based on post-hoc Tukey HSD test)

Table 3. Z-Score values of dehydrated celery root

Sample:	Z-Score values (%)
C.D.	7.60
L.	15.66
O.D.+L.	91.67

The results of Z-Score analysis, table 3, showed that convectively dehydrated, lyophilized and combined dehydrated celery root obtained: 7.60, 15.66 and 91.67% of total quality, respectively.

CONCLUSIONS

The significant differences between different methods of drying of celery root are confirmed by the application of post-hoc Tukey's HSD test at a 95% confidence limit. Osmotic dehydration stage in the combined dehydration method, influenced mineral mater content increase, due to the molasses content influx into the dehydrating celery root material. The calculation of Z-Score Analysis, based on chemical and mineral parameters, points out the best score of celery root dehydration by the combined method, obtaining 91.67%, while celery lyophilisation and convective dehydration obtained only 15.67% and 7.60% of maximal score values, respectively. Presented data suggests that the combined dehydration method is far superior in retaining celery root's desired chemical and mineral properties compared to lyophilisation and convective dehydration.

ACKNOWLEDGEMENTS

This research was funded by The Ministry of Science, Technological Development and Innovations of the Republic of Serbia, grant number: 451-03-66/2024-03/200222, and Provincial Secretariat of Higher Education and Scientific Research, Autonomous Province of Vojvodina, Republic of Serbia, contract number: 000882147 2024 09418 003 000 000 001 04 004.

REFERENCES

AOAC International: Gaithersburg, MD, USA, (2019). Horwitz, W. Official Methods of Analysis of AOAC International, 21st ed.

Baysan U., Imam Harun A.,Koç M., (2024). Evaluation of innovative drying approaches in celery drying considering product quality and drying energy efficiency, Innovative Food Science & Emerging Technologies, 93,103636.

Bruznican, S., De Clercq, H., Eeckhaut, T., Van Huylenbroeck, J., & Geelen, D. (2020). Celery and celeriac: A critical view on present and future breeding. Frontiers in Plant Science, 10, 1699.

Filipović V., Filipović J., Lončar B., Knežević V., Nićetin M., Filipović I. (2022a) Synergetic dehydration method of osmotic treatment in molasses and successive lyophilization of peaches. J. Food Process. Preserv. 46, e16512.

Filipović, V., Lončar, B., Filipović, J., Nićetin, M., Knežević, V., Šeregelj, V., Košutić, M., Bodroža Solarov, M. (2022b). Addition of Combinedly Dehydrated Peach to the Cookies—Technological Quality Testing and Optimization. Foods, 11, 1258.

Kian-Pour N., Akdeniz E., Said Toker O., (2022). Influence of coating-blanching in starch solutions, on the drying kinetics, transport properties, quality parameters, and microstructure of celery root chips. LWT - Food Science and Technology, 160, 113262.

Kręcisz M., Kolniak-Ostek J., Łyczko J., Stępień B. (2023) Evaluation of bioactive compounds, volatile compounds, drying process kinetics and selected physical properties of vacuum impregnation celery dried by different methods, Food Chemistry,413, 135490.

Košutic, M. Extruded Flakes Product Supplemented with Sunflower and Dry Residue of Wild Oregano. Ph.D. Thesis, University of Novi Sad, Faculty of Technology, Novi Sad, Serbia, 2016. Available online: https://nardus.mpn.gov.rs/handle/123456789/6673 (accessed on 11 March 2022).

Košutić, M., Filipović, J., Pezo, L., Plavšić, D., Ivkov, M. (2016). Physical and sensory properties of corn flakes with added dry residue of wild oregano distillation, Journal of the Serbian Chemical Society, 81 (9) 1013–1024.

Kręcisz M., Kolniak-Ostek J., Jacek Łyczko , Stępień B.,(2023). Evaluation of bioactive compounds, volatile compounds, drying process kinetics and selected physical properties of vacuum impregnation celery dried by different methods, Food Chemistry, Volume 413,35490.

Liu, D-K., Xu, C-C., Zhang, L., Ma, H., Chen, X-J., Sui, Y-C., Zhang, H-Z. (2020) Evaluation of bioactive components and antioxidant capacity of four celery (Apium graveolens L.) leaves and petioles, International Journal of Food Properties, 23:1, 1097-1109,

Lončar B., Nićetin M., Filipović V., Knežević V., Pezo L., Šuput D., Kuljanin T. (2021). Osmotic dehydration in sugar beet molasses-food safety and quality benefits. Journal of Hygienic Engineering and Design, 34, 15-20.

Marić L., Malešić E., Jurinjak Tušek A., Benković M., Valinger D., Jurina T., Gajdoš Kljusurić J. (2020) Effects of drying on physical and chemical properties of root vegetables: Artificial neural network modelling, Food and Bioproducts Processing, 119, 148-160.

Olsen H., Aaby K., Borge G.I.A. (2009). Characterization and quantification of flavonoids and hydroxycinnamic acids in curly kale (Brassica oleracea L. Convar. acephala Var. sabellica) by HPLC-DAD-ESI-MSn, Journal of Agricultural Food Chemistry, 57 pp. 2816-2825.

Szarek, N.; Jaworska, G.; Hanus, P. (2024). Profile of phenolic compounds and antioxidant activity of celery (*Apium graveolens*) juices obtained from pulp after α-amylase treatment from *Aspergillus oryzae*. *Molecules*, *29*, 1438.

EVALUATION OF A NOVEL DISPOSABLE AMPEROMETRIC GLYCEROL BIOSENSOR BASED ON A MELDOLAS BLUE-MODIFIED SCREEN-PRINTED CARBON ELECTRODE FOR JUICE BEVERAGE ANALYSIS

S. I. Ekonomou*, A. Crew, O. Doran and J.P. Hart*

Centre for Research in Biosciences, Faculty of Health and Applied Sciences, University of the West of England, Bristol, BS16 1QY

*Corresponding authors:
john.hart@uwe.ac.uk
sotirios.oikonomou@uwe.ac.uk

ABSTRACT

This study details the design and development of an electrochemical biosensor for measuring glycerol, an important compound in food and beverages, due to its role as a naturally occurring triose sugar and food additive (E 422). Monitoring glycerol concentration is essential for ensuring food quality and safety. The biosensor operates by enzymatically oxidizing glycerol using glycerol dehydrogenase (GLDH) in the presence of oxidized Nicotinamide adenine dinucleotide (NAD^+), resulting in the production of its reduced form (NADH) and dihydroxyacetone. The NADH then undergoes electrocatalytic oxidation at a Meldolas Blue-modified Screen-printed Carbon Electrode (MB-SPCE), with the resulting current serving as the analytical response. This response is directly proportional to the glycerol concentration. Standard addition calibration studies using chronoamperometry were performed with glycerol concentrations between 1.0 to 3.0 mM, utilizing only 100 uL of diluted (0.1 M phosphate buffer) grape juice (GJ) directly on the biosensor surface. Calibration plots were constructed by taking current measurements at 100 s after application of the applied potential; this demonstrated that the glycerol biosensor produced a linear response across the concentration range studied. The amperometric biosensor was successfully applied to the measurement of glycerol in commercially available GJ, representing non-alcoholic beverages. These analyses have the ability to be conducted outside the laboratory using commercially available, portable potentiostats. Overall, this approach shows promise to form a platform for the development of novel rapid technology for point-of-test evaluation of glycerol in the production and quality control of non-alcoholic beverages.
Keywords: *Food safety and quality, amperometric glycerol biosensor, chronoamperometry, screen-printed carbon electrode, Meldolas Blue, grape juice*

INTRODUCTION

It has been reported that the consumption of grape juice (GJ) is increasing worldwide because of its sensory characteristics and nutritional value (Bendaali et al., 2022). It is, therefore, important to be able to ascertain the quality of GJ for commercial purposes. Kupina (1984) suggested that glycerol, a triose sugar, is one of the crucial indicators of GJ quality. Caputi *et al.* (1992) have developed a High-Performance Liquid Chromatographic (HPLC) method for the measurement of glycerol, which involves a strong cation exchange analytical column in conjunction with a guard column, together with a refractive index detector. The method required sample preparation, including a membrane filtration step. The evaluation of the method was performed using 12 GJ samples in a collaborative study, and the authors reported that the procedure had been officially recommended (Caputi et al., 1992). Linget *et al.* (1998) reported an alternative HPLC approach for GJ analysis, which could also reliably measure the glycerol GJ concentration. This method involves a complex HPLC system incorporating an on-line clean-up step using a dialysis procedure, enabling simultaneous measurement of other analytes, including amino acids, sugars and organic acids.

While these chromatographic procedures are very reliable, and effective, they have some important drawbacks. HPLC is a high-cost technique, and the operation and maintenance of its instruments require highly skilled technical personnel. The overall analysis time can be quite long, owing to the serial nature of the final measurement step with long elution times; this is particularly the case when many analyses are required. Therefore, alternative

approaches, which offer more rapid analyses, lower costs, and require simpler instrument operation, are highly desirable. One such approach involves the use of electrochemical biosensors, which are fabricated using screen-printed technology. These devices have been effectively employed to address complex analytical challenges in different fields including agri-food, environmental, and biomedical analyses (Gareth Hughes et al., 2016). Biosensors offer significant advantages such as low cost, particularly as they can be mass-produced using carbon materials and can be fabricated easily in a wide range of planar geometries. The surface of the base screen-printed carbon electrodes is readily tailored with a wide range of enzymes and electrocatalysts to produce biosensors with high selectivity, which is a prerequisite for the analysis of complex matrices (Smart et al., 2020). Consequently, it was considered that this approach provides a platform for developing and applying a glycerol biosensor.

In a previous study (Sprules et al., 1994), we developed a sensor based on a screen-printed carbon electrode modified with the electrocatalyst Meldolas Blue (MB-SPCE) and reduced nicotinamide adenine dinucleotide (NADH). This device has the advantage of operating at 0 V (*vs* Ag/AgCl), which results in highly selective measurements. We showed that this device could be converted to a lactate biosensor by immobilizing the enzyme lactate dehydrogenase onto the surface of the MB-SPCE (Sprules, Hartley, et al., 1996). Additionally, it was found that the MB-SPCE could be readily converted into various biosensors by immobilizing a suitable dehydrogenase enzyme, together with the cofactor of oxidized Nicotinamide adenine dinucleotide (NAD^+), for the analyte of interest. For example, an ethanol biosensor was developed to assess the quality of a beverage by immobilizing alcohol dehydrogenase (Sprules, Hart, et al., 1996). In a separate study, a glutamate biosensor was developed by immobilizing glutamate dehydrogenase onto the MB-SPCE surface and was applied to assess food quality (G. Hughes et al., 2016; Gareth Hughes et al., 2014). Bearing the above discussion in mind, a glycerol biosensor was developed by immobilizing glycerol dehydrogenase and NAD^+ onto an MB-SPCE. The intention was to apply the prototype device to determine glycerol in GJ and deduce its performance in this complex matrix. This paper describes the procedure for the fabrication and operation of a glycerol biosensor; its potential for applications in the area of juice beverage analysis is demonstrated using a commercial GJ product. The described approach could have broad applications in the areas of food quality and safety.

MATERIAL AND METHODS

Raw materials, chemicals, and reagents

Purple commercially available GJ was purchased at Tesco's, a UK supermarket chain, and stored at 4°C. GLDH was obtained from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich (Dorset, UK). Deionized water was obtained from a Purite RO200 Stillplus HP System (Oxon, UK). Stock solutions of monosodium, disodium, and trisodium orthophosphate were prepared at a concentration of 0.2 M by dissolving the appropriate mass in deionized water and were then mixed to achieve the desired pH and subsequently diluted in the cell to a working concentration of 0.1 M. Sodium chloride was prepared to a concentration of 1.0 M by dissolving the appropriate mass in deionized water and was diluted in the cell, giving a final concentration of 0.1 M.

Instrumentation

An Emstat Blue potentiostat (PalmSens, The Netherlands) was used for the voltammetric and amperometric measurements. The potentiostat was connected to a PC for data acquisition *via* PS Trace Software.

Gwent Electronic Materials Ltd (Pontypool, UK) supplied all disposable MB-SPCEs. The working electrode (containing MB-SPCE) was fabricated using carbon graphite-based ink with Meldolas Blue (C2030519P5), and the reference electrode was fabricated using Ag/AgCl ink (C2130809D5). The working electrode's area (3 × 3 mm) was defined using electrical insulation tape.

pH measurement
The pH of all samples was recorded using a Testo 205 pH meter (Testo Limited, UK). All solutions were stirred using a colour squid (IKA, UK) and warmed in a HAAKE P5 water bath (Thermo Scientific, UK).

Assessment of glycerol concentration
A commercial glycerol assay kit (Megazyme International Ireland, Ireland) was used to estimate the glycerol concentration in the GJ samples. The amount of NADH was detected by measuring absorbance at 340 nm using a UV-vis spectrophotometer (Thermo Scientific, UK) as described in our previous study (Ekonomou et al., 2024). Initially, the absorbance difference (A1-A2) for both blank and sample was determined. Next, the concentration of glycerol was calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{glycerol} \text{ [g/L]}$$

Where:
V = final volume [mL]
MW = molecular weight of glycerol [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for glycerol:

$$c = \frac{2.34 \times 92.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{glycerol} \text{ [g/L]}$$

The sample was diluted during preparation and multiplied by the dilution factor, F.

Fabrication of glycerol biosensor
A mixture of 10 uL containing 10 U of GLDH and 660 ug of NAD^+ in a 0.1 M PBS, pH 9 solution was drop-coated on the surface of the MB-SPCE working electrodes. The layer was allowed to dry overnight under a vacuum at –0.6 MPa, at 4^{o}C. Next, a 10 uL aliquot of glutaraldehyde (GLA; 0.01% in phosphate buffer) was placed on top of the enzyme/NAD^+ layer and left overnight under vacuum to allow crosslinking to occur. The fabricated biosensors were then refrigerated until they were ready for calibration and GJ analysis.

Standard addition calibration procedure and analysis of GJ
A standard addition calibration study was performed with solutions prepared in 0.1 M PBS pH 9 spiked with 1, 2, and 3 mM glycerol concentrations. The electrochemical technique employed was chronoamperometry, using an applied potential of 0.0 V *vs* Ag/AgCl, preceded by an incubation time of 3 min. Aliquots of 100 uL of GJ and GJ samples spiked with glycerol were directly deposited onto the glycerol biosensor surface, which was warmed to 30°C on a thermostated surface and current measurements were taken at 100 s. A new biosensor was used for each glycerol concentration measurement.
The analysis of GJ was performed in a similar manner to that described above, including an initial dilution step to adjust the concentration of glycerol to be in the linear range. All GJ dilutions were carried out using 0.1 M PBS, pH 9.

Statistical analysis
All data acquired were expressed as mean ± standard deviation (SD). Data were analyzed using the paired two-sample for means t-test with IBM® SPSS® statistics 26 software for macOS (SPSS Inc.) at a 5% level of significance.

RESULTS AND DISCUSSION

Principal of operation of the amperometric glycerol biosensor

The overall sequence of reactions involved during the operation of the amperometric glycerol biosensor is shown in Fig. 1. The biosensor operates based on the enzymatic oxidation of glycerol by GLDH in the presence of NAD^+. This process leads to the production of NADH and dihydroxyacetone. The NADH then undergoes electrocatalytic oxidation at an MB-SPCE, and the resulting electrocatalytic oxidation current is the analytical response. This response is directly proportional to the glycerol concentration. It's important to note that the advantage of using the electrocatalytic oxidation reaction of NADH is that the operating potential is much lower compared to the direct oxidation at bare carbon electrodes. This aspect will be discussed later.

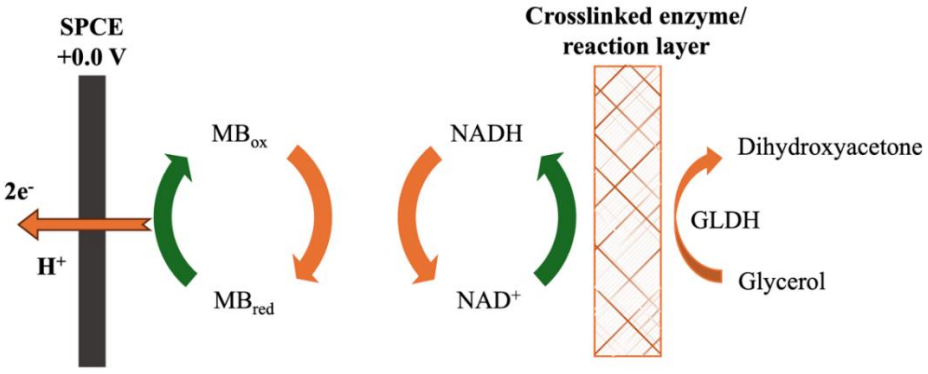


Figure 1. The sequence of reactions involved in the operation of the amperometric glycerol biosensor.

Standard addition calibration study and Grape juice analysis

A standard addition calibration study was conducted using the glycerol biosensor along with chronoamperometry (Fig. 2 A-C). To do this, 100 uL aliquots of GJ to which was added glycerol (1 – 3 mM), were deposited on the working/reference electrodes. Chronoamperometry was carried out with an applied potential of 0.0 V *vs* Ag/AgCl, following a 3 min incubation period. Fig. 2 (A) shows the chronoamperograms obtained for the solutions containing only GJ. Fig. 2 (B) shows the chronoamperograms of GJ samples containing additions 1 mM (blue and green lines), 2 mM (orange and purple lines), and 3 mM (haki and red lines) of the appropriate glycerol additions.

The chronoamperometric currents were collected for all GJ samples and standards at 100s. Fig. 2 (C) shows the resulting calibration plot and the regression equation was calculated to be i (uA) = 0.043C (mM) + 0.0017, with an R^2 value equal to 0.9971. Using the standard addition plot above (Fig. 2C), we calculated the unknown GJ concentrations from the Chronoamperometric responses shown in Fig. 2 (A). This data is further discussed below.

In the present work, two methods, the first using a novel glycerol biosensor with amperometric detection and the second using a commercial glycerol assay kit with colorimetric detection, were compared for the determination of glycerol concentration in GJ. Commercial kits (such as the one used in the current study) have been developed for the determination of glycerol content in GJ. Even though it is a straightforward and convenient enzymatic-colorimetric method, it involves multiple steps, which may lead to spurious data.

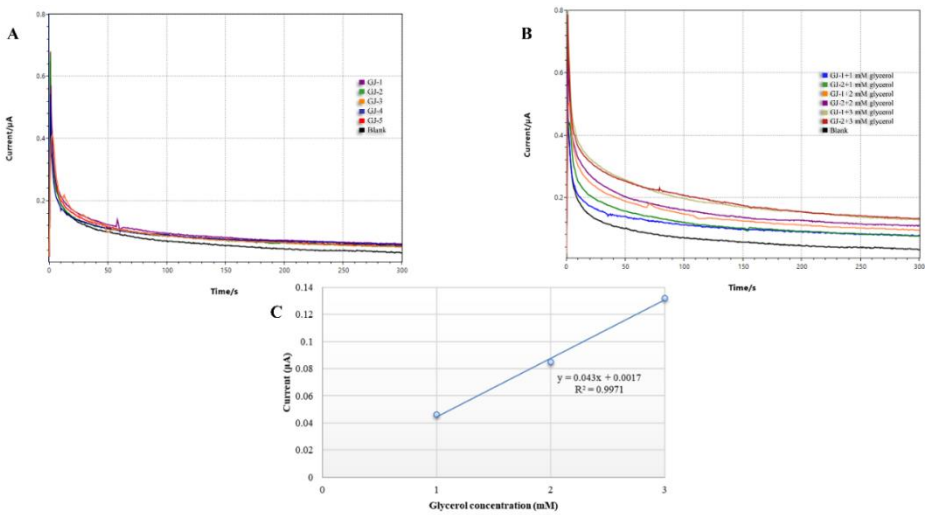


Figure 2. Chronoamperometric responses obtained with the glycerol biosensor for A) GJ (n=5) and B) GJ spiked with 1, 2, and 3 mM of glycerol at 100 – 300 s. C) Presents the standard addition plot for GJ over the concentration range of glycerol added (1 – 3 mM).

Next, a comparison was made with our newly developed glycerol biosensor and a commercially available glycerol kit. Table 1 summarises the glycerol recoveries for the individual GJ samples as well as the mean recovery (1.09 gL^{-1}) and precision data with a relative standard deviation (RSD) equal to 11.52% by the biosensor. This Table also compares the results obtained with the glycerol kit, which resulted in a mean recovery of 0.28 gL^{-1} and an RSD equal to 9.59%. Our results, obtained by the biosensor agrees with the results reported by Csutorás *et al.* (2014) in a similar type of GJ product containing 1.21 gL^{-1}. A similar difference in recovery data in GJ has been reported by de Souza *et al.* (2013) when using two different analytical techniques (enzymatic-amperometric and spectrophotometric). de Souza *et al.* (2013) noted that for all the beverage samples they tested, including various wines and sodas, the glycerol content observed was similar for both methods except for the GJ sample, where half the glycerol content was observed when using the enzymatic-spectrophotometric method (de Souza et al., 2013).

Our results reveal that our novel biosensor can successfully detect glycerol concentration in GJ and can be easily and rapidly used in the food industry to test quality. One explanation for the different results obtained with the enzymatic-spectrophotometric kit compared with our biosensor is related to a high lactate concentration (0.025 – 5 gL^{-1}) naturally occurring in the GJ as reported by Coehlo et al. (2018) testing two commercial GJ samples; this only affects the former analytical approach, which has a different enzyme sequence which may be inhibited by lactate in GJ. In previous work performed by our group, it was ascertained that when working at an applied potential of 0.0 V, no significant interferences were detected in various liquid samples (Sprules, Hart, *et al.*, 1996; Hughes *et al.*, 2014; G. Hughes *et al.*, 2016).

Table 1. Recovery of glycerol content (in gL^{-1}) from GJ using a glycerol biosensor and enzymatic-colorimetric method.

Sample	Recovery (gL^{-1})	
	Biosensor	**Enzymatic-colorimetry**
1	0.91	0.29
2	1.16	0.30
3	1.04	0.30
4	1.04	0.29
5	1.28	0.23
Mean	1.09[a]	0.28[b]
SD (n=5)	0.13	0.03
RSD (%)	11.52	9.59

[a] Different lowercase letters indicate significant differences between methods ($p < 0.05$).

CONCLUSIONS

This paper has demonstrated the possibility of applying a rapid, convenient electrochemical glycerol biosensor for the measurement of glycerol in GJ. It is convenient to use due to its simple geometry, which permits a microlitre volume of liquid sample to be deposited on the biosensor's surface and determined using a chronoamperometric procedure. This offers another advantage for biosensors against spectrophotometric methods, as cloudy and colored solutions can be measured accurately. Such chronoamperometric methods can be performed with small, commercial handheld potentiostats, which allow samples to be analyzed outside of the laboratory. This has the potential to be commercialized and used directly in the food industry setting at the point of production. This approach has the potential to lead to a future platform for developing novel rapid technology for point-of-test evaluation of glycerol in numerous food products and beverages.

ACKNOWLEDGEMENTS

The authors are grateful to the University of the West of England, Bristol for supporting this project.

REFERENCES

Bendaali, Y., Vaquero, C., González, C., & Morata, A. (2022). Elaboration of an organic beverage based on grape juice with positive nutritional properties. *Food Science & Nutrition*, *10*(6), 1768–1779.

Caputi, A., Christensen, E., Biedenweg, N., & Miller, S. (1992). Liquid Chromatographic Method for Determination of Glycerol in Wine and Grape Juice: Collaborative Study. *Journal of AOAC INTERNATIONAL*, *75*(3), 379–383.

Csutorás, C., Hudák, O., Rácz, K., & Rácz, L. (2014). Technological Experiments for the Enhancement of Glycerol Content in High Quality Wines. *Journal of Agricultural Chemistry and Environment*, *03*(02), 48–52.

de Souza, F. C., de Vasconcellos Junior, F. J., Cabral, R. C., Fernández, T. L., & D'Elia, E. (2013). Simple enzymatic methods for glycerol analysis in commercial beverages. *CyTA - Journal of Food*, *11*(3), 270–276.

Ekonomou, S. I., Crew, A., Doran, O., & Hart, J. P. (2024). Development of a Disposable, Amperometric Glycerol Biosensor Based on a Screen-Printed Carbon Electrode, Modified with the Electrocatalyst Meldolas Blue, Coated with Glycerol Dehydrogenase and NAD+: Application to the Analysis of Wine Quality. *Applied Sciences*, *14*(14), 6118.

Hughes, G., Pemberton, R. M., Fielden, P. R., & Hart, J. P. (2016). The design, development and application of electrochemical glutamate biosensors. *TrAC Trends in Analytical Chemistry*, *79*, 106–113.

Hughes, Gareth, Pemberton, R. M., Fielden, P. R., & Hart, J. P. (2014). Development of a Disposable Screen Printed Amperometric Biosensor Based on Glutamate Dehydrogenase, for the Determination of Glutamate

in Clinical and Food Applications Analytical & Bioanalytical Electrochemistry. *Anal. Bioanal. Electrochem*, *6*(4), 435–449.

Hughes, Gareth, Westmacott, K., Honeychurch, K., Crew, A., Pemberton, R., & Hart, J. (2016). Recent Advances in the Fabrication and Application of Screen-Printed Electrochemical (Bio)Sensors Based on Carbon Materials for Biomedical, Agri-Food and Environmental Analyses. *Biosensors*, *6*(4), 50. https://doi.org/10.3390/bios6040050

Kupina, S. A. (1984). Simultaneous Quantitation of Glycerol, Acetic Acid and Ethanol in Grape Juice by High Performance Liquid Chromatography. *American Journal of Enology and Viticulture*, *35*(2), 59–62.

Linget, C., Netter, C., Heems, D., & Vérette, E. (1998). On-line dialysis with HPLC for the automated preparation and analysis of amino acids, sugars and organic acids in grape juice and wines. *Analusis*, *26*(1), 35–39.

Smart, A., Crew, A., Doran, O., & Hart, J. P. (2020). Studies Towards the Development of a Novel, Screen-Printed Carbon-Based, Biosensor for the Measurement of Polyunsaturated Fatty Acids. *Applied Sciences*, *10*(21), 7779.

Sprules, S. D., Hart, J. P., Pittson, R., & Wring, S. A. (1996). Evaluation of a new disposable screen-printed sensor strip for the measurement of NADH and its modification to produce a lactate biosensor employing microliter volumes. *Electroanalysis*, *8*(6), 539–543.

Sprules, S. D., Hart, J. P., Wring, S. A., & Pittson, R. (1994). Development of a disposable amperometric sensor for reduced nicotinamide adenine dinucleotide based on a chemically modified screen-printed carbon electrode. *The Analyst*, *119*(2), 253.

Sprules, S. D., Hartley, I. C., Wedge, R., Hart, J. P., & Pittson, R. (1996). A disposable reagentless screen-printed amperometric biosensor for the measurement of alcohol in beverages. *Analytica Chimica Acta*, *329*(3), 215–221.

EFFECTIVENESS OF ESSENTIAL PLANTS IN THE PREVENTION OF KETOSIS IN DAIRY COWS

Ema Barjaktarić, Marcela Šperanda

Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University, 31000 Osijek, Republic of Croatia

*Corresponding author:
E-mail address: ema.barjaktaric@gmail.com

ABSTRACT

Ketosis, a metabolic disorder often present in dairy cows during the peripartal period, is characterized by low glucose levels and high ketone body levels in the blood. This disorder can significantly reduce milk production and affect the overall health of the cows. Traditional prevention methods include proper nutrition and dietary supplements, but increasing research is focusing on the use of essential plants as an alternative or complementary solution. Essential oils, known for their antioxidant, anti-inflammatory, and antimicrobial properties, can play a significant role in the prevention of ketosis. Plants such as green tea, turmeric, medicinal dandelion contain bioactive compounds that can improve the metabolism of glucose and fats, thereby reducing the risk of developing ketosis. Studies have shown that adding essential oils to the diet of dairy cows can positively affect metabolism and reduce the level of ketone bodies in the blood. For example, green tea (*Camellia sinensis*) contains catechins and other antioxidants that can improve glucose and fat metabolism, potentially reducing the risk of ketosis. Additionally, ginger (*Zingiber officinale*) is known for its antioxidant and anti-inflammatory properties. It can improve digestion and reduce oxidative stress, which can help reduce the risk of ketosis. The combination of different essential oils can have a synergistic effect, providing more comprehensive protection against ketosis. However, the correct dosage and method of application are important to achieve optimal effectiveness without adverse consequences. Although the research results are promising, further studies are needed to determine the optimal conditions for application and the long-term effects of essential plants on the health and productivity of dairy cows. In any case, essential plants represent an interesting and potentially effective option for improving the health and well-being of dairy cows and can become an important part of the strategy for preventing ketosis.
***Keywords**: essential oils, plant extract, phytotherapy prevention of ketosis, dairy cows*

INTRODUCTION

The dairy industry plays a critical role in global agriculture, contributing significantly to the food supply and economy. However, the productivity and health of dairy cows are often compromised by metabolic disorders, among which ketosis stands out as a particularly prevalent and challenging condition. Ketosis typically occurs during the early lactation period when the energy demands for milk production exceed the cow's intake, leading to negative energy balance and the mobilization of body fat. This process results in the accumulation of ketone bodies in the bloodstream, which can lead to reduced milk yield, poor reproductive performance, and in severe cases, can be life-threatening for the cow.

Traditionally, the management of ketosis has involved a combination of dietary adjustments, such as increasing energy density and supplementing with glucose precursors like propylene glycol. However, these strategies are not always effective in preventing the onset of ketosis and may not be sustainable in the long term. Consequently, there has been growing interest in exploring alternative, natural approaches to ketosis prevention, with particular focus on the use of essential plants and their extracts.

METHODS AND RESULTS OF RECENT RESEARCH

Multicomponent plant extract

Ruminants as a result of their evolution, have adapted to consuming a wide variety and large quantities of phytochemicals. According to Durrer et al. (2020) recent studies suggest a strong likelihood that even domesticated ruminants can self-medicate using plants (e.g., *Cichorium intybus* L.), especially in cases of metabolic imbalances. Additionally, there is a long-standing tradition of using medicinal plants such as *Camellia sinensis (L.) Kuntze*, *Gentiana lutea L., Taraxacum officinale F. H. Wick*, and *Trigonella foenum-graecum L*. to treat gastrointestinal and metabolic disorders in cattle. Therefore, it is plausible that herbal remedies could assist cows with subclinical ketosis, even in modern dairy farming environments. However, in vitro and in vivo studies on the use of plant extracts or individual phytochemicals in livestock treatment are limited. A recent study by Dorn et al. (2016) demonstrated that a combination of sodium propionate (SP) and a multicomponent herbal extract (HE) containing *C. sinensis, C. intybus, G. lutea, G. glabra, T. officinale*, *T. foenum-graecum,* and *Z. officinale* provided a slight but significant advantage in improving milk acetone (MAC) in cows with subclinical ketosis compared to using pure SP alone or placebo (PL). The combination also showed a tendency to reduce blood β-hydroxybutyrate (BHB) levels more effectively than pure SP. Therefore, alternative approaches need to be explored. Medicinal plants and their extracts could serve as a therapeutic option for subclinical ketosis in dairy cows (Durrer et al., 2020).

One approach to breaking the vicious cycle of subclinical ketosis involves using bitter substances, such as those from *Cichorium intybus* or *Taraxacum officinale*, or the bitter glycoside gentiopicrin from *Gentiana lutea*, to counteract inappetence. Additionally, the stimulation of gastric secretion and the anti-emetic properties of *Zingiber officinale Roscoe* may be beneficial. Impaired fat metabolism and fat deposition in the liver may be stabilized by glycyrrhizin from *Glycyrrhiza glabra L*., as well as by *T. officinale* and *Trigonella foenum-graecum*. Since the liver is the primary organ affected, cows with subclinical ketosis may also benefit from the hepatoprotective effects of *Z. officinale, C. intybus, G. glabra,* and *T. officinale*.

A recent study by Dorn et al. demonstrated a slight but significant advantage of combining spirulina (SP) with a multicomponent herbal extract (HE) based on *Camellia sinensis, C. intybus, G. lutea, G. glabra, T. officinale, T. foenum-graecum,* and *Z. officinale* compared to using pure SP or a placebo (PL) alone in cows with subclinical ketosis. The combination also showed a trend towards lower blood-BHB levels compared to pure SP (Durrer et al., 2020).

Plant bioactive complex

Kumprechtová et al. (2022) noted that among polyphenols, rosemary has been shown to enhance antioxidant status and decrease lipid peroxidation and mid-lactation dairy cows. However the antioxidant status indicators has measured, such as total antioxidant capacity (TAC) and glutathione peroxidase (GSH-Px), did not show significant improvement with plant bioactive supplementation (TAC: $p = 0.08$ at D+7 and D+14; GSH-Px: $p > 0.1$). Collecting blood samples before the experiment began would have helped confirm the lack of statistical differences among groups prior to the experiment, allowing for a more precise analysis of the supplementation's effects. It is stated found that supplementation with green tea and turmeric extract from three weeks before calving to nine weeks postpartum reduced haptoglobin levels ($p < 0.10$) and liver cholesterol concentration during the first and third weeks postpartum. These findings suggest that the plant bioactive complex may support dairy cow metabolism during the periparturient period. Kumprechtová et al. (2022) observed a reduction in postpartum serum haptoglobin and better stability of serum parameters over time, indicating reduced inflammation. In terms of energy status, there was a reduction in postpartum serum NEFAs, suggesting lower adipose tissue mobilization. Plant bioactive compounds with antioxidant and anti-inflammatory properties administered during the transition period appear to improve health parameters in postpartum dairy cows.

Kumprechtová et al. (2022) state in their work that a plant bioactive complex can help support the metabolism of dairy cows during the peripartum period. A decrease in serum haptoglobin levels after calving and greater stability of serum parameters over time suggest a decrease in inflammation. In addition, the decrease in serum NEFAs after delivery indicates a reduced mobilization of adipose tissue. Overall, plant bioactive compounds with antioxidant and anti-inflammatory properties administered during the transition period appear to improve health outcomes in postpartum dairy cows.

Welted thistle (*Carduus crispus)*

Considering the prophylactic and therapeutic properties of milk thistle (*Silybum marianum)* and milk thistle burdock (*Arctium tomentosum)*, there is a clear need to develop feed additives for high-yield dairy cows that can help maintain metabolic processes at a sufficient level to prevent the onset of ketosis. Milk thistle is a plant rich in essential oils and flavonoids (such as silibinin, silicristin, and silidianin) that provide potent detoxifying, hepatoprotective, antioxidant, and anti-inflammatory effects in humans. The plant also contains alkaloids, saponins, oils, proteins, vitamin K, resins, mucilage, tyramine, histamine, as well as macro- and micronutrients. The leaves, roots, and seeds of milk thistle possess medicinal properties. This plant can be effectively combined with various herbal mixtures and tinctures for treating certain diseases. Clinical pharmacology of hepatoprotectors has shown that milk thistle exhibits antioxidant effects, prevents the development of connective tissue in the liver, and has anti-inflammatory properties. Its beneficial impact extends to both the liver and the gastrointestinal tract. Milk thistle is most effective when used in powder form, as it cleanses liver cells at the micro level (Ovsiienko, 2020).

According to Ovsiienko (2020), two different recipes for the production of feed additives were developed. The first recipe involved creating two types of hay flour from milk thistle and welted thistle, which served as the base for granular feed additives. In the second recipe, hay flour made from milk thistle and welted thistle was mixed with saponite flour and molasses in the following proportions: 94 % hay flour, 3 % saponite flour, and 3 % molasses before granulation.

It was established that a granular feed additive made from hay flour of milk thistle or welted thistle, when used for feeding high-yielding cows during the transit period, reduces the level of ketone bodies by 34.7-33.3 % compared to animals in the control group. Additionally, a feed additive that combines hay flour from milk thistle or welted thistle with saponite flour and molasses further lowers the concentration of ketone bodies by 13 % and 17 %, respectively, compared to the additive made solely from hay flour of milk thistle or welted thistle (Ovsiienko, 2020).

Chinese herbal supplements

Chinese herbal feed additives offer a natural, multifunctional, and non-toxic alternative for reducing disease incidence in dairy cows, without the risks associated with antibiotic use. These additives are primarily derived from the roots, stems, and leaves of plants, which possess natural biological activities. Enzymes in the body convert the molecules from these plants into forms that tissues can more easily utilize. Research indicates that these additives improve disease resistance, promote fattening and weight gain, enhance feed utilization efficiency, and shorten the feeding cycle, providing both safety and economic benefits. The bioactive components in traditional Chinese medicines (TCM) can modulate microbial community function and improve growth and nutrient digestibility in cows. For example, *Pericarpium citri reticulatae* is commonly used in clinical practice to treat nausea, vomiting, and indigestion, while *Radix glycyrrhizae* offers protective effects against damage caused by heat and oxidative stress. In addition to these uses, *Radix glycyrrhizae* has also been employed in the care of dairy cows (Cui, 2022).

Oregano or green tea extracts

According to Stivanin et al. (2019) this study aimed to assess the impact of feeding dairy cows with oregano (*Origanum vulgare)* or green tea (*Camellia sinensis)* extracts on their feeding and social behaviors, intake, and overall health during the transition period. Twenty-four Jersey cows were randomly divided into three groups: a control group (CON), a group receiving 10 g/day of oregano extract (OE), and a group receiving 5 g/day of green tea extract (GT). Throughout the experiment, researchers monitored feeding patterns, social interactions, and health issues. The use of plant extracts did not alter dry matter intake (DMI) before calving, but post-calving, the cows supplemented with oregano extract showed a trend towards an increased DMI by 1,3 kg compared to the control group. The incidence of metabolic disorders and both clinical and subclinical infectious diseases was similar across all groups.

Feeding and social behavior, as well as the frequency of health problems, were observed throughout the experimental period. Addition of herbal supplements did not affect pre-calving dry matter intake; however, oregano supplementation after calving was associated with a 1.3 kg increase in dry matter intake compared to the control group. During the day before calving, oregano supplementation was associated with a 38-minute reduction in time spent lying down and an 8.6-minute reduction in time spent eating concentrate compared to the green tea group. After calving, oregano reduced the time spent on concentrate feeding by 9.8 minutes compared to the green tea group and by 7.5 minutes compared to the control group. In addition, cows receiving oregano had fewer total feeder visits than those in other treatment groups. Before calving, when green tea was included in the diet, cows visited the feeder 3.3 times more often and had 1.2 to 1.6 times fewer social interactions compared to those in the oregano and control groups, respectively. Rates of metabolic disorders and clinical and subclinical infections were comparable across treatments, but cows in the oregano group that had disease showed clinical symptoms or subclinical diagnoses an average of 8 days later than those receiving green tea (Stivanin et al., 2019).

The study found that oregano extract, but not green tea extract, had a tendency to improve DMI and feeding rate in Jersey dairy cows during the early weeks post-calving. The extracts had distinct effects on feeding and social behaviors, including the number of visits to the feeding trough and social and aggressive interactions. Cows supplemented with oregano extract tended to produce more milk and exhibit better feed efficiency compared to those receiving green tea extract. However, the plant extracts did not reduce the occurrence of diseases or metabolic disorders. Given the observed improvements in intake rate, feed efficiency, and social behavior, oregano extract may help enhance cow welfare during the transition period (Stivanin et al., 2019).

Green tea and curcuma extract

In this study, researchers examined whether supplementing dairy cows with a plant-based product containing 95 % green tea and 5 % turmeric extract, both rich in polyphenols, could reduce inflammation and endoplasmic reticulum (ER) stress in the liver during early lactation. Twenty-seven cows were divided into two groups: a control group (n = 14) and a treatment group (n = 13). Both groups were fed a total mixed ration, but the treatment group's ration was supplemented with 0.175 g of the plant product per kg of dry matter from three weeks before calving until nine weeks after. There were no differences in dry matter intake and energy balance between the groups during weeks 2 to 9 postpartum. However, the cows receiving the plant supplement produced more energy-corrected milk during this period and had lower levels of triacylglycerols and cholesterol in their livers at weeks 1 and 3 postpartum compared to the control group ($p < 0.05$). Additionally, the supplemented cows tended to have lower mRNA levels of haptoglobin ($p < 0.10$), though there were no significant differences in the relative mRNA levels of eight genes involved in the unfolded protein response in the liver between the groups. The relative hepatic mRNA concentration of fibroblast growth factor, a stress hormone triggered by various stress conditions, was reduced in the supplemented cows at weeks 1 and 3 postpartum ($p < 0.05$) (Winkler et al., 2015).

The findings of this study suggest that supplementing dairy cows with a plant product made of green tea and turmeric extract from three weeks before calving to nine weeks postpartum had a moderate impact on inflammation but had a lesser effect on ER stress in the liver during early lactation. However, the supplemented cows showed a reduction in hepatic mRNA levels of FGF21, a stress-related hormone, and lower hepatic lipid concentrations. This indicates that the plant product helped reduce metabolic stress in the liver. Additionally, the supplementation led to an increase in energy-corrected milk yield during early lactation. Overall, these results suggest that a plant product containing green tea and turmeric could be beneficial in enhancing milk production and preventing fatty liver syndrome in dairy cows during early lactation (Winkler et al., 2015).

CONCLUSIONS

The conclusion about the effectiveness of essential plants in the prevention of ketosis in dairy cows indicates the significant potential of these herbal remedies as an alternative or additional approach in the management of livestock health. Studies have shown that certain essential plants can have a positive effect on metabolism, improve nutrient intake and reduce the risk of developing ketosis. The use of plant extracts as nutritional supplements can provide a natural way to maintain cow health, reducing the need for synthetic drugs and improving productivity and animal welfare. However, in order to fully understand and optimize the application of these herbs, additional research is necessary that would include long-term effects, optimal doses and interactions with other nutrients and drugs. Based on current knowledge, essential plants represent a promising tool in the ketosis prevention strategy, but their use should be supported and supplemented by conventional methods and recommendations of livestock nutrition and health experts.

REFERENCES

Cui, Y., Shan, Z., Hou, L., Wang, Q., Loor, J. J., & Xu, C. (2022.): Effect of natural Chinese herbal supplements (TCMF4) on lactation performance and serum biomarkers in Peripartal dairy cows. Frontiers in Veterinary Science, 8, 801418.

Dorn, K., Leiber, F., Sundrum, A., Holinger, M., Mayer, P., & Walkenhorst, M. (2016.): A field trial on the effects of pure sodium propionate and a combination with herbal extracts on short term development of subclinical ketosis. Livestock Science, 187, 87-95.

Durrer, M., Mevissen, M., Holinger, M., Hamburger, M., Graf-Schiller, S., Mayer, P., ... & Walkenhorst, M. (2020.): Effects of a Multicomponent Herbal Extract on the Course of Subclinical Ketosis in Dairy Cows–a Blinded Placebo-controlled Field-study. Planta medica, 86(18), 1375-1388.

Kumprechtová, D., Chabrillat, T., Guillaume, S., Kerros, S., Kadek, R., Indrová, E., & Illek, J. (2022.): Effect of Plant Bioactive Compounds Supplemented in Transition Dairy Cows on the Metabolic and Inflammatory Status. Molecules, 27(18), 6092.

Ovsiienko, S. (2020.): Method of preventing high-yielding cow's ketosis. The Scientific Heritage, (55-3), 9-13.

Stivanin, S. C. B., Vizzotto, E. F., de Paris, M., Zanela, M. B., Passos, L. T., Angelo, I. D. V., & Fischer, V. (2019.): Addition of oregano or green tea extracts into the diet for Jersey cows in transition period. Feeding and social behavior, intake and health status. Plant extracts for cows in the transition period. Animal Feed Science and Technology, 257, 114265.

Winkler, A., Gessner, D. K., Koch, C., Romberg, F. J., Dusel, G., Herzog, E., ... & Eder, K. (2015.): Effects of a plant product consisting of green tea and curcuma extract on milk production and the expression of hepatic genes involved in endoplasmic stress response and inflammation in dairy cows. Archives of Animal Nutrition, 69(6), 425-441.

AFLATOXICOSIS IN DOGS - EXAMPLES AND INTERPRETATIONS

Ksenija Nesic*

Scientific Institute of Veterinary Medicine of Serbia, Smolucska 11, 11070 Belgrade, Serbia

*Corresponding author:
E-mail address: ksenija.nesic@nivs.rs

ABSTRACT

The presence of aflatoxins in pet food is often overlooked in practice until an incident occurs, which has unfortunately become increasingly common in recent times. On the other hand, the perception of the danger of these secondary metabolites of molds in the human food chain is generally at a satisfactory level. For both humans and livestock, the maximum permissible levels in food are regulated, so monitoring programs are part of the strategy of state control of food and feed safety in European countries, including Serbia. However, the mycotoxicological situation for pet food is different. Dogs are particularly sensitive to aflatoxins and often suffer fatal consequences. Considering that pets are referred to as "companion animals", it is clear that due to the emotional attachment of the owners, each case represents a very sensitive and complicated challenge that exceeds the financial losses typical for farms. The lack of knowledge about the effects of aflatoxins on the health of dogs and the lack of regulations and controls for this type of feed are the most common causes of recent outbreaks. The detour of contaminated grain from the human food chain into pet food is unacceptable and the frightening consequences can only be avoided by increasing the level of information and educating all stakeholders.The aim of this article is therefore to raise awareness of the importance of controls and conscientious behavior in the pet food industry and to warn feed manufacturers and dog owners about the negative effects that aflatoxins have on these animals.
***Keywords**: aflatoxins, dogs, pet food*

INTRODUCTION

The production of dog food on a global scale has been developing successfully for years and is still making great progress, but it is also a very demanding task. The most important goal is to meet the diverse nutritional needs of dogs, which vary according to breed, age and activity category. But the social status of the dog also contributes to this complexity: the increased bond with humans (to the point of identification) and the strong urban integration which induce the different requirements of both owners and animals (Schleicher et al., 2019).
Dry dog food formulations are based on derivatives of all types of meat and meat products, usually from chicken, beef, pork, but also from other animal species (rabbit, quail, pheasant, etc.). Protein components from soy are also used. Although dogs and cats do not have an absolute dietary requirement for carbohydrates, most pet food manufacturers take advantage of their ability to digest them, and use varying amounts of different grains, such as corn, rice, wheat, barley and sorghum. These feedingstuffs are added due to their low cost and acceptable nutritional value, which do not affect the taste and digestibility of the nutrients. These ingredients are widely used as a source of energy, and additionally of some vitamins, minerals, fiber and fats (Martínez-Martínez et al., 2021). As recently published by Zhou et al. (2024), cereal ingredients make up between 30 and 50% of pet food formulations, in some cases, even reaching 70% (Kempe et al. 2008), and they are also used as fillers to improve croquette consistency (Witaszak et al. 2020).
Although great attention is paid to the production of food for pet animals, there are sometimes disputes regarding its safety and quality. Many potential threats endanger pet food, making it necessary to apply appropriate standards in production. In the EU and Serbia, most food safety criteria for pets are self-regulated by the industry, which encourages manufacturers to follow guides to good practice. The European pet food industry uses the Guide to Good Practice for the Manufacture of Safe Pet Foods (FEDIAF, 2018). Among the various health problems caused by food, the appearance of mycotoxicoses is a great concern. Reports of clinical cases of mycotoxicoses in dogs are scarce, but there is ample

evidence in the literature regarding the geographic distribution of aflatoxigenic fungi and their secondary metabolites in both complete dog feed and ingredients, leading to aflatoxicosis in dogs.
And yet, neither European nor Serbian regulations have been established for mycotoxins in dog food. However, although the legislation on animal feed provides a framework for ensuring that feedstuffs do not present any danger to animal health or to the environment, the main focus of mycotoxin limits are the farming animals and, ultimately, the human food safety. That is, there are no such specific MRLs for grain-based dog and cat foods, as they are grouped as "non-food-producing animals" according the food laws (Macias-Montes et al., 2020). Therefore, pet food is regulated by the maximum mycotoxin contamination levels for all feedstuffs rather than by pet-specific legislation. In the European Union, the list of hazards in the FEDIAF Guide To Good Practice for the Manufacture of Safe Pet foods (2018) includes mycotoxins. The possibility of raw material contamination or final products during transport and storage is emphasized. It is also highlighted that higher risk depends on the geographical origin of incoming material and weather conditions, while the occurrence of risk is linked to the quantity of cereals and vegetables used in the recipes.
Therefore, this paper is intended to raise awareness of the risk to which dogs are exposed in conditions where aflatoxins (but also other mycotoxins) are evidently detected more and more often, while specific regulations do not exist, and to indicate the urgent need for regular control mechanisms.

ETIOLOGY OF AFLATOXICOSIS IN DOGS

Today, the origin of aflatoxins as secondary metabolites of molds in the genus *Aspergillus*, mainly species *A. flavus* and *A. parasiticus,* is very well known. These molds infest cereals, and in conditions of major climate changes they are increasingly present, while mycotoxins generally become an unavoidable reality (Nesic, 2018). As published by Kos et al. (2024), rainy and wet conditions favor the growth and development of *Fusarium* species and the synthesis of *Fusarium* metabolites (e.g. 2014), while *Aspergillus* metabolites are scarce in maize samples from the same year. Conversely, hot and dry conditions increase the prevalence of *Aspergillus* metabolites. The results from Serbia summarized in their 15-year review indicate a strong dependence of contamination on weather conditions providing extensive data particularly for maize.
Cereals are usually integrated into dog feed, especially maize, sorghum, rice, wheat, oats, barley, etc., as a good source of nutrients, but they also present a significant risk for the health of dogs because they are vulnerable to contamination by Aspergillus fungi both in the field and in storage. The situation is complicated by the fact that commercial dry food is used as the sole or main source of the dog diet and all the amount contained on each bag is usually eaten until it is exhausted. If it is contaminated, this may cause a prolonged ingestion of toxic metabolites, and even if at low doses (Hernandez et al., 2020), can induce adverse health effects, in this case named aflatoxicosis. The aflatoxins are primarily hepatotoxic and cause liver damage in animals with aflatoxin B1 being most toxic, followed by aflatoxins G1, B2, and G2. Susceptibility varies with breed, species, age, dose, length of exposure and health and nutritional status.
The presence of mycotoxins affects the quality and safety of food and feed, posing serious consequences for both human and animal health. Unfortunately, in practice, primarily food-producing animals are taken into consideration. Regulations in this area only prescribe maximum permissible limits for humans and farm animals (EU, 2011; Serbia, 2014). All other species are neglected, which does not mean they are spared from adverse effects in practice or do not face problems caused by contamination. On the contrary, neglect puts these species, including dogs, at an even greater disadvantage. Due to the lack of control or ignorance of pet food producers, contaminated grains can end up in dog food, whether intentionally or not.

CLINICAL PICTURE OF AFLATOXICOSIS IN DOGS

Research on the toxicity of aflatoxins in dogs began in the 1960s, but still compared with other species, the effects of aflatoxins in dogs are less well documented (Aquino & Correa, 2011). Yet there are reports showing that the median lethal dose (LD50) of AFB1 for dogs is 0.5–1.5 mg/kg body weight, while clinical manifestations are observed at doses from 60 µg/kg of aflatoxins in feed, as shown by Stenske et al. (2006) in the USA cases from December 2005. Table 1 shows some cases of dog aflatoxicosis described in the literature, although the number of these publications is very limited and rare.

Table 1. Cases of dog aflatoxicosis described in the literature

Content of aflatoxins in feed or feedstuffs (µg/kg)	Number of affected dogs	Mortality (%)	Reference
89.0 – 191 (feed)	4	100	Reis-Gomes et al., 2014
1640 – 1770 (corn)	65	92	Wouters et al., 2013
80 - 300 (feed)	50	68	Bruchim et al., 2012
< 5 – 4946 (feed)	100	96	Arnot et al., 2012
48 – 800	72	36	Dereszynski et al., 2008
223- 579	9	100	Newman et al., 2007

Diagnosing mycotoxicosis is a significant challenge for veterinarians due to the complexity of differential diagnosis. The disease syndromes induced by mycotoxins can easily be mistaken for other health conditions caused by pathogenic microorganisms. Additionally, as said before, symptoms can vary widely depending on the breed, age, health status, and nutritional status of the animal. In all species, the liver is the primary target organ after ingestion of aflatoxin. In dogs, aflatoxicosis presents with digestive, hemodynamic, and nervous system alterations. Digestive symptoms include vomiting, anorexia, hematemesis, hematochezia, and melena. Hemodynamic changes may include ascites, peripheral edema, jaundice, dehydration, decreased blood pressure, hemorrhagic diathesis, and petechiae on the mucous membranes. Clinical signs associated with central nervous system disorders may manifest as depression, vocalization, stupor, seizures, and coma due to hepatic encephalopathy. (Arnot et al., 2012; Bruchim et al., 2012; Dereszynski et al., 2008).

Changes in blood biochemistry are also noted: an increase in the specific activity of liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Coagulation tests in cases of dog aflatoxicosis show a decrease in the blood's ability to clot, evidenced mainly by an increase in prothrombin time (PT) and activated partial thromboplastin time (aPTT), as well as a decrease in antithrombin in plasma, protein C activity, and coagulation factor VII (Prins et al., 2010). Additionally, a decrease in fibrinogen and platelets, delays in coagulation, and disseminated intravascular coagulation (DIC) with depletion of coagulation factors are observed. The hemorrhagic effects of aflatoxins are attributed to their chemical structure, which contains a coumarin ring with an anticoagulant effect (Martínez-Martínez et al., 2021). Liver failure also leads to changes in other parameters (Newman et al., 2007). Hyperbilirubinemia, an increase in total bilirubin, and a decrease in total proteins and albumin can be seen, as well as a decrease in cholesterol concentrations due to cholestasis resulting from fibrosis of the bile ducts.

However, it is often challenging to definitively attribute a disease outbreak to aflatoxins based solely on clinical symptoms, biochemical parameters, and macroscopic findings, as these can be nonspecific and misleading. To accurately diagnose mycotoxicosis, feed analysis and histopathology examinations are essential. Histological assessment of the livers of affected animals, as well as analyzing the feed for mycotoxin content, are critical steps in confirming clinical diagnoses (Aquino and Correa, 2011).

THERAPY OF AFLATOXICOSIS IN DOGS

There is no real therapy for mycotoxicosis. The treatment of aflatoxicosis is exclusively symptomatic and supportive (Bruchim et al., 2012; Stenske et al., 2006). It includes the administration of an electrolyte solution containing additional potassium chloride. Nutritional support is based on total parenteral nutrition with intravenous fluids and transfusions of fresh frozen plasma to correct the dehydration and hypovolemia that occur in these cases. The dogs are also given S-adenosylmethionine and the milk thistle derivative silymarin to treat the liver. Vitamin K1 is administered to promote the activation of blood clotting, as aflatoxins such as coumarin have an anticoagulant effect. In addition to broad-spectrum antibiotics (ampicillin, enrofloxacin) against secondary and systemic infections caused by aflatoxin-mediated immunosuppression, antiemetics and agents to protect the gastrointestinal mucosa (famotidine and sucralfate) are prescribed. Vitamin E is used to prevent lipid peroxidation and damage to liver cell membranes, and L-carnitine reduces oxidative damage (Martínez-Martínez et al., 2021).

CONCLUSIONS

Based on the summarized data, the obtained conclusions are as follows:
A control program for mycotoxins should cover the pet food industry, and the Hazard Analysis Critical Control Point (HACCP) criteria are required to include these contaminants as well. In addition, scientifically based regulations for the acceptable limits of mycotoxins in pet food would be beneficial. However, strict regulations would increase competition with the human food chain, leading to higher costs for pet food and lower profits for the industry. So the right balance needs to be found and implemented.
The safety of food for cats and dogs is of paramount interest to manufacturers, as long-lived healthy consumers contribute to increased sales. Therefore, any mishap regarding product safety or quality can have disastrous consequences for the company's profits, reputation and viability. The health of pets is also of great emotional importance to their owners, which makes pet food manufacturers even more responsible for the safety and quality of their products. That is why conscientious business operations and the application of good production practices must not be questioned.
Nevertheless, owners are not exempt from responsibility for the health of their pets. They must ensure that the pet food is stored in such a way that it is not contaminated with fungi. Open bags should be placed in a clean and dry place that is ventilated and protected from moisture and other environmental influences. The shelf life of commercial products must also be taken into account.
This paper is intended to help producers and owners deal with this, at least sporadically, unavoidable problem, with the ultimate goal of ensuring permanent well-being for man's best animal friend.

ACKNOWLEDGEMENTS

The work is done as part of the Contract No 451-03-66/2024-03/ 200030 with the Serbian Ministry of Science, Technological Development and Innovation.

REFERENCES

Aquino, S., & Corrêa, B., (2011). Aflatoxins in Pet Foods: A Risk to Special Consumers. In I. Torres-Pachecho, *Aflatoxins: Detection, Measurement and Control* (pp. 53-74). IntechOpen.

Arnot, L., Duncan, N., Coetzer, H., & Botha, C., (2012). An outbreak of canine aflatoxicosis in Gauteng province, South Africa. *Journal of the South African Veterinary Association, 83,* 1–4. https://doi.org/10.4102/jsava.v83i1.2

Bruchim, Y., Segev, G., Sela, U., Bdolah, T., Salomon, A., & Aroch, I., (2012). Accidental fatal aflatoxicosis due to contaminated commercial diet in 50 dogs. *Research in Veterinary Science, 93*, 279–287. https://doi.org/10.1016/j.rvsc.2011.07.024

Dereszynski, D., Center, S., Randolph, J., Brooks, M., Hadden, A., Palyada, K., McDonough, S., Messick, J., Stokol, T., Bischoff, K., Gluckman, S., & Sanders, S. Y., (2008). Clinical and clinicopathologic features of dogs that consumed foodborne hepatotoxic aflatoxins: 72 cases (2005–2006). *Journal of the American Veterinary Medical Association, 232,* 1329–1337. https://doi.org/10.2460/javma.232.9.1329

EU (2011). Commission regulation (EU) No 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for nitrite, melamine, Ambrosia spp. and carry-over of certain coccidiostats and histomonostats and consolidating Annexes I and II thereto. *Official Journal of the European Union*, 159/7-24.

FEDIAF, (2018). Guide to Good Practice for the Manufacture of Safe Pet Foods. The European Pet Food Industry, pp. 3–72.

Hernandez, E., Valdivia, A., Cruz, C., Saldaña, M., Quezada, T., Rangel, E., Ortiz, R., Medina, L., & Jaramillo-Juarez, F., (2020). Diagnosis of subclinical aflatoxicosis by biochemical changes in dairy cows under field conditions. *Pakistan Veterinary Journal*, 1–6. https://doi.org/10.29261/PAKVETJ/2020.075

Kempe, R.M., Saastamoinen, M., Hyyppä, S., & Smeds, K. (2008) Composition, digestibility and nutritive value of cereals for dogs. *Agricultural and Food Science, 13*, 5–17. https://doi.org/10.2137/1239099041838067

Kos, J., Radić, B., Lešić, T., Anić, M., Jovanov, P., Šarić, B., & Pleadin, J. (2024). Climate Change and Mycotoxins Trends in Serbia and Croatia: A 15-Year Review. *Foods, 13,* 1391. https://doi.org/10.3390/foods13091391

Martínez-Martínez, L., Valdivia-Flores, A. G., Guerrero-Barrera, A. L., Quezada-Tristán, T., Rangel-Muñoz, E. J., & Ortiz-Martínez, R. (2021). Toxic Effect of Aflatoxins in Dogs Fed Contaminated Commercial Dry Feed: A Review. *Toxins,13,* 65. https://doi.org/10.3390/toxins13010065

Nesic, K., (2018). Mycotoxins – climate impact and steps to prevention based on prediction. *Acta Veterinaria-Beograd, 68 (1),* 1-15. https://doi.org/10.2478/acve-2018-0001

Newman, S.J., Smith, J.R., Stenske, K.A., Newman, L.B., Dunlap, J.R., Imerman, P.M., & Kirk, C.A. (2007). Aflatoxicosis in nine dogs after exposure to contaminated commercial dog food. *Journal of Veterinary Diagnostic Investigation, 19*, 168–175. https://doi.org/10.1177/104063870701900205

Prins, M., Schellens, C.J., van Leeuwen, M.W., Rothuizen, J., Teske, E., (2010). Coagulation disorders in dogs with hepatic disease. *The Veterinary Journal, 185*, 163–168. https://doi.org/10.1016/j.tvjl.2009.05.009

Reis-Gomes, A., Marcolongo-Pereira, C., Sallis, E.S.V., Pereira, D.I.B., Schild, A.L., Faria, R.O., & Meireles, M.C.A. (2014). Aflatoxicosis in dogs in Southern Rio Grande do Sul. *Pesquisa Veterinaria Brasileira, 34,* 162–166. https://doi.org/10.1590/S0100-736X2014000200011

Schleicher, M., Cash, S. B., & Freeman, L. M. (2019). Determinants of pet food purchasing decisions. *The Canadian veterinary journal, 60(6)*, 644-650.

Serbia (2014). Pravilnik o izmeni pravilnika o kvalitetu hrane za životinje. *Official Gazette, 27*.

Stenske, K., Smith, J., Newman, S., Newman, L., Kirk, C. Aflatoxicosis in dogs and dealing with suspected contaminated commercial foods. *Journal of the American Veterinary Medical Association, 228,* 1686–1691. https://doi.org/10.2460/javma.228.11.1686

Witaszak, N., Waśkiewicz, A., Bocianowski, J., & Stępień, Ł. (2020) Contamination of pet food with mycobiota and fusarium mycotoxins-focus on dogs and cats. *Toxins, 12(2),* 130. https://doi.org/10.3390/toxins12020130

Wouters, A.T.B., Casagrande, R.A., Wouters, F., Watanabe, T.T.N., Boabaid, F.M., Cruz, C.E.F., & Driemeier, D., (2013). An outbreak of aflatoxin poisoning in dogs associated with aflatoxin B1–contaminated maize products. *Journal of Veterinary Diagnostic Investigation, 25(2),* 282-287. https://doi.org/10.1177/1040638713477409

Zhou, G., Hu, S., Xie, L., Huang, H., Huang, W., Zheng, Q., & Zhang, N., (2024). Individual and combined occurrences of the prevalent mycotoxins in commercial feline and canine food. *Mycotoxin Research, 40*. https://doi.org/10.1007/s12550-024-00545-2

IMPACT OF MICROPLASTICS FROM THE FOOD CHAIN ON THE REPRODUCTIVE HEALTH

Marcela Šperanda[1]*, Maja Karnaš[1], Mislav Đidara[1], Brigita Popović[1], Mira Pucarević[2], Željka Jeličić Marinković[3], Krunoslav Zmaić[1]

[1]Josip Juraj Strossmayer University of Osijek Faculty of Agrobiotechnical Science Osijek, Vladimira Preloga 1, Osijek, Croatia
[2] Educons University in Sremska Kamenica, Vojvode Putnika 85-87, 21208 Sremska Kamenica, Serbia
[3] Institut for Nature Conservation of Vojvodina Province, Radnička 20a, 21101 Novi Sad, Serbia

*Corresponding author:
E-mail address: marcela.speranda@fazos.hr

ABSTRACT

Microplastics (MPs) enter animals' bodies (digestive, respiratory system and skin) through contaminated water or feed/food. This can lead to the accumulation of plastics in their bodies, which may be transferred to humans through consumption of animal products. MPs have been shown to cause various health issues in all living organisms, such as inflammation, oxidative stress, and cellular damage. Their different distribution routes depend on their physical and chemical properties, and they can also act as a carrier of other harmful substances and microorganisms. MPs have even been discovered in animal products such as milk and eggs. Their harmful effect upon living organisms is still unknow, however, there is evidence of absorbed particles presence in the gonads. As a consequence, more expressed ovarian cysts, more frequent corpora lutea presence, dilatation of the oviducts, lower number of growing follicles, a thinner layer of the granulosa cells in female mouse and rats were noticed. In the testes of male animals an increase in superoxide dismutase (SOD) and malondialdehyde (MDA) activities were described as a result of antioxidative response after MPs exposure. Toxicological research has demonstrated that MPs elicit toxic reactions in pig testicular cells, leading to apoptosis and necrosis of testicular tissue. In mice, presence of MPs is connected with downregulation of testosterone, LH (luteinizing hormone), FSH (follicle-stimulating hormone) and AMH (anti-Mullerian hormone) concentrations. In marine vertebrates, long-term exposure to MPs has been shown to cause weakening of the thyroid endocrine function, leading to a decreased ability to regulate growth, development, metabolism, and reproduction. Data for MPs effect upon livestock is lacking or limited, therefore, more studies should investigate alternative ways in which microplastics are excreted in livestock. In conclusion, microplastics pose a serious threat to animal health, affecting a wide range of species across different environments. Addressing this issue, requires a concerted effort from scientists, policymakers, industries, and the public to reduce plastic pollution and protect animal health.
Keywords*: microplastic, food chain, livestock, reproductive health, endocrine system*

INTRODUCTION

In the last 50 years or more, the production and distribution of plastic has increased, to the point that the current era is referred to as the "Plasticene" (Campanale et al., 2020) or "Plastic age" (Thompson et al., 2009). Between 2010 and 2021, the volume of plastic waste generated per inhabitant increased by about 29% (+8.1 kilos per person). In 2021, only 6,56 million tons of plastic waste were recycled (European Parliament, 2018). Due to the long-term decomposition time of plastic, significant amounts of microplastics and nanoplastisc remains in the environment. These particles can form unintentionally when larger pieces of plastic deteriorate, but they can also be intentionally manufactured and added to products for specific purposes for their beneficial properties, such as texture enhancement, exfoliation, or as emulsifiers. Some of the key products that often contain intentionally added microplastics include cosmetics, cleaning products, paints, coatings, fertilizers and pesticides. According to

the European Chemicals Agency (ECHA) around 145 000 tons of microplastics are estimated to be used in the EU/EEA each year (ECHA, 2023).
Microplastics (MPs) are defined as tiny plastic particles measuring from 1 μm to 5 mm in size (Welden and Lusher, 2020). Multiple studies have confirmed the widespread presence of microplastics in various environmental settings, including remote aquatic and terrestrial locations. (Martins et al., 2020; McIvor et al., 2022; Feng et al., 2023) Almost every day new investigation brings information about detection of MPs in the water, soil, groundwater, drinking water, complete food chain, different parts of organs in aquatic and terrestrial animals, even humans body and liquids (Hurley et al., 2018). The complexity of the problem is evident from the multitude of factors such as affected species, types of MPs, concentrations, routes and durations of exposure, and yet unexplored interactions with environmental substances, microorganisms, medicines and food/feed ingredients.

INFLUENCE OF MPS ON THE LIVING WORLD

Numerous researchers have demonstrated the negative impact of MPs on aquatic animals such as: physical blockages in the digestive tract, which reduce r animal's ability to feed properly; slow growth; increased oxidative and physiological stress; and reduced enzymatic activities due to the adsorption of other harmful pollutants from the water (such as heavy metals and persistent organic pollutants, mainly polyaromatic hydrocarbons). These effects can lead to liver toxicity, disruption of endocrine function, reduced immunity, and finally, harmful effects on the reproductive systems of marine animals (Sutton et al., 2016; Mallik et al., 2021). In contrast, the presence of MPs in terrestrial ecosystems is still understudied. The complexity of distribution patterns and pathways on land is likely the main factor contributing to this issue, along with the identification of aquatic systems as a primary endpoint for MP pollution. Additionally, MPs on land are often difficult to detect due to masking by vegetation and soils (Malizia and Monmany-Garzia, 2019). Some laboratory studies have shown that earthworms can play a crucial role in redistributing MPs, as their actions lead to an increased downward movement of MPs in soil (Rillig et al., 2017). Terrestrial animals including livestock can ingest microplastics through contaminated water or food. This can lead to the accumulation of plastics in their bodies, which may then be transferred to humans through the consumption of animal products. In humans, microplastics have been shown to cause various health issues, such as inflammation, oxidative stress, and cellular damage, primarily due to their physical and chemical properties. The mechanisms of MP's harmful effects are complex because of various entry points in the body (skin, respiratory, digestive system), different distribution routes depending on the chemistry (blood stream, different receptors; Benson at al., 2022, Veen et al., 2022), and their protentional role as carriers of other harmful substances and microorganisms (Ullah et al., 2023). Initially, there was limited concern when MP particles were found in feces, as it was thought that it passed through the digestive system and exited without causing harm (Beriot et al, 2021). However, concern has increased with the realization that MPs can enters the bloodstream and be distributed throughout all organ systems continuing their way up the food chain (Veen et al., 2022). The same authors have found polyvinyl chloride (PVC-P), PE, and polymers of styrene (Styr-P) in pigs and cows' blood. There is evidence that MP particles are transformed by the bovine rumen micropopulation (Wu et al., 2022); plastic polymer type polyethylene was present in the cows feed and polypropylene was found in the manure. The highest concentration of MPs, (0.19 items/g) was found in cows' meat. Predominant polymer types were nylon and fiber cow and sheep tissues (Bahrani et al., 2024). MPs have even been discovered in animal products, such as milk and eggs (Da Costa Filho et al., 2021; Liu et al., 2022). MP concentrations in the egg yolk were higher (8.95 MP egg−1) compared with the egg white (3.40 MP egg−1) (Liu et al., 2022). Therefore, more studies should investigate alternative ways in which microplastics are excreted in livestock (Aardema et al., 2024).
A study on the impact of microplastics on the fungal community during cow manure and sawdust composting found that MPs, particularly polyethylene and polyhydroxyalkanoate,

affected the diversity of the fungal community and increased the number of phytopathogenic fungi in the compost (Zhou et al., 2022).

IMPACT OF MICROPLASTICS ON REPRODUCTIVE HEALTH

A significant amount of plastic is used on farms and enters the environment through many sources: transportation, building constructions, agricultural production, plastic-coated fertilizers and biosolids (Okoffo et al., 2021). Long-term application of biosolids, often leads to the fragmentation of plastic into smaller particles, due to the UV radiation, water and wind (Briasouliss et al., 2015). Animal manure is another source of microplastics due to its widespread use as fertilizer on farmlands across the world. The application of chicken manure compost mixed with NPK fertilizer on agricultural soil for 13 years contributed 1.61 × 10^9 MP particles per hectare, according to Zhang et al., 2022. Additionally, a recent study in the U.S. found that topsoil amended by cattle manure has on average 1.1 ± 0.3 MP particles per gram of dry weight (Beni et al., 2023). Drinking water is also a notable source of MPs in farms which is proven recently in the World Health Organization's (WHO) report on "Microplastic in drinking water". This document confirms that we still do not know how MPs in water influence health, but it may be similar to the act on marine organisms in the oceans (WHO, 2019). However, if we do not know how harmful microplastics are for the living organism, there is evidence that small absorbed particles reach the gonads (Piehl et al., 2018). This accumulation is always connected with reproductive dysfunction. Wei et al. (2022) informed about more expressed ovarian cysts, more frequent *corpora lutea* presence, dilatation of the oviducts, lower number of growing follicles, a thinner layer of the granulosa cells in the female mouse and rats. In the testes of male animals an increase in SOD and MDA activities were described as a result of antioxidative response after MPs exposure (Deng et al. 2021). Toxicological research has demonstrated that MPs elicit toxic reactions in pig testicular cells, leading to apoptosis and necrosis of testicular tissue (Wang et al., 2022).
Microplastics and nanoplastics induce the proinflammatory and prooxidant processes, as well as disrupt reproductive hormone concentrations in male and female animals. Regarding inflammatory effectors, plastic particles have been shown to upregulating the abundance of TNF-α (tumor necrosis factor), interleukin IL-1ß, IL-6, IL-8 and the apoptotic factor caspase-3 (Wang et al., 2022). The hormonal panel showed a consistent downregulation of T4 (testosterone), LH (luteinizing hormone), FSH (follicle-stimulating hormone) and AMH (anti-Mullerian hormone) concentrations (Jin et al., 2022). The same authors had found that accumulation of MPs in testes of mice decreased sperm quality and testosterone concentration. Consequently, inflammation of the testis and male reproductive dysfunction had occurred (Jin et al., 2022). After exposure to polystyrene microplastics, the concentrations of FSH, LH and T4 decreased while estradiol level increased in the serum of male mice. In contrast, the observed hormonal changes in females were the opposite (Wei et al., 2022).

IMPACT OF MICROPLASTICS ON ENDOCRINE SYSTEM

Microplastics often carry harmful chemicals and additives that they have absorbed, including endocrine disrupting compounds (EDCs), such as bisphenols, phthalates, polybrominated and polychlorinated biphenyl ethers, and perfluorinated compounds. They are known to disrupt the hormonal system by mimicking or blocking nuclear receptors like androgen and estrogen receptors (Galloway et al., 2017). One study examined the sorption behaviors of three EDCs on polyamide MPs under simulated environmental conditions, finding that polyamide MPs have the potential to serve as carriers of these EDCs in environmental systems (Lara et al., 2021). In a more recent study, it is indicated that the presence of MPs leads to differences in the adsorption capacity between regular soil and soil contaminated with MPs. A potentially improved retention in soil due to stronger interactions between bisphenol A and the polyamide MP particles was observed as well (Pozo et al., 2024). This

raises concern since increased affinity of EDCs for adsorption onto MPs may result in their higher accumulation, raising the risk of better bioavailability to soil organisms, and subsequently farm animals.
There is limited number of research studies on the direct effects of MPs on the mammalian endocrine system. However, long-term exposure to MPs and associated chemicals has been shown to weaken of thyroid endocrine function, leading to a decreased regulation of growth, development, metabolism, and reproduction in marine vertebrates (Kloas et al., 2009). Nanoplastic particles have been found to not only significantly inhibit the proliferative capability of pig kidney cells, but also to induce inflammation and cell senescence, leading to mitochondrial damage and the accumulation of mitochondrial reactive oxygen species, ultimately resulting in cellular inflammation and death (Lu and Wei, 2024).

CONCLUSIONS

Microplastics pose a serious threat to animal health, affecting a wide range of species across various environments. Addressing this issue requires a concerted effort from scientists, policymakers, industries, and the public to reduce plastic pollution and protect animal health.

ACKNOWLEDGEMENTS

The paper was made in the frame of the project ECO(RE)ACT 2024 HR-RS 00018 approved by the Ministry of Regional Development and EU Funds of the Republic of Croatia.

REFERENCES

Aardema, H., Vethaak, A., Kamstra, J. H., & Legler, J. (2024): Farm animals as a critical link between environmental and human health impacts of micro-and nanoplastics. *Micropl.&Nanopl*. 4, 5 https://doi.org/10.1186/s43591-024-00082-w

Bahrani, F., Mohammadi, A., Dobaradaran, S., De-la-Torre, G. E., Arfaeinia, H., Ramavandi, B., Saeedi, R., & Tekle-Röttering A. (2024): Occurrence of microplastics in edible tissues of livestock (cow and sheep). *Environ Sci Pollut Res Int.*;31(14):22145-22157. doi: 10.1007/s11356-024-32424-9. PMID: 38403824.

Beni, N., Karimifard, S., Gilley, J., Messer, T., Schmidt, A., & Bartelt-Hunt, S. (2023): Higher concentrations of microplastics in runoff from biosolid-amended croplands than manure-amended croplands. *Communications Earth & Environment.* 4;42. 10.1038/s43247-023-00691-y.

Beriot, N., Peek, J., Zornoza, R., Geissen V., &Huerta L. E. (2021): Low density-microplastics detected in sheep faeces and soil: a case study from the intensive vegetable farming in Southeast Spain. *Sci Total Environ.* ;755(Pt1):142653.

Benson, N. U., Agboola, O. D., Fred-Ahmadu, O.H., De-la-Torre G. E., Oluwalana A., & Williams A. (2022): Micro (Nano) plastics prevalence, food web interactions and toxicity assessment in aquatic organisms: A review. *Front Mar Sci* 9:291. doi: 10.3389/fmars.2022.851281

Briassoulis, D., Babou, E., Hiskakis, M., & Kyrikou, I. (2015): Analysis of long-term degradation behavior of polyethylene mulching films with pro-oxidants under real cultivation and soil burial conditions. *Environ. Sci. Pollut. Res*., 22, 2584–2598.

Campanale, C., Massarelli, C., Savino, I., Locaputo, V., & Uricchio, V. F. (2020): A detailed review study on potential effects of microplastics and additives of concern on human health. *Int. J. Environ. Res. Public Health*, 17, 1212.

Da Costa Filho, P. A., Andrey, D., Eriksen, B., Peixoto, R. P., Carreres, B. M., Ambühl M. E., Descarrega J. B., Dubascoux, S., Zbinden, P., Panchaud, A., & Poitevin E. (2021): Detection and characterization of small-sized microplastics (≥ 5 μm) in milk products. *Sci Rep*. 11(1):24046. doi: 10.1038/s41598-021-03458-7. PMID: 34911996; PMCID: PMC8674347.

Deng, Y., Yan, Z., Shen, R., Huang, Y., Ren, H., & Zhang, Y. (2018): Enhanced reproductive toxicities induced by phthalates contaminated microplastics in male mice (Mus musculus). *J. Hazard. Mater*., 406, 124644.

European Parliament (2018): Plastic waste and recycling in the EU: facts and figures,https://www.europarl.europa.eu/topics/en/article/20181212STO21610/plastic-waste-and-recycling-in-the-eu-facts-and-figures, accessed: 15. 7. 2024.

ECHA (2023): Microplastics; https://echa.europa.eu/hot-topics/microplastics; accessed 19.7. 2024.

Feng, S., Lu, H., Yao, T., Tang, M., & Yin C. (2023): Analysis of microplastics in soils on the high-altitude area of the Tibetan Plateau: Multiple environmental factors. *Sci Total Environ*.; 857(Pt 1):159399. doi:10.1016/j.scitotenv.2022.159399

Galloway, T. S., Cole, M., & Lewis, C. Interactions of microplastic debris throughout the marine ecosystem. *Nat. Ecol. Evol.*, 1 (2017), pp. 1-8, 10.1038/s41559-017-0116

Hurley, R. R., Lusher, A. L., Olsen, M., & Nizzetto L. (2018): Validation of a method for extracting microplastics from complex, organic-rich, environment matrices. *Environ Sci Technol*., 52(13):7409–17.

Kloas, W., Urbatzka, R., Opitz, R., Würtz, S., Behrends, T., Hermelink, B., Hofmann, F, Jagnytsch, O, Kroupova H, Lorenz, C., Neumann, N., Pietsch, C., Trubiroha, A., Van Ballegooy, C., Wiedemann, C., & Lutz I. (2009): Endocrine disruption in aquatic vertebrates. *Ann New York Acad Sci* 1163(1):187–200. doi: 10.1111/j.1749-6632.2009.04453.x

Jin, H., Yan, M., Pan, C., Liu Z., Sha X., Jiang C., Li L., Pan M., Li D., Han X., & Ding J (2022): Chronic exposure to polystyrene microplastics induced male reproductive toxicity and decreased testosterone levels via the LH-mediated LHR/cAMP/PKA/StAR pathway. *Part Fibre Toxicol* 19, 13 https://doi.org/10.1186/s12989-022-00453-2

Lara, L. Z., Bertoldi, C., Alves, N.M., & Fernandes, A .N. (2021): Sorption of endocrine disrupting compounds onto polyamide microplastics under different environmental conditions: Behavior and mechanism, *Science of The Total Environment,* 796, p. 148983. 10.1016/j.scitotenv.2021.148983

Liu, Q., Chen, Z., Chen, Y., Yang, F., Yao, W,. & Xie, Y. (2022): Microplastics contamination in eggs: Detection, occurrence and status. *Food Chem.* 397, 133771. 10.1016/j.foodchem.2022.133771

Lu G., & Wei, S. (2024): Nanoplastics trigger the aging and inflammation of porcine kidney cells. Toxicology., 506, 153870. doi:10.1016/j.tox.2024.153870

Mallik, A., Xavier, K.A.M., Naidu, B., & Nayak, B. (2021): Ecotoxicological and physiological risks of microplastics on fish and their possible mitigation measures. *Science of The Total Environment*. 779; 146433. 10.1016/j.scitotenv.2021.146433.

Malizia, A., & Monmany-Garzia A. C. (2019): Terrestrial ecologists should stop ignoring plastic pollution in the Anthropocene time. *Sci Total Environ* 668:1025-1029. doi:10.1016/j.scitotenv.2019.03.044

Martins, I., Rodríguez, Y., & Pham, C. (2020): Trace elements in microplastics stranded on beaches of remote islands in the NE Atlantic. *Marine Pollution Bulletin*. 156; 111270. 10.1016/j.marpolbul.2020.111270.

McIvor, A., Pires, R., Lopes, C., Raimundo, J., Campos, P., Pais, M., Canning-Clode, J., & Dinis, A. (2022): Assessing microplastic exposure of the Critically Endangered Mediterranean Monk Seal (*Monachus monachus*) on a remote oceanic island. *Science of The Total Environment*. 856; 159077. 10.1016/j.scitotenv.2022.159077.

Okoffo, E. D., O'Brien, S., O'Brien, J.W., Tscharke, B.J., & Thomas, K.V., (2019): Wastewater Treatment Plants as a Source of Plastics in the Environment: A Review of Occurrence, Methods for Identification, Quantification and Fate. *Environ. Sci*., 5, 1908–1931.

Piehl, S., Leibner, A., Löder, M.G.J., Dris, R., Bogner C. & Laforsch C. (2018): Identification and quantification of macro- and microplastics on an agricultural farmland. *Sci Rep* 8, 17950 DOI:10.1038/s41598-018-36172-y

Pozo, L., Mejías, C., Santos, J., Martín, Bueno, J., Aparicio, I. & Alonso, E. (2024): Influence of microplastic contamination on the dissipation of endocrine disrupting chemicals in soil environment. *Environmental Pollution*. 2024; 349; 123919. 10.1016/j.envpol.2024.123919.

Rillig, M. C., Ziersch, L., & Hempel, S. (2017): Microplastic transport in soil by earthworms. *Sci Rep.,* 7(1):1362. doi:10.1038/s41598-017-01594-7

Sutton, R., Mason, S. A., Stanek, S. K., Willis-Norton, E., Wren, I. F., & Box, C. (2016): Microplastic contamination in the San Francisco Bay, California, USA. *Mar. Pollut. Bull.* 109, 230–235. https://doi.org/10.1016/j.marpolbul.2016.05.077.

Thompson, R. C., Swan, S. H., Moore, C. J. & vom Saal, F. S. (2009): Our plastic age. *Phil. Trans. R. Soc*. B3641973–1976 http://doi.org/10.1098/rstb.2009.0054

Ullah, S., Ahmad, S., Guo, X., Ullah S., Ullah S., Nabi G., & Wanghe K. (2023) A review of the endocrine disrupting effects of micro and nano plastic and their associated chemicals in mammals. *Front. Endocrinol.* 13:1084236. doi: 10.3389/fendo.2022.1084236:

Veen van der I., Mourik van L. M., Velzen van M. J. M., Groenewoud Q. R. & Leslie H. A. (2022): Plastic Particles in Livestock Feed, Milk, Meat and Blood - A Pilot Study. Department of Environment & Health, Vrije Universiteit Amsterdam. 2022.

Wang, X., Zhang, X., Sun, K., Wang, S. & Gong, D. (2022): Polystyrene microplastics induce apoptosis and necroptosis in swine testis cells via ROS/MAPK/HIF1α pathway. *Environmental Toxicology*; 37(10): 2483-2492. doi:10.1002/tox.23611

World Health Organization. Microplastics in Drinking-Water; World Health Organization: Geneva, Switzerland, 2019; ISBN 9789241516198.

Wei, Z., Wang, Y., Wang, S., Xie, J., Han, Q. & Chen, M. (2022): Comparing the effects of polystyrene microplastics exposure on reproduction and fertility in male and female mice. Toxicology, 465, 153059.

Welden N. A. & Lusher A. (2020): Microplastics. Plastic Waste and Recycling. In: Plastic Waste and Recycling, Letcher TM (Ed.), Academic Press, 223–249. doi:10.1016/b978-0-12-817880-5.00009-8.

Wu, R., Cai, Y., Chen, Y., Yang, Y., Xing, S., & Liao, X. (2021): Occurrence of microplastic in livestock and poultry manure in South China. *Environ Pollut*. 277:116790.

Zhang, J., Wang, X., Xue, W., Xu, L., Ding, W., Zhao, M., Liu, S., Zou, G. & Chen, Y. (2022): Microplastics pollution in soil increases dramatically with long-term application of organic composts in a wheat–maize rotation. *J. Clean. Prod.,* 356, 131889, 10.1016/j.jclepro.2022.131889

Zhou, Y., Sun, Y., Liu, J., Ren, X., Zhang, Z. & Wang, Q. (2022): Effects of microplastics on humification and fungal community during cow manure composting. *Sci. Total Environ*., 803, 150029, 10.1016/j.scitotenv.2021.150029

HIGH PRESSURE PROCESSING OF FERMENTED SAUSAGES APPLIED AT THE EARLY STAGE OF RIPENING REDUCES LACTIC ACID BACTERIA HETEROFERMENTATIVE ACTIVITY

Ducic, M.[1*], Barcenilla C.[2], Cobo-Diaz, J.[2], Lopez, M.[2,3], Alvarez-Ordonez, A.[2,3], Prieto, M.[2,3]

[1]Faculty of Agriculture, University of Novi Sad, Trg D. Obradovića 8, 21000 Novi Sad, Serbia
[2]IFaculty of Veterinary Medicine, University of Leon, Campus de Vegazana, 24071 Leon, Spain
[3]I Institute of Food Science and Technology University of Leon, Calle *de* la Serna, nº 58 Campus de Vegazana, 24071 Leon, Spain

*Corresponding author:
E-mail address: jamerika.miroslav@gmail.com

ABSTRACT

Lactic acid bacteria in fermented sausages contribute to fermentation by several positive effects, like acidification, desirable degradation of initial sausage components, competitiveness and production of inhibitory compounds such as bacteriocins. Heteroferementative microbiota, in addition to lactic acid, produce to a lesser extent also acetic and formic acids, hydrogen peroxide, carbon dioxide and ethanol. However, higher quantities of these substances can lead to flavor, color and textural problems of the final products, like sour taste, hole formation and/or grey discoloration of sausages. Among additional processing measures for improving quality, high pressure technology is used mainly to increase shelf life and the safety of meat products. The aim of the study was to examine heterofermentative activity in dry fermented sausages produced without or with starter culture and processed by high pressure at the early stage of ripening. Application of high pressure at the optimal moment reduced significantly counts of colonies with heterofermentative activity in products without starter culture. Percentage of heterofermentative microbiota decreased from 50.6% to 6.5% in the total population of lactic acid bacteria in pressurized sausages, compared to untreated counterparts. Also, ratio of microbial community members assessed by amplicon 16S rRNA metataxonomics show that in total population percentage of obligate heterofermentative *Leuconostoc* genus clearly decreased (23.3% to 11.6%), while the percentage of *Latilactobacillus* increased (14.9% to 31.3%) in final products after high pressure treatment. Expectedly, in sausages with starter culture (pressurized or not) no colonies with heterofermentative activity were detected. The patterns of changes of main physicochemical parameters (pH and a_w) in all sausages during ripening were usual for this type of products. Results showed that the application of high pressure at the optimal moment of the sausage ripening induces reduction of indigenous lactic acid bacteria heterofermentative activity.
***Keywords**: lactic acid bacteria, high pressure processing, sausages, heterofermentation, preservation*

INTRODUCTION

Lactic acid bacteria (LAB) in fermented sausages contribute to fermentation by several positive effects, like acidification, desirable degradation of initial sausage components, competitiveness and production of inhibitory compounds such as bacteriocins. Heteroferementative microbiota, in addition to lactic acid, produce to a lesser extent also acetic and formic acids, hydrogen peroxide, carbon dioxide, ethanol and other substances which contribute positively to the safety and sensorial quality of fermented products. However, higher quantities of these substances can lead to flavor, textural and color problems like sour taste, hole formation and/or grey discoloration of sausages (Labadie, 2007; Koch, 2004). Among additional processing measures for improving quality, high pressure as a novel technology is used mainly at the final stage of production to increase shelf life and the safety of meat products. Application of high pressure just after fermentative microbiota reach maximum levels is a promising new approach in processing of dry fermented sausages and there are no previous studies about impact of this new approach on heterofermentative activity of lactic acid bacteria in such treated products. So, the aim of the study was to examine heterofermentative activity in Spanish dry fermented sausages

produced without or with starter culture and processed by high pressure at the early stage of ripening.

MATERIAL AND METHODS

The composition of chorizo type of sausages was: pork meat (72%), pork back fat (21%), spices (3.5 %, paprika, garlic, oregano), NaCl (1.6%) and Ligavi 384® (1.8 % dextrin and dextrose 82.5%,pork proteins 17.5%), The batter was stuffed into natural pork casings (32-34 mm diameter). Half of the sausages were produced with starter (*Latilactobacillus sakei*, *Pediococcus acidilactici*, and *Staphylococcus carnosus*) and another half as artisanal (without starter). Experimental design is presented in Figure 1:

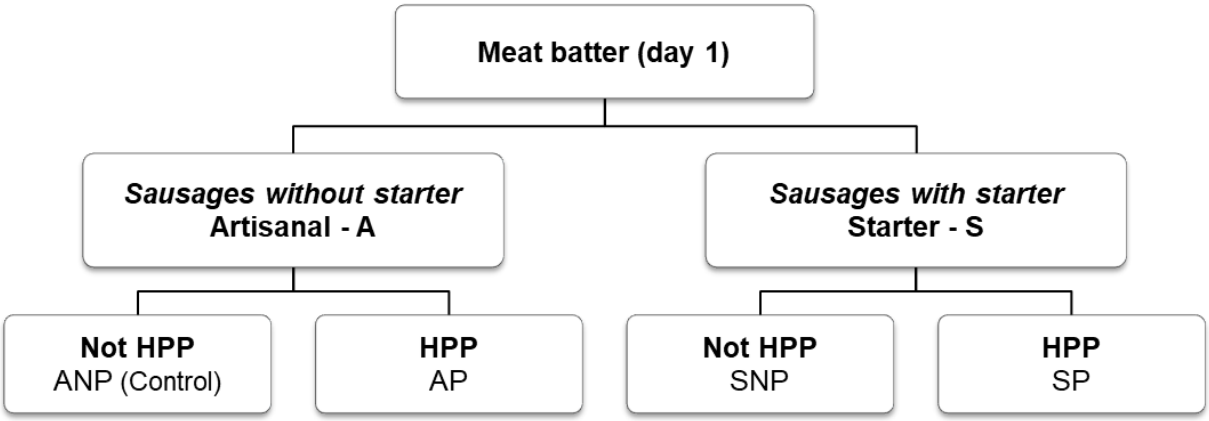


Figure 1: Scheme of the experiment

Legend: ANP - Sausages without starter and without HPP; AP - sausages without starter and with HPP; SNP - sausages with starter and without HPP; SP - sausages with starter and with HPP

Sausages were stored in a ripening chamber with temperature and humidity control (12-14°C; 75% RH) for 5 weeks. Physico-chemical analyses were performed on sausages at the start (day 1), at the mid-process (day 18) and at the end of the process (day 36). Examination of physico-chemical parameters in sausages comprised of determination of a_w and pH using LAB swift-a_w Euro-plug&BAT set equipment (Novasina) and pH meter, Crison pH 25 (Crison Instruments), respectively.

High pressure treatment (300 MPa, 5 min) was applied on sausages after 7 days from the beginning of the process, i.e. when LAB have already grown to their maximum level. For that purpose industrial hydrostatic pressure unit (Wave 6000/135, Hyperbaric) was used. After high pressure processing (HPP) sausages were returned to the ripening chamber for ripening. All sausages (pressurized or not) were microbiologicaly examined after 36 days of production.

Lactic acid bacteria counts were determined on Lactic Acid Bacteria Count Plates (3M Health Care) at 30°C for 48 h (AFNOR validated method 3M 01/19-11/17). Heterofermentative activity of LAB was also evaluated on LAB Count Plates and its manifestation was presented as colonies with bubbles, in accordance with the aforementioned producer interpretation guide.

Evolution of microbial communities was assessed by amplicon 16S rRNA metataxonomics. Amplicon sequencing of the 16S rRNA gene was done with the Illumina Miseq platform using 300 bp pair-end sequencing. The primers used were S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Carrasco et al., 2020), which amplify the V3-V4 hypervariable region of the 16S rRNA gene, giving an average amplicon length of 430 bp.
PCR amplification, preparation of Miseq libraries, and sequencing were performed at the sequencing platform of Centro de Investigacion Biomedica de La Rioja (CIBIR), Spain.

RESULTS AND DISCUSSION

Levels of pH in control (ANP) and sausages with starter (pressurized or not) were similar during ripening, while in pressurized sausages without starter values became somewhat higher probably due to inhibition of indigenous microbiota by HPP to a moderate extent.

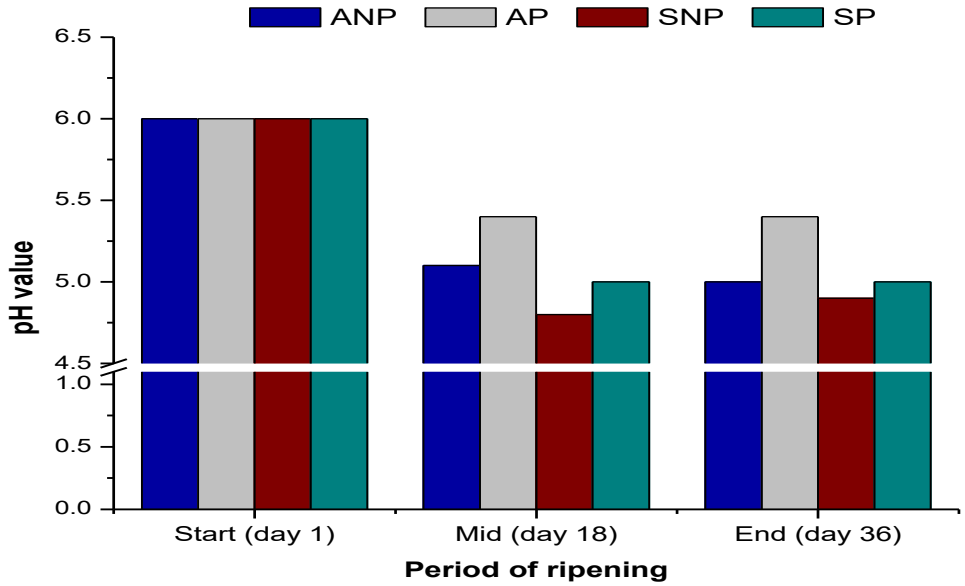


Figure 2. pH value in sausages

The water activity in all sausages gradually decreased during ripening reaching final values below 0.9 at the end of the production (Figure 3). These results are similar with previous findings regarding traditional Spanish type of sausages manufactured at low temperature and relative humidity conditions (Latorre-Moratalla et al., 2010). However, presence of starter culture and HPP induced in sausages more intensive decrease of water activity during ripening probably due to increase in denaturation of proteins with consequently easier evacuation of water.

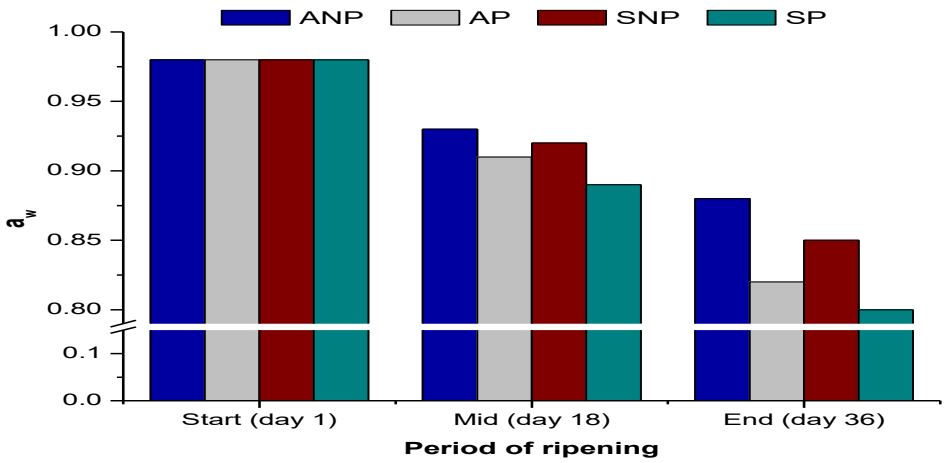


Figure 3. Water activity in sausages

Application of high pressure after 7 days of ripening markedly reduced counts of colonies with heterofermentative activity in products without starter culture (Table 1). Percentage of heterofermentative microbiota decreased from 50.6% to 6.5% in the total population of lactic acid bacteria in pressurized sausages, compared to untreated counterparts. Expectedly, in sausages with starter culture (pressurized or not) no colonies with heterofermentative activity were detected.

Table 1. Count of lactic acid bacteria in dry fermented sausages at the end of production

Lactic Acid Bacteria	**Unit**	**Batches of sausages**			
		ANP	**AP**	**SNP**	**SP**
Number of all colonies	log10 ± SD	9.4 ± 0.3	8.9 ± 0.4	8.7 ± 0.4	7.4 ± 0.4
Number of colonies with bubbles	log10 ± SD	9.1 ± 0.3	7.8 ± 0.5	NF	NF
Percentage of colonies with bubbles in all colonies	%	50.6	6.5	ND	ND

NF – Not found; ND – Not determined

The ratio of microbial community members indicates that in total population percentage of obligate heterofermentative *Leuconostoc* genus clearly decreased (from 23.3% to 11.6%), while the percentage of homofermentative *Latilactobacillus* increased (from 14.9% to 31.3%) in final products, after high pressure treatment (Table 2). In sausages with starter culture *Latilactobacillus* genus decreased from 94.3% to 86.6% after HPP, probably due to the effect of pressurization on selected starter strain, which consequent increased competition of indigenous genera in sausage matrix during the remaining ripening period. In sausages with starter culture presence of *Leuconostoc* genus was negligible, weather products were pressurized or not.

Table 2. Ratio of Latilactobacillus and Leuconstoc in dry fermented sausages at the end of production

Percentage of genus in microbiota community	Batches of sausages			
	ANP	**AP**	**SNP**	**SP**
Latilactobacillus	14.9%	31.3%	94.3%	86.6%
Leuconostoc	23.3%	11.6%	0.06%	0.04%

CONCLUSIONS

The patterns of changes of main physicochemical parameters in all sausages (pressurized or not) during ripening were usual for this type of product.
The results showed that applying high pressure at the optimal moment during sausage ripening induces a reduction in the heterofermentative activity of indigenous lactic acid bacteria and their potentially negative effects on the quality of these products.

ACKNOWLEDGEMENTS

The financial support of the Ministry of Education, Science, and Technological Development of the Republic of Serbia (no. 451-03-66/2024-03) and MASTER (project from the EU Horizon 2020 research and innovation programme (no 818368) is gratefully acknowledged.

REFERENCES

Carrasco, J., García-Delgado, C., Lavega, R., Tello, M. L., Toro, M. D., Barba-Vicente, V., Rodrigez-Cruz, M.S., Sanchez-Martin, M. J., Perez, M. & Preston, G. M. (2020). Holistic assessment of the microbiome dynamics in the substrates used for commercial champignon (Agaricus bisporus) cultivation. *Microbial Biotechnology, 13*(6), 1933–1947. https://doi.org/10.1111/1751-7915.13639

French Association for Standardization (AFNOR) (2017) *Validation of alternative analysis methods.* 3M™ Petrifilm™ Lactic Acid Bacteria Count Plate. Paris, France.

Koch, G. (2004). Biopreservation. In W.K. Jensen, C. Devine & M. Dikeman (Eds.), *Encyclopedia of meat sciences* (pp. 68–74). Oxford: Elsevier.

Labadie, J. (2007). Spoilage microorganisms: risks and control. In F. Toldra, Y. H. Hui, I. Astiasaran, WK, Nip, J.G. Sebranek, ET. F. Silveira, L.H. Stahnke, R. Talon (Eds.) *Handbook of fermented meat and poultry* (pp. 421-427). Ames: Blackwell Publishing

Latorre-Moratalla, M.L., Bover-Cid, S. & Vidal-Carou, M.C. (2010). Technological conditions influence aminogenesis during spontaneous sausage fermentation. *Meat Science*, *85*, 537-541.

MICROPLASTICS CONTAMINATION IN AGRICULTURAL SYSTEMS

Mislav Đidara[1]*, Marcela Šperanda[1], Brigita Popović[1], Nataša Stojić[2], Laszlo Galambos[3],

[1]Josip Juraj Strossmayer University of Osijek Faculty of Agrobiotechnical Sciences Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia
[2]Educons University in Sremska Kamenica, Vojvode Putnika 85-87, 21208 Sremska Kamenica, Serbia
[3] Institute for Nature Conservation of Vojvodina Province, Radnička 20a, 21101 Novi Sad, Serbia

*Corresponding author:
E-mail address: mdidara@fazos.hr

ABSTRACT

Microplastics have become a major environmental concern, contaminating water, soil, and air, and impacting crop quality, food safety, and human health. They enter agricultural systems through irrigation water, soil amendments, flooding, and atmospheric deposition. Microplastics can alter soil structure and fertility, and plants can absorb them, leading to food chain contamination. Domestic animals ingest microplastics through contaminated feed, further exacerbating the issue. Addressing microplastic contamination in agriculture requires policy measures, sustainable practices, waste management, and public education. The EU's strategies, such as the European Green Deal and the EU Plastics Strategy, aim to reduce environmental impacts through regulations like REACH and the Single-Use Plastics Directive, promoting biodegradable alternatives and enhancing recycling. Monitoring microplastics in irrigation water and soil is essential for understanding contamination levels. Standardized detection methods ensure consistent data and reliable assessments. Microplastics pose food safety risks as they accumulate in animal tissues and carry harmful chemicals like persistent organic pollutants and heavy metals. Ingested by humans, these chemicals can cause inflammation, oxidative stress, and endocrine disruption, increasing health risks. The ECO(RE)ACT project, funded by Interreg IPA Croatia-Serbia, aims to reduce microplastics contamination and enhance climate change resilience in Croatia and Serbia through agricultural plastic waste management, ecosystem monitoring, and innovative mitigation solutions. By leveraging cross-border expertise, the project seeks to reduce microplastics contamination and promote sustainable agricultural practices.
***Keywords**: Microplastics contamination, Agricultural systems, Food safety, Environmental health*

INTRODUCTION

Microplastics have become a significant environmental issue in recent years. They originate from the breakdown of larger plastic waste or the use of plastic microbeads in products (Rocha-Santos & Duarte, 2015). These tiny particles are ubiquitous, contaminating water, soil, and even the air we breathe. Their presence in agricultural systems directly threatens crop quality and food safety, ultimately impacting human health.

Microplastics enter agricultural systems through various pathways, including contaminated irrigation water, soil amendments like compost and sludge, flooding of agricultural fields, and atmospheric deposition. These particles can accumulate in the soil, affecting its structure and fertility (Rai et al., 2023). Moreover, plants can absorb them, entering the food chain and posing risks to human health (Baho et al., 2021). Domestic animals can also ingest microplastics through contaminated feed, exacerbating the issue. The omnipresence of microplastics in environment necessitates a comprehensive approach for effective solution.

POLICY FRAMEWORK AND NECESSARY INFRASTRUCTURE FOR REDUCING MICROPLASTICS

Addressing microplastic pollution in agriculture requires a combination of policy measures, sustainable practices, improved waste management, and public education.

Governments must play a crucial role in reducing microplastic contamination through regulations that limit plastic use and improve waste management practices. The European Union has implemented a multi-faceted approach to combat microplastic pollution,

integrating strict policy frameworks, sustainable agricultural practices, enhanced waste management strategies, and comprehensive public education initiatives. At the heart of these efforts lies the European Green Deal (European Commission, Directorate-General for Communication, 2021), launched in 2019 to guide the EU towards climate neutrality by 2050 and promote a circular economy that minimizes environmental impact across all sectors. Central to this plan is the EU Plastics Strategy, introduced in 2018, aimed at reassessing the lifecycle of plastics from production to disposal to mitigate their harmful effects on ecosystems.

The EU regulatory framework includes REACH regulations (Regulation (EC) No 1907/2006) and the Single-Use Plastics Directive (Directive (EU) 2019/904), complementing efforts to combat microplastic pollution. The REACH regulation, supported by Commission Regulation (EU) 2023/2055, bans synthetic polymer microbeads in products and mandates producers to ensure their safe disposal. Simultaneously, the Single-Use Plastics Directive restricts plastics contributing to microplastic pollution, promotes alternatives, and strengthens recycling efforts to reduce environmental impact.

Encouraging agricultural producers to reduce plastic and microplastic usage requires a comprehensive approach (Briassoulis, 2023). Governments can incentivize changes through financial incentives and regulatory frameworks that encourage the use of biodegradable alternatives and limit harmful plastics. Educational campaigns are essential for raising awareness among farmers about the environmental impacts of plastics and microplastics, providing training and showcasing successful sustainable practices. Technological innovations, such as biodegradable mulches and precision agriculture, play a crucial role in resource optimization and reducing plastic use (Kiran et al., 2022).

Improving recycling infrastructure tailored to agricultural plastics and promoting circular economy practices further supports sustainable waste management. Collaboration with industry stakeholders, community engagement, and funding for research and development are also critical to fostering innovation and ensuring the widespread adoption of sustainable practices in the agricultural sector. This integrated approach would reduce environmental harm while enhancing the efficiency and resilience of farms in changing climatic conditions (Briassoulis, 2023).

Effective waste management is crucial for reducing the release of microplastics into the environment (Kiran et al., 2022). Advancing recycling systems to ensure proper processing and reuse of plastic can reduce the amount of plastic waste degrading into microplastics. Additionally, reducing reliance on single-use plastics and promoting the use of biodegradable alternatives can further decrease the amount of plastic waste entering the environment (Rafiq & Xu, 2023).

Raising public awareness about the sources and impacts of microplastics is crucial for driving behavioral change. Educational campaigns can inform consumers about the importance of reducing plastic consumption and encourage them to make more sustainable choices. By promoting a culture of environmental responsibility, we can collectively reduce the demand for plastic products and decrease overall microplastic production (Briassoulis, 2023).

MONITORING MICROPLASTICS IN IRRIGATION WATER AND SOIL

Effective monitoring of microplastics is essential for understanding the extent of contamination and implementing appropriate reduction measures. Regular monitoring programs can track changes in microplastic levels over time and help identify contamination sources. Detecting the presence of microplastics in irrigation water sources and soil poses significant challenges due to the small size of microplastic particles, their widespread distribution, and complex detection methods (Pérez-Reverón et al., 2022).

Detecting microplastics in water samples requires specialized techniques such as filtration, separation, and spectroscopic analysis, which can be labor-intensive and costly (Rocha-Santos & Duarte, 2015). Furthermore, variability in sampling methods and environmental factors complicates data interpretation and comparison between studies (Fan et al., 2023).

Microplastics in soil can affect its structure and fertility, potentially impacting crop yields and quality (Baho et al., 2021). In soil, microplastics can enter through wastewater, flooding, or atmospheric deposition, posing challenges in detection and quantification. Soil samples may contain a diverse range of organic and inorganic materials that interfere with microplastic extraction and analysis, making reliable measurements difficult to achieve. Techniques such as separation, chemical digestion, and microscopy require careful validation to ensure reliable results (Rocha-Santos & Duarte, 2015).

Standardized protocols for sampling and analysis are crucial for reducing variability and ensuring data consistency on microplastics in different areas. Efforts are needed to develop reliable methodologies, establish baseline data, and assess long-term trends in microplastic contamination (Fan et al., 2023). Addressing these challenges is critical for understanding the full extent of microplastic pollution, assessing ecological risks, and formulating strategies to reduce their environmental impact.

MICROPLASTICS AS FOOD CONTAMINANT AFFECTING HUMAN HEALTH

Microplastics in the environment can ultimately affect human health through the food we consume. Monitoring microplastics should include animal feed and animal-derived food products, as their presence in these food sources poses significant food safety risks due to potential human health impacts. Microplastics enter animal feed through various pathways such as contaminated water sources, agricultural soil, or the air (Li et al., 2023). These tiny particles can accumulate in animal tissues over time, presenting a potential risk for humans who consume meat, dairy, and fish products. For instance, fish and other seafood can ingest microplastics directly from their aquatic environment, while livestock may ingest them through contaminated feed and water (Li et al., 2023).

Microplastic particles can contain or absorb harmful chemicals such as persistent organic pollutants (POPs) and heavy metals (Rai et al., 2023). POPs, which include substances like polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), are known for their long-lasting presence in the environment and potential to bioaccumulate in living organisms (Rai et al., 2023). Heavy metals like lead, cadmium, and mercury can also adhere to microplastic surfaces. These chemicals are toxic and can cause a range of health issues when ingested.

When humans consume animal products contaminated with microplastics and their associated chemicals, there are several potential health risks. Ingested microplastics can cause inflammation in the gastrointestinal tract, leading to various health problems, including leaky gut syndrome, which can allow harmful substances to enter the bloodstream (Li et al., 2023). Microplastics and their chemical contaminants can induce oxidative stress, leading to cellular damage, which is linked to numerous health conditions, including neurodegenerative diseases, cardiovascular diseases, and aging (Li et al., 2023). Many chemicals associated with microplastics, such as bisphenol A (BPA) and phthalates, are known endocrine disruptors, interfering with hormone function and potentially causing reproductive issues, developmental problems, and increased cancer risk. Some chemicals found in microplastics are carcinogenic, and prolonged exposure to these chemicals, even in small amounts, could increase cancer risk over time (Li et al., 2023).

PIONEERING ECOSYSTEM-BASED MICROPLASTICS MONITORING IN CROSS-BORDER REGION

Given the outlined problems mentioned above, the project “Pioneering ecosystem-based microplastics contamination reduction and climate change resilience in cross-border region ECO(RE)ACT” has been designed to address the critical issues of microplastics contamination within the cross-border region of Croatia and Serbia. This joint endeavor brings together three institutions: Josip Juraj Strossmayer University of Osijek Faculty of Agrobiotechnical Sciences Osijek (Croatia), Educons University's Faculty of Environmental

Protection (Serbia) and the Institute for Nature Conservation of Vojvodina Province (Serbia). Project is financed by Interreg IPA Croatia-Serbia.

The project aims to pioneer the reduction of microplastics contamination and enhance climate change resilience in a cross-border region through an ecosystem-based approach. It focuses on three main areas to achieve its objectives.

Firstly, the project develops a comprehensive strategy to raise awareness and implement sustainable practices for agricultural plastic waste management. This includes setting up coordination mechanisms, conducting a situational analysis, creating a joint strategy, and engaging stakeholders through effective communication efforts.

Secondly, the project creates collective solutions for monitoring microplastics in ecosystems. This involves organizing knowledge exchange workshops, identifying key control points for monitoring, and executing a pilot program to test these solutions.

Lastly, the project l pilots innovative solutions to mitigate microplastics contamination from agricultural production. It establishes a specialized Centre for Microplastics to serve as a research and mitigation hub, develop a certification process for assessing microplastics contamination, and implement educational initiatives to raise awareness among the public and stakeholders.

By leveraging the complementary expertise of its partners in agricultural and environmental sciences, the project aims to develop climate-resilient strategies. These efforts will ensure that microplastics detection capabilities and monitoring control points are adapted to changing environmental conditions. The cross-border approach will strengthen regional cooperation to address the complex challenges posed by microplastics pollution and climate change impacts, ultimately aiming for a measurable reduction in contamination levels and widespread adoption of sustainable practices in the agricultural sector.

CONCLUSIONS

The omnipresence of microplastics in the environment, particularly in agricultural systems, poses a significant threat to ecosystems and human health. Addressing this issue requires a comprehensive approach involving policy measures, sustainable agricultural practices, improved waste management, and public education. By implementing these strategies and establishing effective monitoring programs, the impact of microplastics can be mitigated and ensure a healthier, cleaner future for all.

REFERENCES

Baho, D. L., Bundschuh, M. and Futter, M. N. (2021). Microplastics in terrestrial ecosystems: Moving beyond the state of the art to minimize the risk of ecological surprise. *Global Change Biology, 27(17)*, 3969-3986.

Briassoulis, D. (2023). Agricultural plastics as a potential threat to food security, health, and environment through soil pollution by microplastics: Problem definition. *Science of The Total Environment, 892,* 164533.

Commission Regulation (EU) 2023/2055 of 25 September 2023 amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards synthetic polymer microparticles

Directive (EU) 2019/904 of the European Parliament and of the Council of 5 June 2019 on the reduction of the impact of certain plastic products on the environment

European Commission, Directorate-General for Communication, (2021) European green deal: delivering on our targets. Publications Office of the European Union. https://data.europa.eu/doi/10.2775/373022

Fan, W., Qiu, C., Qu, Q., Hu, X., Mu, L., Gao, Z. and Tang, X. (2023). Sources and identification of microplastics in soils. *Soil & Environmental Health, 1(2),* 100019.

Kiran, B. R., Kopperi, H., Venkata Mohan, S. (2022). Micro/nano-plastics occurrence, identification, risk analysis and mitigation: challenges and perspectives. *Reviews in environmental science and biotechnology*, 21(1), 169-203.

Li, Y., Tao, L., Wang, Q., Wang, F., Li, G. and Song, M. (2023). Potential health impact of microplastics: a review of environmental distribution, human exposure, and toxic effects. *Environment & Health, 1(4),* 249-257.

Pérez-Reverón, R., González-Sálamo, J., Hernández-Sánchez, C., González-Pleiter, M., Hernández-Borges, J. and Díaz-Peña, F.J., (2022). Recycled wastewater as a potential source of microplastics in irrigated soils from an arid-insular territory (Fuerteventura, Spain). *Science of The Total Environment, 817,* 152830.

Rafiq, A. & Xu, J.L. (2023). Microplastics in waste management systems: a review of analytical methods, challenges and prospects. *Waste Management, 171,* 54-70.

Rai, M., Pant, G., Pant, K., Aloo, B.N., Kumar, G., Singh, H.B. and Tripathi, V. (2023). Microplastic pollution in terrestrial ecosystems and its interaction with other soil pollutants: a potential threat to soil ecosystem sustainability. *Resources, 12(6),* 67.

Regulation (EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

Rocha-Santos, T. & Duarte, A. C. (2015). A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. *Trends in analytical chemistry*, 65, 47-53.

BIOSTIMULATION OF CRUCIFEROUS FOODS FOR HUMAN HEALTH

Cristina García-Viguera, Sonia Medina, Raúl Domínguez, and Diego A. Moreno*

Laboratorio de Fitoquímica y Alimentos Saludables (LabFAS), CEBAS, CSIC, Campus Universitario de Espinardo – 25, 30100, Murcia, Spain

*Corresponding and presenting author:
E-mail address: dmoreno@cebas.csic.es

ABSTRACT

Cruciferous vegetables (Brassicaceae) are rich in nutrients (vitamins, minerals, folates, etc.) and bioactive compounds (glucosinolates/isothiocyanates – GSL/ITC and phenolic compounds, vitamins, and minerals), associated with their health-promoting potential in modulation of several types of cancer and chronic non-communicable diseases, in which a chronic inflammatory condition is present at metabolic and neurological level. The current research lines on metabolite farming includes multidisciplinary work integrating the food chain – from seed to food and health – evaluating the influence of pre- and post-harvest factors to enrich foods in bioactive phytochemicals for human health, using sustainable practices. In this activity, we evaluated different varieties of cruciferous germinating seeds (red cabbage, red radish, broccoli, mustards, etc.) under controlled environmental conditions of hydroponics using priming, elicitors a selective LED light illumination to obtain sprouts/microgreens enriched in bioactive compounds, as sources of extracts and bioaccessible fractions of metabolites to investigate their anti-inflammatory and chemopreventive effects. The evaluation of bioactivity allowed to demonstrate significant antiinflammatory effects of sulforaphane (at therapeutic dosages), without any toxic effect, with higher potency than the parental glucosinolates (glucoraphanin, glucoraphenin) or phenolic compounds. The bioaccesible fractions of GSL/ITCs of red varieties of sprouts (radish and cabbage) demonstrated health-promoting effects on cellular models of chronic inflammation. This research line continues evolving with the study of the effects of GSL/ITC from cruciferous foods in adult population with overweight, to investigate on the physiological, metabolic, and microbiome factors involved in this chronic disease.
***Keywords**: Brassica, biofactory, bioactives, food, inflammation, obesity*

INTRODUCTION

The vegetables, microgreens, and derived ingredients of the cruciferous family (Brassicaceae) are rich in nutrients and bioactive compounds (glucosinolates/isothiocyanates (GSL/ITC), flavonol glycosides, cinnamoyl derivatives, vitamins, carotenoids, and minerals). According to the vast preclinical and clinical available research, the bioactive forms, ITC and indoles, as well as phenolic metabolites, are the responsible of their activity as health-promoters, against several chronic conditions and a diverse list of cancers and degenerative diseases, with a common pathological state of chronic inflammation (Costa-Pérez et al., 2023). The consumption of fresh broccoli sprouts enriched in GSL/ITC demonstrated effective modulation of the inflammation markers in overweight and obese adults (López-Chillón et al., 2019). This positive result triggered the interest to develop, evaluate, and optimize the production of novel ingredients and food products enriched in bioactive compounds from Brassicas for human health and wellbeing.

The biostimulation of the secondary metabolism of the growing sprouts, seedlings, and microgreens, is a sustainable and plausible strategy to enrich fresh foods in beneficial compounds (GSL/ITC) using a biofactory approach. The objective of delivering the necessary daily dosage of bioactive compound from a natural and fresh food will facilitate the nutrition and clinical studies, and the ingredients derived from Brassicas are effective anti-inflammatory agents with a great potential for the future (Ruiz-Alcaráz et al., 2022). The current research lines on metabolite farming includes multidisciplinary work integrating the food chain – from seed to food and health – evaluating the influence of pre- and post-harvest factors to enrich foods in bioactive phytochemicals for human health, using sustainable practices. In this activity, we evaluated different varieties of cruciferous germinating seeds

(red cabbage, red radish, broccoli, mustards, etc.) under controlled environmental conditions of hydroponics using priming, elicitors a selective LED light illumination to obtain sprouts/microgreens enriched in bioactive compounds, as sources of extracts and bioaccessible fractions of metabolites to investigate their anti-inflammatory and chemopreventive effects.

MATERIAL AND METHODS

The seeds of the different varieties of crucifers (Brassica oleracea var. italica L. cv. Calabrese; Raphanus sativus var. sativus L var. Sango and Rambo; Sinapis alba L., Brassica oleracea, var. capitata f. rubra; B. nigra) were supplied by Intersemillas S.A. (Loriguilla, Valencia, Spain). The cleaning and imbibition conditions for the initial germimation (Fig. 1.) as well as the biostimulation with different priming and elicitors are well described elsewhere (Abellán et al., 2021; Baenas et al., 2014; Hernández-Cánovas et al., 2021) (Figure1).

Figure 1. Cruciferous sprouts in growth Chamber at CEBAS-CSIC, detail of red cabbage sprouts and 96-well plate for in vitro anti-inflammatory tests with Brassica extracts.

The protocols of bioaccesibility of the sprouts using freeze-dried plant material is detailed in Abellán et al. (2021), following the INFOGEST accepted methodology without modifications. The set-up and establishment of the cell model for inflammation using HL-60 cells is described in García-Ibañez et al. (2023). The results (average± SD, n = 3) were analysed for ANOVA and Tukey Multiple Range Test ($P< 0.01$) using statistical analysis software SPSS 25.0 (LEAD Technologies, Inc., Chicago, IL, U.S.A.)

RESULTS AND DISCUSSION

The use of elicitors for biostimulating Brassicaceae species to enhance their phytochemical quality was a sustainable and effective strategy to obtain sprouts of different varieties with a range of bioactive compounds of interest for health-promoting purposes (Baenas et al., 2014; López-Chillón et al., 2019).

The selection (optimization) of the priming treaments for the induction of germination with different substances and stressors, in combination with the biostimulation (abiotic and biotic, elicitors including natural hormones, salts, etc.) (Baenas et al., 2014), under different conditions of LED illumination (broad-spectrum or monochromatic) (Guijarro-Real et al., 2022; Hernández-Cánovas et al., 2021), is a sustainable alternative to genetic modification, to obtain foods enriched in bioactives and plant-derived ingredients using sustainable practices.

The current research lines on metabolite farming includes multidisciplinary work integrating the food chain – from seed to food and health – evaluating the influence of pre- and post-harvest factors to enrich foods in bioactive phytochemicals for human health, using sustainable practices. In this activity, we evaluated different varieties of cruciferous germinating seeds (red cabbage, red radish, broccoli, mustards, etc.) under controlled environmental conditions of hydroponics using priming, elicitors a selective LED light illumination to obtain sprouts/microgreens enriched in bioactive compounds, as sources of extracts and bioaccessible fractions of metabolites to investigate their anti-inflammatory and chemopreventive effects

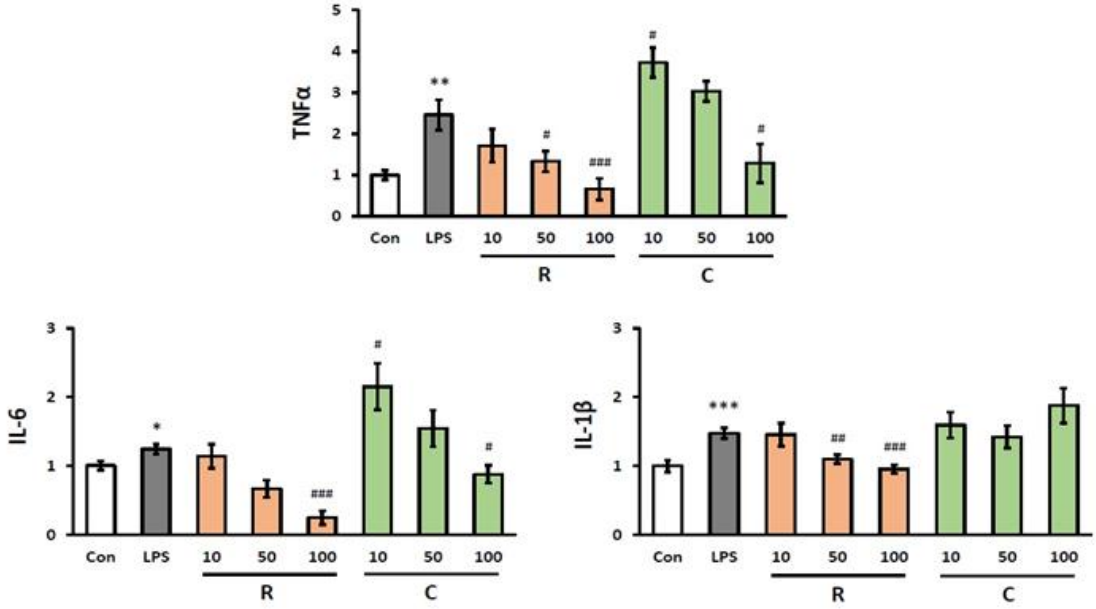


*Figure 2. Protein expression (ELISA) TNF-α, IL-6, and IL-1β of HL-60 differenciated cells and with LPS stimulus, treated with bioaccesible fraction of cruciferous sprouts of red radish (R) or red cabbage (C) (µg/mL). Significance at *p < 0.05, ** p < 0.01, *** p < 0.001 with respect to LPS. TNF-α, tumour necrosis factor alpha; IL-6, Interleukin 6; IL-1 β, Interleukin 1 beta; LPS, lipopolysaccharide (Extracted and modified from García-Ibañez et al., 2023).*

The evaluation of bioactivity allowed to demonstrate significant antiinflammatory effects of sulforaphane (at therapeutic dosages µg/mL) (Figure 2), without any toxic effect, with higher potency than the parental glucosinolates (glucoraphanin, glucoraphenin) or phenolic compounds (Ruiz-Alcaráz et al. 2022). The bioaccesibility of the phytochemicals of interest (GSL/ITC and phenolic compounds) was also characterized in order to drive the functional evaluation of the different varieties in functional studies (Abellán et al., 2021).

CONCLUSIONS

The strategy of biofactory developing microgreens enriched in bioactive and health-promoting GSL/ITC with biostimulation of the metabolism and synthesis of beneficial compounds is compatible with new demands of high quality and fresh foods of acceptable safety, quality and taste also packed with beneficial ingredients. The biostimulation of plant-foods for human health is a research line with great potential for the future of food and nutrition sciences. The bioaccessible fraction of cruciferous foods and derived products can be included in dietary interventions as coadjuvants for the modulation of chronic conditions and treating different diseases, given their potential anti-inflammatory effect on chronic

inflammation (Costa-Pérez et al., 2023; García-Ibañez et al., 2023). The current challenge is to evaluate the use of ingredients enriched in glucosinolates (e.g. glucoraphanin as source of sulforaphane) to evaluate the influence on energy metabolism, inflammation status and markers, body composition, and microbiome in adults with overweight and obesity (Project "SANO").

ACKNOWLEDGEMENTS

This work was supported at least in part by the Project "SANO" CPP2022-009528, with funds of the MCIU/AEI/10.13039/501100011033 and the European Union "NextGenerationEU"/PRTR". Additionally, authors would like to thank the support of the AGROALNEXT Program, granted by MCIN with NextGenerationEU funds and the "Fundación Seneca" – Agency for Science and Technology of the Murcia Region (CARM), project PRTR-C17.I1.

REFERENCES

Abellán Á., Domínguez-Perles R., García-Viguera C., Moreno D.A. (2021). Evidence on the Bioaccessibility of Glucosinolates and Breakdown Products of Cruciferous Sprouts by Simulated In Vitro Gastrointestinal Digestion. *International Journal Molecular Sciences*, *22,* Art.11046.

Baenas, N., García-Viguera, C., Moreno, D.A. (2014). Biotic elicitors effectively increase the glucosinolates content in Brassicaceae sprouts. *Journal Agricultural Food Chemistry, 62*, 1881-1889.

Costa-Pérez A., Núñez-Gómez V., Baenas N., Di Pede G., Achour M., Manach C., Mena P., Del Rio D., García-Viguera C., Moreno D.A., Domínguez-Perles R. (2023). Systematic Review on the Metabolic Interest of Glucosinolates and Their Bioactive Derivatives for Human Health. *Nutrients, 15,* Art. 1424.

Garcia-Ibañez P., Núñez-Sánchez M.A., Oliva-Bolarín A., Martínez-Sánchez M.A., Ramos-Molina B., Ruiz-Alcaraz A.J., Moreno D.A. (2023). Anti-inflammatory potential of digested Brassica sprout extracts in human macrophage-like HL-60 cells. *Food & Function, 14,* 112-121.

Hernández-Cánovas L., Abellán-Victorio Á., Moreno D.A. (2021). The Quality and Glucosinolate Composition of Cruciferous Sprouts under Elicitor Treatments Using MeJA and LED Lights. *Proceedings (MDPI) 70*, Art. 67.

López-Chillón M.T., Carazo-Díaz C., Prieto-Merino D., Zafrilla P., Moreno D.A., Villaño D. (2019). Effects of long-term consumption of broccoli sprouts on inflammatory markers in overweight subjects. *Clinical Nutrition, 38*, 745-752.

Ruiz-Alcaraz A.J., Martínez-Sánchez M.A., García-Peñarrubia P., Martinez-Esparza M., Ramos-Molina B., Moreno D.A. (2022). Analysis of the anti-inflammatory potential of Brassica bioactive compounds in a human macrophage-like cell model derived from HL-60 cells. *Biomedicine. & Pharmacotherapy*. *149,* Art. 112804.

CHEMICAL CHARACTERISTICS, ANTIOXIDANT ACTIVITIES AND GLUCOSINOLATES OF *Capparis spinose* VARIED BY SOIL SUBSTRATES

Elham Yousefi[1], Diego A. Moreno[2]*, Anastasia D. Chatzichristidi[2], Mehdi Abedi[1], Tahereh A. Aghajanzadeh[3]

[1]Department of Range Management, Faculty of Natural Resources and Marine Sciences, Tarbiat Modares University, Noor, Mazandaran Province, Iran
[2]Laboratorio de Fitoquímica y Alimentos Saludables (LabFAS), CEBAS, CSIC, Campus Universitario de Espinardo −25, 30100 Murcia, Spain
[3]Department of Plant Sciences, Faculty of Science, University of Mazandaran, Babolsar, Iran

*Corresponding author:
E-mail addresses: dmoreno@cebas.csic.es

ABSTRACT

Dryland ecosystems contain different soil conditions that can affect plants' chemical properties. Capparis spinosa, an important economic species, can thrive in various soil conditions, yet there is limited information about the effects of environmental conditions on its chemical properties. In this study, we collected *C. spinosa* from both gypsum and non-gypsum soils in southern Iran, where this species's largest and most economically important population exists. We considered different parts of the plants, including petals, sepals, flags, pistils, fruits, leaves, and seeds. We then determined the total phenol content, total flavonoid content, antioxidant activities (DPPH, FRAP, ABTS), and Glucocapparin in both soil conditions. Based on the findings, plant parts and sites significantly impacted most of the parameters. The highest amount of Glucocapparin was found in the pistil, while the lowest amount was found in the petals. Petals had the highest total phenol content, while the lowest was in the seeds. The highest total flavonoid content was in the leaves and fruit, and the lowest was in the seeds. The largest amount of DPPH was found in the leaves, while the lowest amount was in the pistils. As gypsum levels increased, the amount of DPPH also increased in various plant parts. There was no significant difference between the two sites for FRAP, with the highest amount found in the sepals. Overall, the gypsum sites showed higher values for most of the parameters. The PCA results showed that pistils were associated with Glucocapparin, petals with DPPH, and the leaf and sepal with total phenol, FRAP, ABTS, and total flavonoids. Total phenol had correlations with most of the factors. Additionally, different glucosinolate compounds were found in various parts of the plants. Our results indicate that *C.spinosa* has valuable chemical compounds in both soil conditions.
***Keywords**:* Gypsum soil, *Caper*, biochemical trait, Glucosinolate

INTRODUCTION

Drylands are harsh environments with aridity and highly available elements, to which plants have adapted by developing a high diversity of chemical traits (Gross et al., 2024). Gypsum soils contain large amounts of gypsum (CaSO42H2O), Ca, S, and Mg (Palacio et al., 2014). Plant adaptation, particularly for secondary metabolites to gypsum soils, is less well understood. The soil conditions of gypsum influence plants' chemical properties and secondary metabolites. Species that grow in gypsum soil may have higher phenolic and flavonoid content compared (Bautista et al., 2016). Comparing gypsovags in two gypsum and non-gypsum soils showed higher phenolic compounds and antioxidant activity in non-gypsum soil (Çekiç et al., 2018). Additionally, sulfur increased total glucosinolate levels in *Brassica rapa* (Li et al., 2007). Previous studies were mainly conducted on agricultural land and the Brassicaceae family. To our knowledge, there are no studies on environmental factors affecting *C. spinosa* bioactive compounds and glucosinolate in natural ecosystems.

Glucosinolates are natural substances in many plants, particularly in the Brassicaceae, Caricaceae, and Capparaceae families (Connolly et al., 2021). However, there are limited studies on Capparaceae. *Capparis spinosa* is a well-known species of the Capparidaceae family found in the Mediterranean region and holds ecological and

economic significance (Chedraoui et al., 2017). *Capparis spinosa* is a plant with high nutritional and bioactive value (Annaz et al., 2022). It has high polyphenol content and antioxidant activity in different parts, particularly buds (Grimalt et al., 2019). Glucosinolate compounds such as glucocapparin, glucobrassicin, neo-glucobrassicin, and hydroxy-glucobrassicin are present in various parts of *C. spinosa* (Ahmed et al., 1972; Maldini et al., 2016). To our knowledge, there are no studies on different plant parts, especially flower parts, for polyphenols, antioxidant activity, and glucosinolate in gypsum habitats.

The largest population of C. spinosa for economic purposes is in the southwest of Zagros in the Kazeron region of Iran, with a high content of gypsum in the soil in most localities and high support for the economy of local people. This study aimed to investigate 1) what is the effect of gypsum soil on glucosinolate and antioxidant activity of *C. spinosa*; 2) how chemical compositions in *C. spinosa* leaves, flowers, and fruits change in gypsum and non-gypsum soils; and 3) how the chemical status of Caper bush in the different soils from an agri-food perspective.

MATERIALS AND METHODS

Study area and plant sampling

The sampling sites were located in the Abkenar rangeland (N 29º 27' 22.722", E 51º 45' 37.536"; Kazerun city, Fars province, Iran). Two sites with similar climatic and topographic conditions, including gypsum and non-gypsum sites, were chosen, with the gypsum site being a relict gypsum mine. Three replications of flowers, leaves, and fruit samples were taken at random from both sites from healthy *C. spinosa* shrubs in the summer season and transported to the laboratory; then, the leaves and fruits were dried using a freeze drier at -80 and ground. Seven plant parts were considered for biochemical analysis, including sepal, petal, pistil, flag, leaves, fruit, and seed.

Measurement of leaf, fruit, and flower biochemical parameters

The hydroalcoholic extracts were prepared to analyze the total polyphenol content and antioxidant capacity of formulations of *C. spinosa*. Total phenolic content (TPC) was determined by the Folin-Ciocalto colorimetric method using gallic acid as standard (Singleton et al., 1999). Total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method and quercetin as a standard (Akkol et al., 2008). DPPH free radical scavenging activity was determined using the Brand-Williams et al. (1995) method with minor modifications. ABTS radical scavenging activity was measured using the Re et al. (1999) method with slight modifications. The FRAP assay was developed by Benzie and Strain (1996) to measure the Ferric reducing antioxidant power assay.Glucosinolates hydromethanolic extraction and chromatographic analysis was carried following the methodology described in Abellán et al. (2021) for intact glucosinolates, as described elsewhere.

To assess the effects of sites, plant parts, and their interactions on the biochemical parameters of C. spinosa, we used a GLMM and compared the means using Tukey HSD. Principle component analysis (PCA) was used to evaluate the relations between biochemical traits and correlations between the characteristics. PERMANOVA was also performed to test for significant differences in biochemical composition between treatments.

RESULTS

Both the sites and plant parts significantly affected the chemical properties, except for FRAP and Glucocapparin. Plant parts had the highest effects among the properties, except for ABTS, where sites had the highest impact (Table 1). The highest TPC values were found in the flag and fruit, while the lowest values were observed in the seed and pistil. TPC levels for petals, pistils, and fruits significantly increased in gypsum soil,

whereas levels for sepal and flag decreased (Fig. 1). The highest TFC was observed in the leaves at both sites, and the seeds at both sites showed the lowest TFC values. In the gypsum site, the TFC increased in the leaves, petals, and flags, while it decreased in the fruits and sepals (Fig.1).

Table 1. Results of GLMMs for the effects of the sites, plant parts treatments, and their interactions on chemical characters. Bold values indicate significant effects of treatments.

	Sites(S)		Parts (P)		S × P	
Treatments	**F**	**P**	**F**	**P**	**F**	**P**
TPC (GAE mg/kg FW)	**34.01**	**<.001**	**20101.62**	**<.001**	**328.41**	**<.001**
TFC (QU mg/kg FW)	**95.16**	**<.001**	**740.07**	**<.001**	**310.14**	**<.001**
ABTS (%)	**253.40**	**<.001**	**117.07**	**<.001**	**205.25**	**<.001**
DPPH (%)	**21.03**	**<.001**	**91.27**	**<.001**	**9.58**	**<.001**
FRAP	0.49	0.491	**14.16**	**<.001**	1.42	0.242
Glucocapparin (μmol/gDW)	0.003	0.946	**37.12**	**<.001**	**4.85**	**0.002**

Table 2. GLS content for different parts of C. spinosa in two sites. For calculations of percentage the sum of low-concentration GLSs divided by total GLSs (sum of low-concentration GLS+ Glucocapparin)

Parts	**Sites**	**n-propyl-glucosinolate**	**n-butyl-glucosinolate**	**glucobrassicin**	**Neo-glucobrassicin**	**Hydroxy-glucobrassicin**	**Total GLSs**	**low-concentration GLS %**
Pistil	**Gypsum**	21.00	2.97	0.09	ND	1.79	61.66	41.92
	Non-gypsum	ND	ND	ND	ND	0.19	35.79	0.53
Sepal	**Gypsum**	10.05	2.37	ND	ND	ND	41.81	29.71
	Non-gypsum	ND	ND	ND	ND	ND	31.58	0
Flag	**Gypsum**	2.74	ND	ND	ND	ND	26.01	9.47
	Non-gypsum	ND	ND	ND	ND	ND	22.1	0
Petal	**Gypsum**	3.42	1.38	0.62	ND	ND	27.07	20
	Non-gypsum	ND	ND	ND	ND	ND	22.8	0
Fruit	**Gypsum**	ND	ND	ND	ND	0.75	34.1	2.2
	Non-gypsum	ND	ND	ND	ND	ND	27.6	0
Leaf	**Gypsum**	ND	ND	1.29	3.60	0.19	36.7	13.84
	Non-gypsum	5.33	ND	0.57	0.84	ND	37.9	18.30
Seed	**Gypsum**	ND	ND	ND	ND	ND	24.2	0
	Non-gypsum	ND	ND	ND	ND	ND	29.6	0

*Table 3. The correlation coefficient between biochemical characters for C. spinosa. * P < 0.1, *P < 0.05, ***P < 0.0001.*

Trait	**TPC**	**TFC**	**ABTS**	**DPPH**	**FRAP**	**Glucocapparin**
TPC (GAE mg/kg FW)	**1**					
TFC (QU mg/kg FW)	**0.65*** **	**1**				
ABTS (%)	**0.55*** **	**0.53*** **	**1**			
DPPH (%)	**0.35***	0.30	0.23	**1**		
FRAP	0.25	**0.62*** **	0.15	**0.37***	**1**	
Glucocapparin (μmol/gDW)	**-0.35***	0.13	-0.11	**-0.63*** **	0.01	**1**

*Table 4. Correlations between chemical parameters for C.spinosa and principal component axes. *P < 0.05, ***P < 0.001.*

Traits	Principal component				
	Dim. 1	**Dim. 2**	**Dim. 3**	**Dim. 4**	**Dim. 5**
TPC (GAE mg/kg FW)	**0.82*** **	-0.01	-0.35	-0.43	0.01
TFC (QU mg/kg FW)	**0.81*** **	**0.50*** **	0.07	-0.09	0.18
ABTS (%)	**0.67*** **	0.18	-0.55	0.44	-0.14
DPPH (%)	**0.66*** **	**-0.56*** **	0.32	0.23	0.31
FRAP	**0.60*** **	0.30	0.68	0.03	-0.29
Glucocapparin (µmol/gDW)	**-0.38***	**0.88*** **	0.04	0.10	0.22

Table 5. Examining the significant effects of the biochemical in the sites, plant parts and their interactions using the PERMANOVA

Treatments	Df	Sum of Sq.	F	R^2	P-value
Part	1	184.51	44.8	0.69	**0.001**
Site	1	22.04	10.8	0.28	**0.001**
Part×Site	1	23.27	13.3	0.21	**0.001**

The antioxidant activities varied significantly among different treatments. The highest and lowest FRAP values were observed at both sites in the leaf and pistil, respectively. Other parts generally showed no significant differences between them. (Fig.1). High DPPH values were recorded for the seed, leaf, fruit, petal, and flags. However, the sepal and pistil exhibited significantly lower values than the other parts. In the gypsum site, the pistil, leaf, and seed showed a significant increase compared to the non-gypsum site (Fig.1). When considering ABTS, the flag from the non-gypsum site showed the highest value, while the flag from the gypsum site showed the lowest value. The ABTS values for the seed, leaf, and fruit from the gypsum site were significantly increased, while the flag exhibited a decrease in the gypsum site (Fig.1).

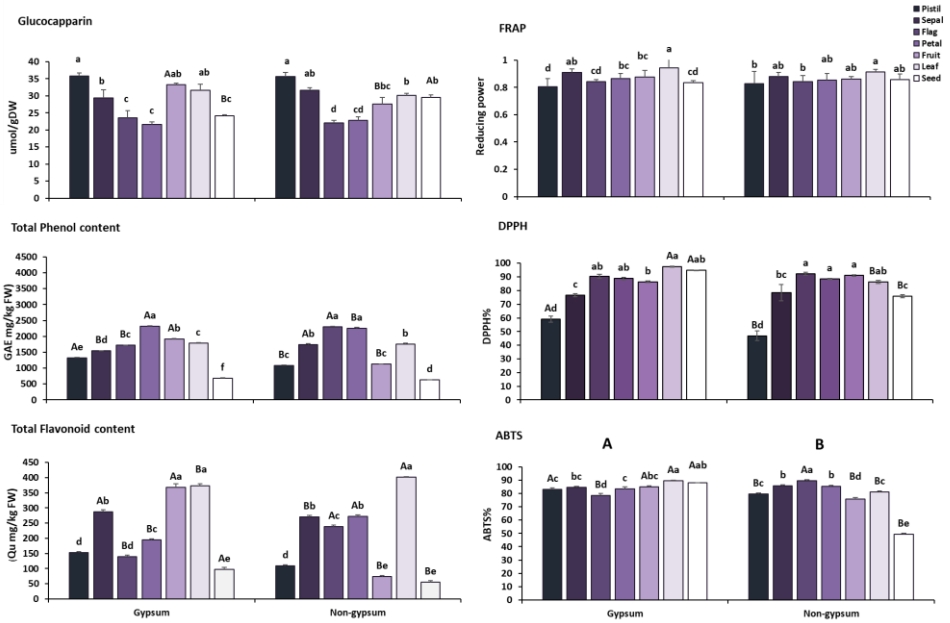


Figure 1. Effects of the sites and plant parts treatments on biochemical parameters (mean ± SE, n = 3). Uppercase letters are the results of the T- tests for the sites and lower-case letters are the results of the Tukey test for the plant parts.

The highest values of Glucocapparin were observed in the pistil, sepal, leaf, and fruit, while the petal and flag showed lower values in both sites. There were contrasting increases and decreases in the fruit and seed in the gypsum site compared to the non-gypsum site (Fig.1). Most glucosinolate compounds were found in low concentrations in the plant parts of the gypsum site, except for the leaf and pistil, which were also observed in the non-gypsum site (Table 2).

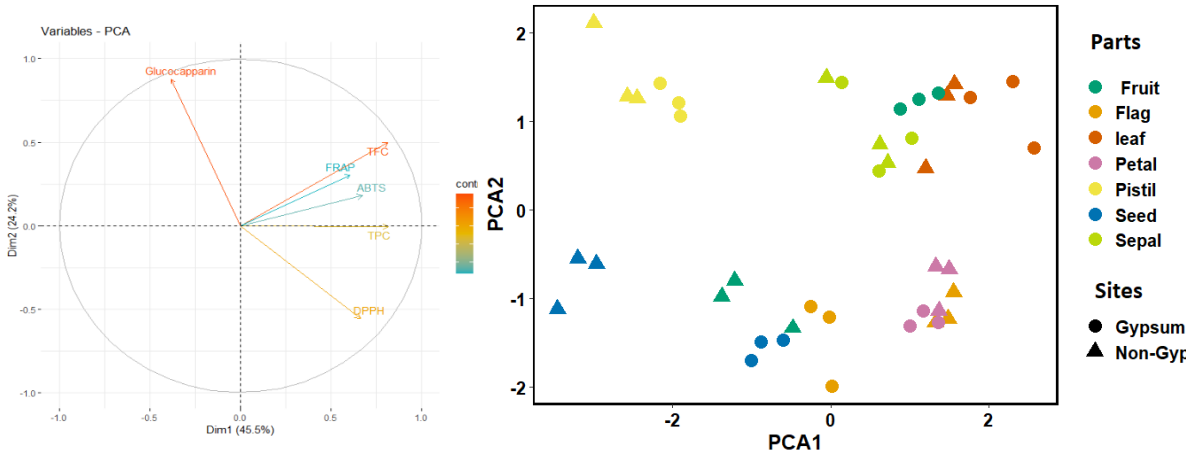


Figure 2. PCA results for seven plant parts in 2 sites

Our results showed positive correlations between most parameters, except for glucocapparin, which negatively correlated with TPC and DPPH. (Table 2). The principal component analysis (PCA) results show that the first axis accounts for 45.5% of the variations. In comparison, the second axis accounts for 24.2% of the variations (Fig.2). All parameters exhibited a strong positive correlation with Dim.1, except for glucocapparin, which showed a negative correlation. TFC and glucocapparin displayed strong positive correlations, while DPPH showed negative correlations with Dim.2 (Table 3). Sites, plant parts, and their interactions significantly affected the biochemical compositions, according to the PERMANOVA results (Table 4).

DISCUSSION

The research findings indicate that *C. spinosa* contains high levels of bioactive and chemical compounds in various parts, regardless of soil type. The chemical composition varied primarily in different plant parts and was less affected by soil type. Glucosinolates were high in all plant parts, with Glucocapparin being the predominant compound. Although gypsum did not increase Glucocapparin levels, it strongly impacted other glucosinolates in the respective sites.

C. spinosa had higher antioxidant activity and glucosinolate compounds in gypsum soil

This study showed that gypsum soil demonstrated higher antioxidant activity of ABTS and DPPH and higher phenolic and flavonoid content in most parts. These results contradict Çekiç et al.'s (2018) findings, which indicated that three studied gypsovags have lower antioxidant and phenolic content than non-gypsum soil.

Additionally, gypsum soil influences glucosinolates. Glucocapparin was the dominant compound, consistent with Matthäus and Özcan (2005). Glucosinolates with low concentrations were observed mainly in gypsum, except for a few leaf and pistil observations in non-gypsum. The increases in antioxidant activity and glucosinolates could be linked to higher environmental stress, such as drought and the high sulfur content of gypsum soil (Escudero et al., 2015). More studies are needed on gypsum habitat species to draw better conclusions.

Flower parts show very diverse antioxidant activity and glucosinolate compounds
All plant parts showed high antioxidant activity, with the leaves demonstrating the highest values and the seeds and pistils showing the lowest, regardless of the site. consistent to (Bhoyar et al., 2018). Moreover, there was substantial glucocapparin content in every part of the plant. There were high values for the fruit and leaves, and the flower sections had varying amounts that ranged from highest to lowest, consistent with (Maldini et al., 2016). Furthermore, taking into account glucosinolates in low concentration, observations were made of n-propyl-glucosinolate and glucobrassicin in flower and leaf, hydroxy-glucobrassicin in flower, leaf, and fruit, and N-butyl-glucosinolate only in flower and Neo-glucobrassicin only in leaf consistent to (Maldini et al., 2016). The total content of glucosinolates ranged between 22.1 *µ*mol/g (flag of Non-gypsum) and 61.6 *µ*mol/g (pistil of gypsum), with an average of 32.7 (µmol/g DW), which is considerable in comparison with other glucosinolate-containing plants from Brassicaceae (Matthäus & Luftmann, 2000).
Our study used mature flowers rather than buds, which may account for some variations in the results. Our results are the first to provide data on flower sections, including petals, sepals, pistils, and flags. Our results revealed surprisingly different compounds in flower sections, with variable amounts observed at two sites.

CONCLUSIONS

C. spinosa (caper bush) can thrive in various soil conditions, particularly gypsum. This suggests that C. spinosa exhibits stronger bioactive and antioxidant activity and higher levels of GLSs in gypsum soil due to the more challenging conditions of this habitat. The differing results in flower sections indicate the need for further study of flowers better to understand the plant's response to environmental conditions and to identify plant parts with high bioactive compounds. The high quality of fruits and seeds in both soil conditions indicates that this species could be widely used for restoring harsh lands for agricultural food production and ensuring sustainable food security in the face of global warming, which is expected to impact cropping areas worldwide.

ACKNOWLEDGEMENTS

This study forms part of AGROALNEXT (Agroalnext_2022_027) programme and was partially supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat Valenciana.

REFERENCES

Abellán Á., Domínguez-Perles R., García-Viguera C., Moreno D.A. (2021). Evidence on the Bioaccessibility of Glucosinolates and Breakdown Products of Cruciferous Sprouts by Simulated In Vitro Gastrointestinal Digestion. *International Journal Molecular Sciences*, *22,* Art.11046.

Ahmed, Z.F., Rizk, A.M., Hammouda, F.M. and El-Nasr, M.S., 1972. Glucosinolates of Egyptian *Capparis* species. *Phytochemistry*, *11*(1), pp.251-256.

Akkol, E. K., Göger, F., Koşar, M., & Başer, K. H. C. (2008). Phenolic composition and biological activities of Salvia halophila and Salvia virgata from Turkey. Food Chemistry, 108(3), 942-949.

Annaz, H., Sane, Y., Bitchagno, G.T.M., Ben Bakrim, W., Drissi, B., Mahdi, I., El Bouhssini, M. and Sobeh, M., 2022. Caper (*Capparis spinosa* L.): an updated review on its phytochemistry, nutritional value, traditional uses, and therapeutic potential. *Frontiers in pharmacology*, *13*, p.878749.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry, 239(1), 70-76.

Bhoyar, M.S., Mishra, G.P., Naik, P.K. and Singh, S.B., (2018). Evaluation of antioxidant capacities and total polyphenols in various edible parts of *Capparis spinosa* L. collected from trans-Himalayas. *Defence Life Science Journal*, *3*(2), pp.140-145.

Boscaiu, M., Bautista, I., Lidón, A., Llinares, J., Lull, C., Donat, P., Mayoral, O.,Vicente, O., 2013. Environmental-dependent proline accumulation in plants living on gypsum soils. Acta Physiol Plant, 35(7),2193-2204. https://doi.org/10.1007/s11738-013-1256-3.

Brand-Williams, W., Cuvelier, M.-E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, *28*(1), 25–30.

Çekiç, F.Ö., Özdeniz, E., Öktem, M., Kurt, L. and Keleş, Y., 2018. The role of biochemical regulation on the adaptation of gypsophile and gypsovag species. *Biochemical Systematics and Ecology*, *81*, pp.12-16.

Chedraoui, S., Abi-Rizk, A., El-Beyrouthy, M., Chalak, L., Ouaini, N. and Rajjou, L., 2017. *Capparis spinosa* L. in a systematic review: A xerophilous species of multi values and promising potentialities for agrosystems under the threat of global warming. *Frontiers in Plant science*, *8*, p.1845.

Connolly, E.L., Sim, M., Travica, N., Marx, W., Beasy, G., Lynch, G.S., Bondonno, C.P., Lewis, J.R., Hodgson, J.M. and Blekkenhorst, L.C., 2021. Glucosinolates from cruciferous vegetables and their potential role in chronic disease: Investigating the preclinical and clinical evidence. *Frontiers in pharmacology*, *12*, p.767975.

Grimalt, M., Legua, P., Hernández, F., Amorós, A. and Almansa, M.S., 2019. Antioxidant activity and bioactive compounds contents in different stages of flower bud development from three Spanish caper (*Capparis spinosa*) cultivars. *The Horticulture Journal*, *88*(3), pp.410-419.

Gross, N., Maestre, F. T., Liancourt, P., Berdugo, M., Martin, R., Gozalo, B., ... & Le Bagousse-Pinguet, Y. (2024). Unforeseen plant phenotypic diversity in a dry and grazed world. *Nature*, 1-7.

Li, S., Schonhof, I., Krumbein, A., Li, L., Stützel, H. and Schreiner, M., 2007. Glucosinolate concentration in turnip (*Brassica rapa* ssp. rapifera L.) roots as affected by nitrogen and sulfur supply. *Journal of Agricultural and Food Chemistry*, *55*(21), 8452-8457.

Maldini, M., Foddai, M., Natella, F., Addis, R., Chessa, M., Petretto, G.L., Tuberoso, C.I. and Pintore, G., 2016. Metabolomic study of wild and cultivated caper (*Capparis spinosa* L.) from different areas of Sardinia and their comparative evaluation. *Journal of Mass Spectrometry*, *51*(9), pp.716-728.

Matthäus, B., & Özcan, M. (2005). Glucosinolates and fatty acid, sterol, and tocopherol composition of seed oils from *Capparis spinosa* Var. spinosa and *Capparis ovata* Desf. Var. canescens (Coss.) Heywood. *Journal of agricultural and food chemistry*, *53*(18), 7136-7141.

Matthäus, B.; Luftmann, H. Glucosinolates in members of the family Brassicaceae: separation and identification by LC/ESIMS-MS. *Journal Agricultural Food Chemistry.* 2000, *48*, 2234-2239.

Palacio, S., Azorín, J., Montserrat-Martí, G. and Ferrio, J.P., 2014. The crystallization water of gypsum rocks is a relevant water source for plants. *Nature communications*, *5*(1), p.4660.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine, 26*(9-10), 1231-1237.

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In Methods in enzymology. 299, 152–178. Elsevier.

FRUIT PRODUCTS AND ITS POTENTIAL PREVENTIVE PROPERTIES AGAINST CHRONIC NON-COMMUNICABLE DISEASES

Uroš Čakar[1]*, Mirjana Čolović[2], Nikolina Živković[3], Ivan Stanković[1], Aleksandar Petrović[3], Danijela Krstić[4], Brižita Đorđević[1]

[1] Department of Bromatology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11000 Belgrade, Serbia
[2] Department of Physical Chemistry, Vinča Institute of Nuclear sciences - National Institute of the Republic of Serbia, Mike Petrovića Alasa 12-14
11351 Vinča, Belgrade, Serbia
[3] Department of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Belgrade-Zemun, Serbia
[4] Institute of Medical Chemistry, Faculty of Medicine, University of Belgrade, Dr Subotića 8, 11000 Belgrade, Serbia

*Corresponding author:
E-mail address: urosc@pharmacy.bg.ac.rs

ABSTRACT

Oxidative stress is a main cause for development of chronic non-communicable diseases. It can be prevented by antioxidants, especially those from berry fruit and its products. Blueberry is rich source of nutrients and biologically active compounds, but unfortunately it is not available in the fresh form during the whole year. Blueberry wine is a product obtained after alcoholic fermentation of blueberries, and during this process in wine are preserved all thermo labile biologically active compounds. This study is aimed to show phenolic profile, antioxidant properties and activity of fruit wine on enzymatic systems in vitro and lipid peroxidation. Blueberry wines were produced in different controlled microvinification procedures. Phenolic profile was analyzed by UPLC/MS-MS system. Total phenolic content (TPC), antioxidant and antiradical activity were analyzed by spectrophotometric methods. Level of lipid peroxidation (malondialdehyde (MDA) level) and activity of enzymes of antioxidant protection superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were evaluated on synaptosomes isolated from the brain of Wistar albino rats. Microvinification procedure significantly affected on phenolic profile, antioxidant and antiradical activity. Blueberry wines showed ability to activate enzymes of antioxidant protection and decrease MDA level in synaptosomes in which was experimentally induced oxidative stress. The most predominant group of phenolic compounds were phenolic acids and flavonoids. The TPC as well as FRAP values were significantly different among different microvinification. In oxidative stress induced synaptosomes which were treated with blueberry wine values for MDA were decreased while SOD, GPx and CAT activities were increased. As a rich source of antioxidants blueberry wine could prevent oxidative stress which is responsible for development of chronic non-communicable disease.
Keywords*: blueberry wine, oxidative stress, phenolic compounds, antioxidant properties, enzymes of antioxidant protection*

INTRODUCTION

Berry fruit are very popular part of diet during the spring and summer. During the season it is possible to consume it as a fresh, while different processed forms are available during whole year. Compare to other berry fruit which have lighter colour skin (pink, light red, and red) than blueberry, it is possible to highlight blueberry as a higher source of natural active compounds which exhibit antioxidant properties (Stull et al., 2010; Johnson et al., 2011). From the phenolic antioxidants in the blueberry is possible to point out phenolic acids which are represented in both fruits and their derived products. Phenolic acids are presented as a derivatives of hydroxycinnamic and hydroxybenzoic acids (Torres et al., 1987; Meyer et al., 1998). Phenolic antioxidants have been reported to have various positive effects on the human health like anti-inflammatory, anti-carcinogenic effects and protective effects on neuronal cells (Ji et al., 2024). These compounds possess strong ability to scavenge free radicals, donate hydrogen, chelate metals, break radical chain reactions, and quench singlet

oxygen *in vitro* and *in vivo* (Mamani-Matsuda *et al.*, 2006; Dai and Mumper, 2010). Phenolic compounds from blueberry wine can interact with enzymes of antioxidant protection which represent first line of antioxidant defense. This mechanism is activated when in human body imbalance is in the favor of ROS (Karademir Catalgol *et al.* 2007). Enzymes which are responsible for this activity are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which are indispensable in the entire human body. Their defense mechanism is directed to $O_2^{\cdot -}$ and H_2O_2. Antioxidant defense system in human body acts to suppress the formation of reactive species being able to damage cells (Altuntas *et al.* 2002).
Beside phenolic acids there are also other phenolic (anthocyanins, flavonols, flavanols and stilbens) and non-phenolic (carotenoids) active principles in this fruit which all together contribute to the health beneficial effects (Xiang and Ning, 2008). During the processing of blueberry into the wine, all those natural active compounds retain in the final product.
The aim of this study was to evaluate phenolic profile and *in vitro*, antioxidant, antiradical and protective effects of blueberry wines against oxidative stress conditions in isolated rat synaptosomes as a model system.

MATERIAL AND METHODS

Blueberry (*Vaccinium myrtilus*) originated from Rudnik mountain, Republic of Serbia.
In microvinification were conducted experiments which were divided into the two groups with and without addition of sugar before start of fermentation. Fruit was disintegrated in both groups. To avoid activity of unwanted microorganisms 10g of $K_2S_2O_5$ per 100 kg was added into the fruit must. The control was without added sugar. Total soluble solids (expressed in °Brix) were measured in the fruit must in both groups. Aiming to increase total soluble solids of must up to 20.5 °Brix, sugar was added in the second set. The obtained fruit must were inoculated with the pure strain *Saccharomyces cerevisiae* of the selected wine yeast Lievito Secco (Enartis, Italy) and Lalvin 71B (Lallemand, France) at the dose of 20 g per 100 kg. All experiments in microvinification were conducted in watts with the pigeage system (Hromil, Kovilj-Serbia). Alcohol fermentation was conducted at 20°C over 7 to 10 days. During this process, the must was stirred twice a day. After fermentation, each fruit wine was separated from the must by sedimentation. Afterwards, they were racked off the lees and kept at 12°C for the next six months, until further studies.
In all samples of blueberry wine were evaluated physicochemical properties. The pH value was evaluated by using pH meter, while total titratable acids (TTA) were obtained after titration with NaOH. Total soluble solids (TSS, expressed in °Brix) were measured in the fruit juice using the refractometer. The alcohol concentration was determined by the alcohol density meter after samples distillation. The strength by volume (vol. %) was calculated using 20°C/20°C tables (OIV, 2009).
All chemicals and reagents of analytical grade were purchased from Sigma Aldrich (Steinheim, Germany). Aiming to decrease the influence of the matrix during phenolic identification, solid-phase extraction (SPE) was applied, Oasis HLB 6CC 200 mg cartridges (Waters, Milford, MA, USA). UPLC/MS-MS analysis was performed using a Waters Acquity Ultra Performance H-Class System (Waters, Milford, MA, USA). UPLC separation was achieved on the column compartment with ZORBAX Eclipse XDB C18 column (150 mm×4.6 mm; 5 µm). During analysis, the column was kept at 25 °C while mobile phase flow-rate at 0.7 mL/min and injection volume was 10 µL (Gođevac *et al.*, 2009). Phenolic compounds were identified by comparing their retention times (t_R) and mass spectra with the relevant standards. IntelliStart program (Waters, Milford, MA, USA; 2005) provided parameters that were used for quantification. UPLC was coupled with a triple quadrupole mass spectrometer Acquity TQD (Waters, Milford, MA, USA) with the software MassLynx 4.1 (Waters, Milford, MA, USA; 2005) which was used for data acquisition and processing. Finally, the ionization source conditions were as followed: capillary voltage of 3.5 kV, source temperature of 150°C and desolvation temperature of 450°C, with a flow rate of 900 L/h. Nitrogen and argon were used as cone and collision gases, respectively.

CAT activity was measured by the H_2O_2 degradation assay (Čolović *et al.* 2015), and expressed as U/mg of protein. Total SOD activity was determined by using commercially available SOD assay kit (Sigma-Aldrich). The activity of GPx was measured in a coupled enzyme method by measuring the decrease of NADPH at 340nm (Čolović et al. 2015). Results were expressed as U/mg protein. The level of MDA was estimated as the concentration of thiobarbituric acid reactive product [8]. Results were expressed as moll of thiobarbituric acid – reactive substance (MDA equivalent)/mg protein using a standard curve of 1,1,3,3-tetramethoxypropane.
Redox potential of the fruit wine samples was determined using the Ferric Reducing Ability of Plasma (FRAP) test (Benzie and Strain, 1996). The obtained results were expressed in mmol/L Fe^{2+}. Anti-DPPH radical activity of the fruit wine samples was evaluated as previously described (Blois, 1958). The obtained results were expressed as a reciprocal value I (%) multiplied by 100. Total phenolic content (TPC) of the fruit wine samples was estimated by the Folin–Ciocalteu (FC) method using gallic acid as a standard (Woraratphoka *et al.*, 2007). The results were expressed in mg/L of gallic acid equivalents (mg GAE/L).
Statistical analysis was conducted by using the software SPSS Statistic V22.0 (IBM, Chicago, IL, USA; 2014); t-test for the paired samples; one-way and two-way ANOVA, with Tukey post hoc test for subgroup differences among drupe fruit species.

RESULTS AND DISCUSSION

Physico-chemical properties were evaluated for all fruit wine samples (Table 1). One-way ANOVA revealed significantly higher TSS and alcohol content in the wine samples made with addition of sugar before fermentation. The results for pH and TTA were similar. TSS is important parameter for prediction of alcohol content (Vol. %) in the wine, while pH and TTA directly affect its flavor and aroma.

Table 1. Physico-chemical properties of fruit wine samples

Type of Wine	**Total Soluble Solids must** (°Brix)	**pH**	**Total Titratable Acids** (malic acid g/L)	**Alcohol content** (Vol. %)
Enartis yeast	14.51 ±0.35	2.83 ±0.15	6.82 ±0.27	8.31 ±0.23
Sugar+ Enartis yeast	19.51 ±0.45[a*]	2.85 ±0.17	7.51 ±0.31	11.45 ±0.32[a*]
Lalemand yeast	14.32 ±0.54	2.83 ±0.21	6.58 ±0.45	8.21 ±0.21
Sugar+ Lalemand yeast	18.77 ±0.61[a*]	2.87 ±0.11	7.72 ±0.25	11.27 ±0.35[a*]

a*-significantly different from the wine without sugar addition before fermentation

Fruit wine samples produced without sugar were compared with those made with sugar (Table 2). The addition of sugar significantly affected the content of determined phenolic compounds ($p<0.05$). A higher content of sugar before fermentation was responsible for enhance alcohol concentration in the final product (Table 2). As an extracting agent, alcohol contributed to the enriched phenolic content of the fruit wine samples. The influences of different yeast during the vinification procedure did not affected phenolic content.

Table 2. Content of selected phenolic acids in the analyzed fruit wine samples (µg/mL)

Type of Wine	Caffeic acid	*p*-Coumaric acid	Chlorogenic acid	Gallic acid	Protocatehuic acid	Catechin	Epicatechin
Enartis yeast	93.78 ±5.11	1.67 ±0.21	721.43 ±7.31	54.27 ±2.31	75.24 ±1.43	34.21 ±1.25	43.51 ±1.53
Sugar+ Enartis yeast	127.37 ±7.45	4.15 ±0.27	783.25 ±10.41	75.34 ±3.12	121.47 ±5.32	43.24 ±1.72	57.21 ±1.55
Lalemand yeast	91.53 ±4.17	1.78 ±0.15	725.32 ±10.43	51.34 ±1.85	78.52 ±1.32	32.75 ±1.78	44.81 ±1.82
Sugar+ Lalemand yeast	128.45 ±8.23	3.86 ±0.23	785.51 ±8.21	73.21 ±4.51	123.32 ±5.77	42.35 ±1.82	55.32 ±1.45

The most predominant phenolic compound in blueberry wine was chlorogenic acid which content was (721.43 – 785.51 µg/mL). It is important to point out content of two other phenolic compounds, caffeic and *p*-coumaric acids. Their content in analyzed wine samples were in line with literature data which highlighted blueberries as a rich source of this two phenolic acids (Häkkinen et al., 1999; Zadernowski et al., 2005). Gallic acid was also detected in significant content. The most abundant hydroxybenzoic acid derivative was gallic acid. Its content was reported in the study which analyzed blueberry fruit (Zadernowski et al., 2005). In comparison with our results it was also detected in analyzed wine samples *p*-hydroxybenzoic acid (Häkkinen et al. 1999). Hydroxycinnamic were more abundant than hydroxybenzoic acid derivatives, as described before (Zadernowski et al., 2005). Epicatechin and catechin were also identified, as expected (Liwei et al., 2003). Blueberry wines were enriched with epicatechin ($p < 0.05$). Such a finding is well supported by a Dutch study (Arts et al., 2000).

Table 3. The values for FRAP, TPC and DPPH of analyzed fruit wine samples

Type of Wine	FRAP (mmol/L Fe^{2+})	Total Phenolic Content (TPC) (mg GAE/L)	DPPH IC_{50} (%)
Enartis yeast	72.51 ±1.85	2285.31 ±35.52	1.58 ±0.05
Sugar+ Enartis yeast	88.45 ±1.72[a*]	2532.71 ±38.53[a*]	1.51 ±0.02
Lalemand yeast	73.26 ±1.75	2293.22 ±31.11	1.61 ±0.03
Sugar+ Lalemand yeast	87.51 ±2.07[a*]	2575.61 ±37.41[a*]	1.53 ±0.02

a*-significantly different from the wine without sugar addition before fermentation

The TPC, antioxidant and antiradical properties was affected by the applied vinification procedures. Fruit wines produced with addition of sugar showed better antioxidant and antiradical properties, as well as higher TPC (Table 3). Literature data indicate that blueberry showed high total phenolic content and antioxidant properties (Angeles et al., 2022; Gómez et al., 2021). Quantitative differences throughout literature data may depend on the selection of cultivars, different climate conditions and/or sample preparation (Halvorsen et al., 2002). High values for FRAP and DPPH were observed for blueberry wine. Both parameters most likely depend on the cumulative (synergistic) effect of various compounds present in fruit wines. Generally speaking, berry fruits possess a good antioxidant potential, but to a varying degree. Higher alcohol level improved the extraction of phenolic compounds leading to the enhanced antioxidant potential of the final product. In a agreement to our results literature data highgihted high values for redox potential of blueberry (Vasantha Rupasinghe and Clegg 2007).

Detection of oxidative stress parameters was conducted in the synaptosomal preparations which were *in vitro* treated with H_2O_2 to induce oxidative stress, and wine samples were added to evaluate their protective effects. All investigate wine samples showed ability to induce increase of synaptosomal activity of antioxidant enzymes (SOD, CAT, and GPx). Also

was observed decrease of MDA content compared to single H_2O_2 treatment. Statistically significant difference ($p<0.05$) was shown for controls and synaptosoms treated with blueberry wines for all four parameters. The obtained enzyme activation and decreased lipid peroxidation suggest that the wines intensify defense against ROS induced by H_2O_2, and prevent the membrane lipid damage resulting from the produced ROS abundance. Ability of blueberry wine to decrease oxidative stress in synaptosomes exposed to H_2O_2 is a result of synergistic effect of all antioxidant compounds, which are present in this product. It is possible to highlight among all those compounds, activity of phenolic acids and flavonoids as key bioactive compounds detected in fruit wines. Additionally, technological process of production during which was added sugar before start of fermentation significantly affected on the content of phenolic compounds. Obtained results related to antioxidant, antiradical and protective effect against oxidative stress can be explained by the fact that sugar is responsible for the higher alcohol content, which has a role in the extraction of natural active compounds from solid parts of the fruit.

Table 4. Results for MDA, CAT, SOD and GPx activity in rat synaptosomes jointly treated with H_2O_2 and the blueberry wines.

Type of Wine	**MDA** (nmol/mg)	**CAT** (U/mg protein)	**SOD** (U/mg protein)	**GPx** (U/mg protein)
Enartis yeast	2.31^{c*}	0.0052^{c*}	5.73^{c*}	0.0152^{c*}
Sugar+ Enartis yeast	1.42^{a*c*}	0.0062^{a*c*}	6.48^{a*c*}	0.0175^{a*c*}
Lalemand yeast	2.45^{c*}	0.0050^{c*}	5.78^{c*}	0.0157^{c*}
Sugar+ Lalemand yeast	1.51^{b*c*}	0.0063^{b*c*}	6.58^{b*c*}	0.0178^{b*c*}

a-statistically significant difference compared to wines obtained in vinification Enartis yeast
b-statistically significant difference compared to wines obtained in vinification Lalemand yeast
c-statistically significant difference compared to appropriate
$MDA_{control}$=3.81; $CAT_{control}$=0.0045; $SOD_{control}$=4.78; $GPx_{control}$=0.0147

High antioxidant activity of blueberry wine against ROS in oxidative stress induced synaptosomes is in line with the study which highlighted blueberry as a berry fruit with high antioxidant activity (Čakar et al., 2018). In accordance to our result is possible to highlight the study which indicated blueberry extracts as a good for decrease MDA level in the brains of experimental animals (Miller and Shukitt-Hale 2012). This study showed that blueberry wines are rich source of phenolic acids and flavonoids. Protective effect of flavonoids against ROS is highlighted in the study in which were cell lines exposed to herbal extract rich in flavonoid aglycones, free radical generation and MDA levels decreased, whereas an increase in SOD, catalase, and GPx activities was noticed. The observed activities confirm the protective effects of flavonoid aglycones, which is in agreement with their antioxidant properties (Sun et al., 2011).

CONCLUSIONS

This study showed that blueberry wines can be considered as a rich source of phenolic compounds-phenolic acids and flavonoids. During the production process microfinification procedure significantly influenced on antioxidant, antiradical properties and phenolic compound content. Blueberry wine showed ability to decrease MDA content and increase activity of enzymes of antioxidant protection CAT, GPx and SOD which is result of synergistic effect of phenolic and non-phenolic compounds which are responsible for protection against ROS.

ACKNOWLEDGEMENTS

This work was supported by the agreement number 451-03-66/2024-03/200161 between Faculty of Pharmacy, University of Belgrade and Ministry of science, technological development and innovation of Republic of Serbia.

REFERENCES

Altuntas, I., Kilinc, I., Orhan, H., Demirel, R., Koylu, H., Delibas N. (2004) The effects of diazinon on lipid peroxidation and antioxidant enzymes in erythrocytes in vitro. *Human & Experimental Toxicology*, 23, 9-13.

Angeles Varo, M., Martin-Gomez, J., Serratosa, M. & Merida, J. (2022). Effect of potassium metabisulphite and potassium bicarbonate on color, phenolic compounds, vitamin C and antioxidant activity of blueberry wine. *LWT*, 163, 113585

Arts, I., van de Putte, B., Hollman, P. (2000). Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *Journal of Agricultural and Food Chemistry*, 48, 1746–1751.

Benzie, I.F.F. & Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry*, 239, 70–76.

Blois, M.S. (1958). Antioxidant Determinations by the Use of a Stable Free Radical. *Nature*, 181, 1199.

Čakar, U., Petrović, A., Janković, M., Pejin, B., Vajs, V., Čakar, M., Djordjević, B. (2018). Differentiation of wines made from berry and drupe fruits according to their phenolic profiles. *European Journal of Horticultural Sciences*, 83, 49–61.

Čolović, M., Vasić V., Avramović, N., Gajić, M., Djurić, D., Krstić, D. (2015) In vitro evaluation of neurotoxicity potential and oxidative stress responses of diazinon and its degradation products in rat brain synaptosomes. *Toxicology Letters*, 233, 29-37.

Dai, J. & Mumper, R.J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*, 15, 7313–7352.

Gođevac, D., Tešević, V., Vajs, V., Milosavljević, S. & Stanković, M. (2009). Antioxidant properties of raspberry seed extracts on micronucleus distribution in peripheral blood lymphocytes. *Food and Chemical Toxicology*, 47, 2853–2859.

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., Törrönen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International*, 32, 345–353.

Halvorsen, B., Holte, K., Myhrstad, M., Barikmo, I., Hvattum, E., Remberg, S., Wold, A., Haffner, K., Baugerød, H., Andersen, L., et al. (2002). A systematic screening of total antioxidants in dietary plants. *Journal of Nutrition*, 132, 461–471.

Ji, C., Wang, R., Wang, D., Luo, Y., Huang., Z., Liu, J. & Cheng, A. (2024). Effect of berry-derived phenolic products on blood lipid profiles: A systematic review and meta-analysis, *Food Bioscience,* 58, 103607

Johnson, M., Lucius, A., Meyer, T., de Mejia, E.G. (2011). Cultivar evaluation and effect of fermentation on antioxidant capacity and *in vitro* inhibition of α-amylase and α-glucosidase by Highbush Blueberry (*Vaccinium corombosum*). *Journal of Agricultural and Food Chemistry*. *59*, 8923– 8930.

Karademir Catalgol, B., Ozden, S., Alpertunga, B. (2007). Effects of trichlorfon on malondialdehyde and antioxidant system in human erythrocytes. *Toxicology in Vitro*, 21, 1538–1544.

Liwei, G., Kelm, M., Hammerstone, J., Beecher, G., Holden, J., Haytowitz, D., and Prior, R. (2003). Screening of foods containing proanthocyanidins and their structural characterization using LC MS/MS and thiolytic degradation. *Journal of Agricultural and Food Chemistry*, 51, 7513–7521.

Mamani-Matsuda, M., Kauss, T., Al-Kharrat, A., Rambert, J., Fawaz, F., Thiolat, D., Moynet, D., Coves, S., Malvy, D. & Mossalayi, M.D. (2006). Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. *Biochemical Pharmacology*, 72, 1304–1310.

Martín-Gómez, J., García-Martínez, T., Ángeles Varo, M., Mérida, J. & Serratosa, M. (2021). Phenolic compounds, antioxidant activity and color in the fermentation of mixed blueberry and grape juice with different yeasts. *LWT*, 146, 111661.

Meyer, A., Donovan, J., Pearson, D., Waterhouse, A., Frankel, E. (1998). Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation in vitro. *Journal of Agricultural and Food Chemistry*, 46, 1783–1787.

Miller, M. and Shukitt-Hale, B. (2012). Berry fruit enhances beneficial signaling in the brain. *Journal of Agricultural and Food Chemistry*, 60, 5709–5715.

Organisation Internationale de la Vigne et du Vin (OIV) (2009). Compendium of international methods of wine and must analysis. Paris, France.

Stull, A., Cash, K., Johnson, W., Champagne, C., Cefalu, W. (2010). Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *Journal of Nutrition* 140, 1764–1768.

Sun, L., Zhang, J., Lu, X., Zhang, L., Zhang, Y. (2011). Evaluation to the antioxidant activity of total flavonoids extract from persimmon (*Diospyros kaki* L.) leaves. *Food and Chemical Toxicology*, 49, 2689–2696.

Torres, A., Mau-Lastovicka, T., Rezaaiyan, R. (1987). Total phenolics and high-performance liquid chromatography of phenolic acids of avocado. *Journal of Agricultural and Food Chemistry*, 35, 921–925.

Vasantha Rupasinghe, H., and Clegg, S. (2007). Total antioxidant capacity, total phenolic content, mineral elements, and histamine concentrations in wines of different fruit sources. *Journal of Food Composition and Analysis*, 20, 133–137.

Woraratphoka, J., Intarapichet, K.-O. & Indrapichate, K. (2007). Phenolic compounds and antioxidative properties of selected wines from the northeast of Thailand. Food Chemistry, 104, 1485–1490.

Xiang, Z. & Ning, Z. (2008). Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honeysuckle. *LWT - Food Science and Technology*, 41, 1189–1203.

Zadernowski, R., Naczk, M., Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *Journal of Agricultural and Food Chemistry*, 53, 2118–2124.

POTENTIAL BIOAGENTS FOR MAIZE SEED BIOPRIMING

Aleksandra Janković*, Mladen Petreš, Tatjana Dudaš, Marta Loc, Teodora Mihaljfi, Dragana Budakov, Mila Grahovac

Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: aleksandra.jankovic@polj.edu.rs

ABSTRACT

Chemical seed treatments are still the most common tool against seed and soil borne plant diseases. Due to their negative impact on the environment, human and animal health, it is necessary to find other solutions. Seed biopriming is an advanced seed treatment technique that can be a sustainable alternative. The aim of this research was to examine the effect of different microbial biocontrol agents on reduction of seedborne pathogens in maize. Microorganisms that were used as biopriming agents were: *Bacillus amyloliquefaciens* (BA1), *Bacillus amyloliquefaciens* (ABO2), *Pichia* sp. (P1), *Trichoderma asperellum* (T251/21), and mixture of five *Trichoderma harzianum* isolates. Two samples of maize seeds previously confirmed to be naturally contaminated with *Fusarium* spp. (over 5%) were used as a plant material. Biopriming was performed by submerging the seeds in cell suspension of the agent. Subsequently, the seeds were placed on Potato Dextrose Agar medium and incubated at room temperature for 7 days. Infection rate and germination parameters were rated to determine effects of the biopriming. The highest reduction of infection rate in both seed samples was detected on seeds primed by *T. harzianum* mixture and *T. asperellum* (0% infected seeds), while the lowest reduction was recorded in treatments with *B. amyloliquefaciens* (BA1) (6.5% infected seeds). Analysis of variance revealed the existence of significant differences ($p \leq 0.05$) in the length of the primary root and mesocotyl between the treatments. The shortest primary root and mesocotyl in both samples were observed in treatment with *B. amyloliquefaciens* (ABO2) (16.38 mm and 26.4 mm, respectively), while the longest primary root and mesocotyl were measured after biopriming with *Pichia* sp. (P1) (58.25 mm, 40.03 mm, respectively). The results show that the application of *Bacillus*, *Trichoderma* and *Pichia* bioagents can benefit control of maize seed pathogens when applied as seed treatment, while *Pichia* sp. can also significantly improve root and mesocotyl development.
***Keywords**: biocontrol, seed biopriming, maize*

INTRODUCTION

Maize (*Zea mays* L.) is the most versatile and leading cereal in production volume. It plays an increasing and diverse role in global agri-food systems. Maize is primarily used as a feed globally (56.3% of production), but is also significant as food crop (12.8%), besides other non-food uses (19.6%) (Erenstein et al., 2022). The world's largest maize producers are the USA, followed by China and Brazil, whose production exceeds 60% of the total world production (FAOSTAT, 2022). In the Republic of Serbia, in the last three years, maize occupies the largest area among cereals (over 35%) (Statistical Office of the Republic of Serbia, 2024). According to FAOSTAT data, maize took the first place in production volume among all commodities produced in the Republic of Serbia in 2022 (4,283,293,000 t). Maize cultivation has risen over time, and the crop demand is projected to double by 2050 (Camera et al., 2019). Due to its wide distribution and use, maize as an agricultural crop is given significant attention, and great efforts are made to improve its production. Among many causes that affect maize yield, fungal diseases are one of the main factors responsible for losses. Numerous fungi species cause damage, but only a few cause significant maize losses. Most non-treated maize seeds are declared to be contaminated with seed-borne fungi, including *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Bipolaris maydis*, and *Rhizopus* spp., leading to low-field emergence, reduced crop vigor, increased seedling diseases, and low productivity (Erasto et al., 2023). Increasing public awareness of the environmental and human health risks associated with chemical pesticides led to an increase in interest and acceptance of alternative strategies in crop production. Since the beginning of

agricultural practices, seed priming has been demonstrated to enhance seed quality, germination rate, stress resistance, and crop yield (Singh et al., 2020).
Seed priming is considered to be an easy, highly effective, low cost and low-risk technique. Within the priming technology, biopriming is gaining more and more importance. Seed biopriming involves immersing the seeds in a solution containing bioagents to improve the quality of the seeds. This technique of seed treatment can be applied to control many seed- and soil-borne pathogens (Reddy & Reddy, 2013). Potential bioagents for maize seed biopriming are bacterial bioagents *Pseudomonas sihuinsis*, *Bacillus aerophilus*, *Pseudomonas stutzeri*, *Enterobacter cloacae*, and fungal bioagents *Trichoderma harzianum*, and *Trichoderma afroharzianum* (Afrouz et al., 2023; Chidanandappa & Singh, 2024). Maize seed biopriming shows positive effects, not only in terms of suppressing pathogens but also affecting seed quality.
This research aimed to examine the effect of different microbial biocontrol agents on the reduction of seedborne pathogens in maize. Also, the aim of this paper was to observe the effect of applied bioagents on the root and mesocotyl length of maize.

MATERIAL AND METHODS

In the experiment, as biopriming agents, following beneficial microorganisms were used: *Bacillus amyloliquefaciens* (strain BA1 and ABO2), *Pichia* sp. (strain P1), *Trichoderma asperellum* (isolate T251/21) and *Trichoderma harzianum* (mix of isolates T2, T5, T6, T12 and T13). Bacterial strain *B. amyloliquefaciens* were revitalized by seeding on nutrient agar (NA), while fungal isolates *T. asperellum* and *T. harzianum*, and yeast *Pichia* spp. strains were revitalized by seeding on Potato Dextrose Agar (PDA), after which the inoculum of the selected strains was prepared. The bacterial strain inoculum (ABO2) was prepared by inoculating the Liquid Nutrient Broth (HB, Torlak) with an overnight bacterial culture of the previously revitalized strain *B. amyloliquefaciens* ABO2. The bacterial inoculum was incubated for 24h on a horizontal shaker (222DS Benchtop Shaking Incubator Labnet, USA) with external mixing (150 rpm) at room temperature. After that, 10% (v/v) of the previously prepared inoculum was added to the optimized nutrient medium for cultivation (5 g of sucrose, 1.47 g of yeast extract, 5 g of KH_2PO_4, distilled water up to 1000 ml), and then cultivation performed on a horizontal shaker (222DS Benchtop Shaking Incubator Labnet, USA) with external mixing (150 rpm), at room temperature for 70h. The concentration of bacteria *B. amyloliquefaciens* used in this experiment was 1×10^8 CFU/ml for strain ABO2 and 1×10^{15} CFU/g for strain BA1 (this strain has already been formulated in powder form). Fungi *T. asperellum* (isolate T251/21) and five *T. harzianum* isolates (T2, T5, T6, T12 and T13) were grown on Potato Dextrose Agar (PDA, composition 4 g of potatoes from 250 g of boiled potatoes, 20 g of dextrose, 18 g agar, distilled water up to 1000 ml) at 25±0.5 °C, in the dark, for ten days. Using a sterile inoculation loop to scrape mycelium growing on PDA medium, colonies were transferred to 1000 ml of distilled water until reaching a conidial concentration of 4.7×10^8 CFU/ml, while Tween 20 (0.5 ml per 1000 ml of distilled water) was applied as a solubilizing agent. The final concentration of the conidia suspension was checked under a microscope using a hemocytometer. After 48h on PDA medium, actively growing yeast strain *Pichia* spp. was transferred, taking two loopfuls of culture, in 1000 ml prepared Potato Dextrose Broth medium (PDB, composition 4 g of potatoes from 250 g of boiled potatoes, 20 g of dextrose, distilled water up to 1000 ml). After 48 hours of incubation on a horizontal shaker (222DS Benchtop Shaking Incubator Labnet, USA) with external mixing (150 rpm) at room temperature, the suspension (1×10^7 CFU/ml) was used for seed priming.

Testing of the prepared bioagents was carried out under *in vitro* conditions. These were two maize samples collected from the producer of maize seed, randomly selected based on the seed infection level with *Fusarium* spp. The level of 5% is a threshold for maize seed infection with *Fusarium* spp. determined by the "Regulations on the Health Inspection of Crops and Facilities for the Production of Seeds, Seedlings, and Plant Material, and the

Health Inspection of Seeds, Seedlings, and Plant Material" (Official Gazette of the Republic of Serbia, 107/2008). Seeds were sterilized with sodium hypochlorite (5% NaOCl) and rinsed several times with sterile distilled water. The experimental design comprised six different treatments in three replications. Seeds treated with sterile distilled water served as control. The maize seeds were bioprimed for two hours. After biopriming, the seeds were arranged in Petri dishes Ø 150 mm on PDA medium using sterile tweezers. One Petri dish represented one replicate. In each Petri dish, 50 seeds were placed at an equal distance from each other and incubated at room temperature.

The effect of seed biopriming on pathogens control, as well as seed quality parameters, was evaluated seven days later. The incidence of maize seeds infected was expressed as the percentage of seeds from which fungi species were isolated. The developed mycelium and reproductive structures of the fungus were observed under a microscope to identify the pathogen. After visual assessment and observation under a microscope, 20 seedlings from each Petri dish were selected, and the primary root and mesocotyl lengths were measured. The sum of the primary root and mesocotyl length was considered as seedling length. Length was the expressed in millimeters. Data collected were statistically analyzed using the Analysis of Variance (ANOVA) in Statistica software (version 14.0). Differences between means were determined using the Least Significant Difference (LSD) test at p=0.05 level.

RESULTS AND DISCUSSION

After seven days of incubation, an examination of petri dishes revealed a different percentage of infected seeds between treatments. The highest percentage of infected seeds in both hybrid samples was in the untreated control (24% and 18% infected seeds). Among applied bioagents, the lowest reduction of infection rate in both hybrid samples was recorded in treatments with *B. amyloliquefaciens* (strain BA1) (5% and 8%). In treatments with bioagents *T. asperellum* and *T. harzianum*, no infected seeds were detected, while the average infection rate in treatments with *Pichia* sp. and *B. amyloliquefaciens* (ABO2) were 1% and 3%, respectively (Table 1).

Table 1. The infection rate of maize seeds primed with bioagents

	Infection rate (%)		
Bioagent	**Sample 1**	**Sample 2**	**Average**
BA1	5	8	6.5
ABO2	6	0	3
P1	1	1	1
T251/21	0	0	0
T. mix	0	0	0
Control	24	18	21

Figure 1 shows the Petri dishes with maize seeds after seven days of priming with potential bioagents. The occurence of *Fusarium* spp. and *Bipolaris* spp. was determined. *Fusarium* spp. was recorded in treatments BA1 (2%), ABO2 (3%), and control (7.5%), while the occurrence of *Bipolaris* spp. was registered in treatments P1 (1%), BA1 (3%) and control (10%). In addition to the positive effect of the *Trichoderma* bioagents on the infection rate, the external colonization of maize seeds is also visible. It indicates the shortcomings of the application of these bioagents.

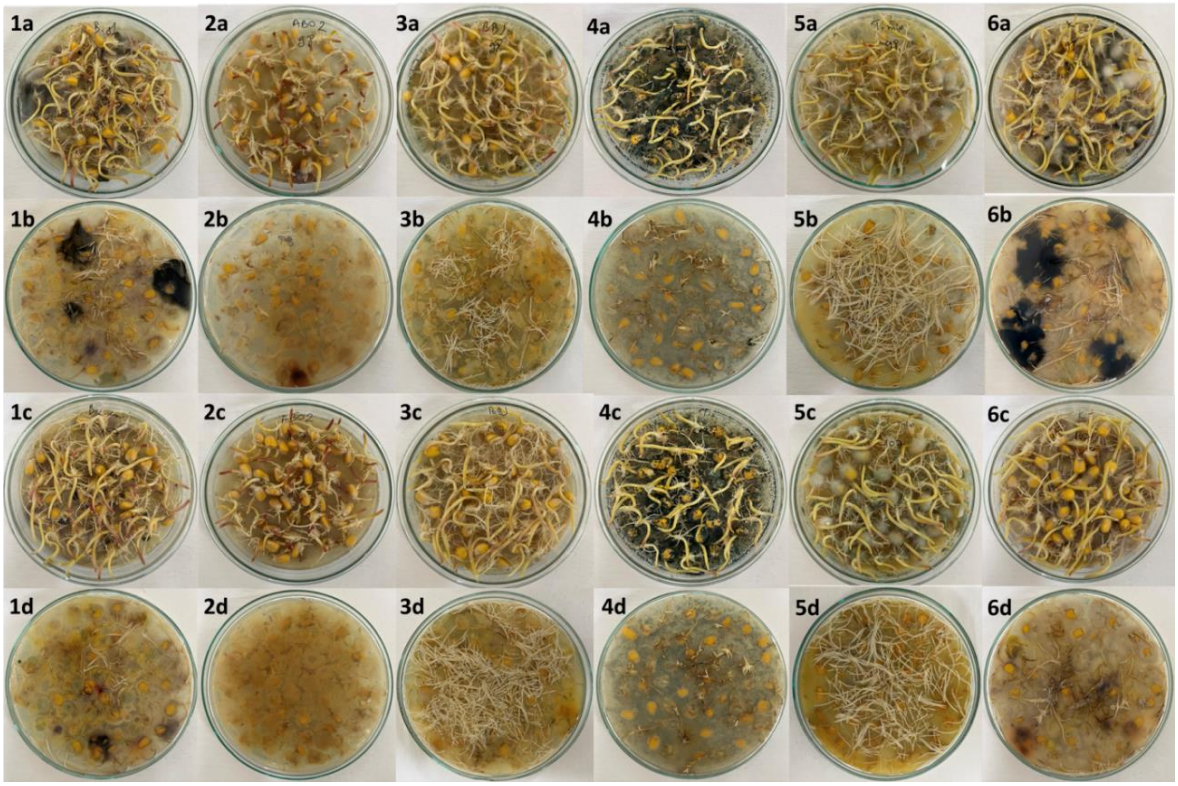


Figure 1. Comparative view of 2 samples of primed hybrid P0217 maize seeds after 7 days (a – view from top side (Sample 1), b – view from bottom side (Sample 1), c - view from top side (Sample 2), d – view from bottom side (Sample 2), 1 – BA1, 2 – ABO2, 3 – P1, 4 – T251/21, 5 – T. mix, 6 – Control)

By measuring the primary root and mesocotyl length of maize seedlings, differences between treatments were recorded. Among all applied bioagents, the highest root length (average 58.25 mm) was recorded when using *Pichia* sp. (P1), while the shortest root length (average 16.38 mm) was noticed using *B. amyloliquefaciens* (ABO2). Compared with the control, when applying *Pichia* sp. and *T. harzianum* (T. mix), an increase in root length was observed (11.54% and 1.96%, respectively), however, not statistically significant. Other bioagents had opposite effect on the root length. The longest mesocotyl was found when the bioagent *Pichia* sp. was applied (average 40.03 mm), while the shortest mesocotyl was rated in treatment with bioagent *B. amyloliquefaciens* (ABO2) (average 26.4 mm). In both samples, statistically significant difference was recorded when applied bioagent *B. amyloliquefaciens* (ABO2), while using *T. asperellum* (T251/21) and *B. amyloliquefaciens* (BA1) in one sample statistically significant difference was noted. The effect of other bioagents was not statistically significant (Figure 2).

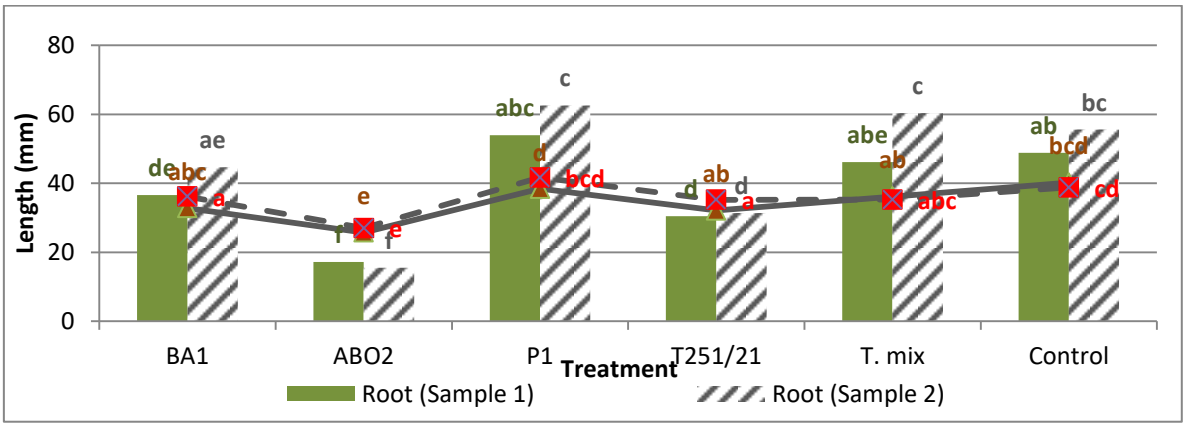


Figure 2. Influence of potential bioagents on primary root and mesocotyl length of maize

Most species of *Trichoderma* have been associated with biocontrol strategies against plant pathogenic fungi. Based on the results of Chandra Nayaka et al. (2010), maize seed treatment with the microbial agent *T. harzianum* effectively reduced *Fusarium verticillioides* incidence. Ferrigo et al. (2020) announced that *T. harzianum* applied to seeds can reduce both *F. verticillioides* and *F. graminearum* disease incidence and severity. These results are in agreement with our work, where it was found that when applying bioagents based on *T. asperellum* and *T. harzianum*, the presence of *Fusarium* was not determined in comparison with the control. Several studies have confirmed the positive influence of biopriming of maize seeds on seedling length. Maize seed primed with *Trichoderma* spp. and *Bacillus* spp. significantly affect plant height, root length, fresh weight, and dry weight (Mutetwa et al., 2019). López-Coria et al. (2016) observed that *T. asperellum*, similar to other *Trichoderma* spp. stimulated shoot and root growth, when it is used for biopriming. Contreras-Cornejo et al. (2009) claimed that biopriming with *Trichoderma* spp. can increase the shoot elongation, particularly of the mesocotyl, while Shaffique et al. (2022) declared that maize seed biopriming with *Enterobacter* sp. enhanced average mesocotyl and root length. In a study by Chidanandappa and Singh (2024), fungal bioagents *T. harzianum* and *T. afroharzianum* and bacterial bioagent *Pseudomonas sihuinsis* significantly increased germination, root:shoot ratio, and seedling vigor, which ultimately enhanced the plant growth and development.

CONCLUSIONS

Biological seed treatments may provide an alternative to chemical control and can save yields to feed the increasing world population while reducing environmental risks and health hazards. As presented, *Bacillus*, *Trichoderma*, and *Pichia* bioagents can benefit the control of maize seed pathogens when applied as a seed treatment, whereby bioagents based on *Trichoderma* expressed the highest potential. Significant differences between applied bioagents on the germination parameters were found. It was confirmed that priming seeds with *Pichia* spp. and *T. harzianum* bioagents can improve root and mesocotyl development, while bioagents *B. amyloliquefaciens* had opposite effect. To realize the application of bioagents for maize seed biopriming, further research of their influence on pathogen control and seed quality are necessary.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant Nos: 451-03-65/2024-03/ 200117, and 451-03-66/2024-03/ 200117).

REFERENCES

Afrouz, M., Sayyed, R. Z., Fazeli-Nasab, B., Piri, R., Almalki, W., & Fitriatin, B. N. (2023). Seed bio-priming with beneficial *Trichoderma harzianum* alleviates cold stress in maize. *PeerJ*, *11*, e15644.

Camera, J. N., Forcelini, C. A., Koefender, J., Golle, D. P., Schoffel, A., & Deuner, C. C. (2019). Reaction of maize hybrids to Northern corn leaf blight and common rust, and chemical control of Northern corn leaf blight. *Arquivos do Instituto Biológico*, *86*, e0082018.

Chandra Nayaka, S., Niranjana, S. R., Uday Shankar, A. C., Niranjan Raj, S., Reddy, M. S., Prakash, H. S., & Mortensen, C. N. (2010). Seed biopriming with novel strain of *Trichoderma harzianum* for the control of toxigenic *Fusarium verticillioides* and fumonisins in maize. *Archives of Phytopathology and Plant Protection*, *43*(3), 264-282.

Chidanandappa, Singh (2024). Enhancing Maize Seed Vigour through Seed Biopriming Using Bioagents. *Journal of Advances in Biology & Biotechnology*, *27*(2), 48-56.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Cortés-Penagos, C., & López-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant physiology*, *149*(3), 1579-1592.

Erasto, R., Kilasi, N., & Madege, R. R. (2023). Prevalence and management of phytopathogenic seed-borne fungi of maize. *Seeds*, *2*(1), 30-42.

Erenstein, O., Jaleta, M., Sonder, K., Mottaleb, K., & Prasanna, B. M. (2022). Global maize production, consumption and trade: trends and R&D implications. Food security, 14(5), 1295-1319.

FAOSTAT. (2022). https://www.fao.org/faostat/en/#data/QCL/visualize Accessed 25 August 2024.

Ferrigo, D., Mondin, M., Ladurner, E., Fiorentini, F., Causin, R., & Raiola, A. (2020). Effect of seed biopriming with *Trichoderma harzianum* strain INAT11 on *Fusarium* ear rot and *Gibberella* ear rot diseases. *Biological Control*, *147*, 104286.

López-Coria, M., Hernández-Mendoza, J. L., & Sánchez-Nieto, S. (2016). *Trichoderma asperellum* induces maize seedling growth by activating the plasma membrane H+-ATPase. *Molecular Plant-Microbe Interactions*, *29*(10), 797-806.

Mutetwa, M., Nyaera, K., Masaka, T., Mtaita, & T. A. (2019). Effect of bio priming seeds with microbial based bio fertilizers on growth of maize seedlings. *International Journal of Research and Review*, *6*(10), 281-288.

Nyandi, M. S., & Pepó, P. (2024). *Aspergillus* and *Fusarium* Mycotoxin Contamination in Maize (*Zea mays* L.): The Interplay of Nitrogen Fertilization and Hybrids Selection. *Toxins*, *16*(7).

Official Gazette of the Republic of Serbia, 107/2008. https://pravno-informacioni-sistem.rs/eli/rep/sgrs/ministarstva/pravilnik/2006/39/2 Accesssed on 24 August 2024.

Oren, L., Ezrati, S., Cohen, D., & Sharon, A. (2003). Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Applied and environmental microbiology*, *69*(3), 1695-1701.

Reddy, P. P., & Reddy, P. P. (2013). Bio-priming of seeds. *Recent advances in crop protection*, 83-90.

Shaffique, S., Khan, M. A., Wani, S. H., Imran, M., Kang, S. M., Pande, A., Adhikari, A., Kwon, E. H., & Lee, I. J. (2022). Biopriming of maize seeds with a novel bacterial strain SH-6 to enhance drought tolerance in South Korea. *Plants*, *11*(13), 1674.

Singh, H. B. (2016). Seed biopriming: a comprehensive approach towards agricultural sustainability. Indian Phytopathol, 69(3), 203-209.

STATISTICA (Data Analysis Software System) (1984-2020). v.14.0.0.15., TIBCO Stat-Soft, Inc., USA (www.statsoft.com)

Statistical Office of the Republic of Serbia. https://www.stat.gov.rs/ Accessed 22 August 2024.

DEVELOPMENT OF GREEN ANALYTICAL METHOD COMBINED SELECTIVE EXTRACTION AND LIQUID CHROMATOGRAPHY FOR ANALYSIS OF FOOD SAMPLES

Andrea Špačková*, Lucia Krasňanská, Katarína Hroboňová

Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 81237 Bratislava, Slovakia

*Corresponding author:
E-mail address: andrea.spackova@stuba.sk

ABSTRACT

Analysis of food involves sample preparation, analysis, and detection of major food components (e.g. carbohydrates, phenolic compounds, aroma compounds, etc.) and miscellaneous components (preservatives, colorants, and other). A large number of different analytical methods have been developed to analyze the properties of food because of the complexity and diversity of food components. Increased method selectivity can be achieved by applying appropriate extraction technique. Molecularly imprinted polymers (MIPs) are synthetic materials that specifically recognize and selectively adsorb certain target molecules, template or their structural analogues. MIPs are a promising tool for the extraction of substances from complex samples. The aim of this work was to develop green analytical method combined with selective extraction and liquid chromatography for analysis of citrus fruits (lime, grapefruit, tangerine, and other). Deionized water, methanol, and deep eutectic solvent-like mixtures were tested for extraction, a solid phase extraction with MIP-based sorbents was used for analytes cleaning and reversed phased high performance liquid chromatography method for extract analysis. The proposed method demonstrated a linear range of 50-1000 ng.mL^{-1}, the limits of detection in the range of 8.2-44 ng.mL^{-1} for selected coumarins, and the recovery more than 85% (RSD ≤ 4.8%). Umbelliferone was detected in lime and in grapefruit, herniarin was detected in lime. The environmental character of the analytical method was evaluated using AGREE, GAPI, and Analytical Eco-Scale.
Keywords*: citrus fruits, coumarins, MIP-based extraction, HPLC*

INTRODUCTION

Natural materials are rich sources of bioactive compounds. They are beneficial in treating a wide range of diseases, they emphasize the smell or taste of the natural product, food, food supplements, cosmetics, and other.
Coumarins, a type of secondary plant metabolites, play a significant role in the biochemistry and physiology of plants. Chemically, coumarins are organic heterocyclic compounds composed of a benzene ring connected to a pyrone ring.
Citrus fruits and citrus peel (e.g., lemon peel) are highly valued in the food, pharmaceutical, and cosmetic industries due to their nutritional, medicinal, and fragrance properties (Saini et al., 2022). Citrus peels, which constitute 40-50% of the fruit's total mass are often regarded as waste, contain valuable bioactive compounds, including coumarins. Natural lemon peel powder, for instance, has been used as a catalyst in synthesizing coumarin-based compounds (Gómez-Mejía et al., 2019; Lončar et al., 2020). Research indicates higher concentrations of coumarins, such as umbelliferone and herniarin (Fig. 1) in citrus peels compared to the edible parts of the fruit (Andrade et al., 2023). These compounds have demonstrated various health benefits: umbelliferone has antituberculotic, anti-inflammatory, antidiabetic, antibacterial, antifungal, and antioxidant properties, while herniarin shows anti-inflammatory, antioxidant, anti-proliferative, and antinociceptive effects. Despite these benefits, high concentrations can cause skin, eye, or respiratory irritation, warranting further pharmacological research (Porras-Dávila et al., 2023).

Figure 1. Chemical structure of umbelliferone and herniarin

Utilizing citrus fruits and peel effectively requires a reliable and reproducible analytical method for targeted analysis and quality evaluation. Sample pretreatment is essential for analyte determination. Advanced techniques, such as ultrasound-assisted extraction (UAE) (Kumar et al., 2021), microwave-assisted extraction (MAE) (Gómez-Mejía et al., 2023), and supercritical fluid extraction (SFE) (Anticona et al., 2020) were used for isolation of coumarins from different citrus fruits. The choice of extraction solvent depends on the nature of the compounds being extracted, the desired purity, and environmental considerations. Ethanol, methanol, dichloromethane, and *n*-hexane are the most used solvents, however greener solvents, like supercritical carbon dioxide and deep eutectic solvents (DES) are introduced due to their efficiency and sustainability (Anticoma et al., 2020). Solid phase extraction (SPE) was commonly used for primary extract purification and analyte preconcentration. In addition to traditional sorbents, a MIP-based sorbents are incorporated in new methods due to the high selectivity, chemical and mechanical resistance, and reusability, making them suitable for extracting specific compounds from complex samples (Chen et al., 2016).

This study focuses on the qualitative and quantitative analysis of citrus peel such as lime, grapefruit, tangerine peel and others. The sample pretreatment procedures incorporating solvent extraction with different solvents and solid phase extraction with a selective MIP-based sorbent was developed.

MATERIAL AND METHODS

Methanol (HPLC grade) and acetic acid (for analysis grade) were supplied by Centralchem (Bratislava, Slovakia). The analytical standards umbelliferone (7-hydroxycoumarin) and herniarin (7-methoxycoumarin) (both 98 %) were purchased from Sigma Aldrich (USA). Chemicals for preparation of DES-like mixtures, choline chloride (≥ 98 %), and 1,3-propanediol (98 %) were purchased from Sigma Aldrich (Schnelldorf, Germany), and L-lactic acid (90 % solution) was supplied by VWR International (Bratislava, Slovakia). Citrus samples were purchased from a local supermarket. Samples were cut into small pieces and stored in a freezer before analysis. DES 1 and DES 2 consisted of choline chloride : L-lactic acid, 1:4, mol/mol and choline chloride : L-lactic acid : 1,3-propanediol, 1:2:1, mol/mol/mol, respectively.

Ultrasound-assisted extraction

Chopped citrus peel (1 g) was mixed with 3 mL of extraction solvent. UAE was realized in an ultrasonic bath (45 kHz) at a constant temperature (40 °C) for 10 minutes. After extraction, the mixture was centrifuged for 1 minute at 3000 rpm.

Solid phase extraction

Primary aqueous UAE sample extract (1.5 mL; obtained under optimal conditions) was mixed with 0.1 g of Fe_3O_4@MIP-umbelliferone) sorbent (Machyňáková et al., 2017) previously preconditioned with methanol : acetic acid (9:1, v/v) and water (1.0 mL). The mixture was stirred at 180 rpm for 30 minutes at 23 °C. Afterward, the sorbent was separated using an external magnet, washed with 1 mL of water for 5 minutes at 180 rpm, and dried at 90 °C. Subsequently, Fe_3O_4@MIP was mixed with 1.5 mL of methanol : acetic acid (9:1, v/v) and stirred for 30 minutes at 180 rpm. The supernatant was magnetically separated and analyzed by HPLC-FLD.

Analysis was performed on HPLC system (series 1200, Agilent Technologies, Germany) equipped with a binary pump, automatic injector, column oven, and fluorescence detector

(FLD). A Kinetex C18 analytical column (100 mm × 4.6 mm, 5 µm, Phenomenex, USA) was used for separation of coumarins. The mobile phase consisted of 1% aqueous acetic acid solution (A) and 1% acetic acid in methanol (B). The gradient elution program was as follows: 0-12 min 30-45% B in A, 12-12.5 min 45-100% B, 12.5-14.5 min 100% B. The flow rate was 1.0 $mL.min^{-1}$, an injection volume was 20 µL, and the column temperature was 23 °C. All analytes were monitored at 320 nm and 450 nm as excitation and emission wavelength, respectively.

RESULTS AND DISCUSSION

Ultrasound-assisted extraction combined with solid phase extraction using a molecularly imprinted polymeric sorbent selective for coumarins was carried out to extract analytes form citrus peel. The sample pretreatment procedures were optimized to produce an extract suitable for HPLC-FLD analysis.

Solvent extraction is the most used sample pretreatment technique for solid matrices to separate or isolate compounds. Solvent selection plays a key role in achieving high extraction efficiency. In the work, the methanol, deionized water, DES 1 : water (3:1, v/v), DES 2 were tested. The most suitable solvents for the extraction of herniarin and umbelliferone was deionized water and a mixture of DES 1 : water (3:1, v/v) (Figure 2). Both solvents are ecological, but the preparation of the DES 1 mixture is more complex. Extracts in deionized water are also preferable for subsequent purification using SPE. Optimization of extraction temperature from 23 to 40 °C shows that a higher temperature allows the extraction of coumarins more effectively. In general, an increased temperature often allows a faster extraction, where the analyte molecules diffuse faster into the extraction solvent. Optimization of the extraction time was evaluated in time interval 10-30 minutes reaching the maximal extraction yield at 10 minutes. Optimal conditions for extracting coumarin derivatives, umbelliferone, and herniarin, were established as UAE with deionized water at 40 °C for 10 minutes. These conditions were suitable for obtaining primary extracts from lime peel and was applied for other citrus fruits (e.g., lemon, orange, pomelo, grapefruit). Advanced technique such as UAE, is "greener" and allows efficient extraction in shorter times (several minutes).

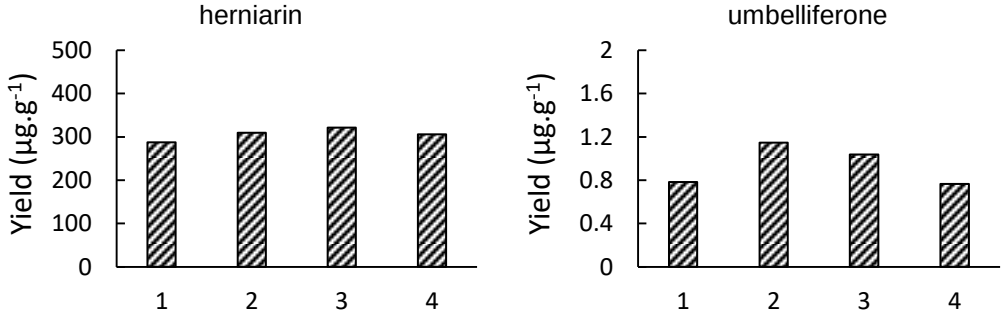


Figure 2. Optimization of extraction solvent for UAE of lime (n = 3, RSD < 8.5 %)
1 – methanol; 2 – deionized water; 3 – DES 1 : water (3:1, v/v); 4 – DES 2

SPE with different type of sorbents were used for primary extract purification and analytes concentration. Sorbents of C18 type, polymeric sorbent selective for phenolic compounds and lab-made MIPs selective for coumarins were tested. MIP-umbelliferone was filled in SPE column or magnetic MIPs (Fe_3O_4@MIP-umbelliferone) was used in batch extraction. Based on the higher recovery results, a batch extraction on Fe_3O_4@MIP-umbelliferone was selected. The recoveries were 93% and 87% (RSD ≤ 4.8%, n=3) for umbelliferone and herniarin, respectively. The Fe_3O_4@MIP extraction method offers advantages such as reduced solvent volume and simple extraction procedure.

The applicability of the method for coumarins isolation and quantification was investigated by extracting different citrus peel samples, lime, grapefruit, tangerine, pomelo, and others. The extracts were analyzed by the HPLC-FLD method, which reached the limits of detection 8.2-44 ng.mL^{-1} for targeted coumarins. Umbelliferone was detected in the lime (Figure 3), grapefruit, tangerine and pomelo sample, while herniarin was detected in lime. Amount of umbelliferone varied from 0.5 to 1.1 µg.g^{-1}, however herniarin content was higher (450 µg.g^{-1}). Citrus fruits, e.g., orange, lemon, grapefruit, pomelo, and various lime species such as Persian lime and Mexican lime vary in structural diversity and composition (Goh et al., 2022).

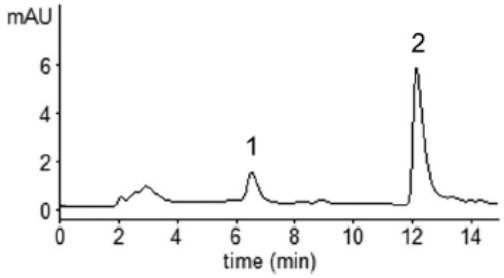
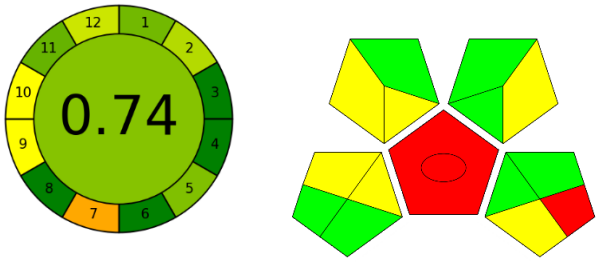


Figure 3. HPLC-FLD chromatograms of lime peel extract tread by Fe_3O_4@MIP batch solid phase extraction. Legend: 1- umbelliferone, 2- herniarin

Proposed method combining UAE extraction, Fe_3O_4@MIP extract cleaning and coumarins concentration, and HPLC-FLD separation of selected coumarins. The approaches contribute to the environmental friendliness of the method were applied. These involve the use of ecological solvents - water or DES, devices with low energy consumption (UAE), and reusable sorbents for SPE. To assess the impact of the proposed procedures on the environment AGREE, GAPI, and Analytical Eco-Scale (Bartolo et al., 2024) tools were used, which represent a comprehensive approaches to evaluating the environmental friendliness of the analytical method. AGREE and GAPI evaluates the environmental impact across the main stages: general method type, sample collection, sample preparation, reagents and solvents required, and instrumentation. The Analytical Eco-Scale is based on assigning penalty points to individual parts of the analytical precedure that are not in accordance with the ideal green analysis. Figure 4 shows the environmental evaluations of the method. AGREE score is higher than 0.7 (Fig. 4a) which indicates ecologically acceptable method. In case of GAPI the most parts, seven, are green, six yellow and two red (Fig. 4b), which means method is ecologically acceptable. The Analytical Eco-Scale score was higher than 80 (Fig. 4c) indicating excellent green analytical method. The biggest impact on the greenness of the approaches was the amount of waste, used solvents, and sample preparation techniques.

a b c



		Penalty points
Solvents	Methanol (8 mL)	6
	Acetic acid (1 mL)	2
	Deionised water (3 mL)	0
Instrumentation	HPLC-FLD	1
	UAE	1
	SPE	1
	Centrifuge	1
	Occupational risks	0
	Waste (15 mL)	6
Total number of penalty points		18
Score		**82**

Figure 4. Evaluation of the environmental impact of the proposed method for the analysis of coumarins in citrus fruits with AGREE (a), GAPI (b), and Analytical Eco-Scale (c)

CONCLUSIONS

The analysis of citrus fruits peel was conducted to evaluate its suitability for various applications, including food, cosmetics, or as a source of bioactive or other value-added compounds. Efficient, simple, and time-saving analytical method that meets the requirements for high-throughput screening and ecological sustainability can enhance the wider use of natural materials. This study shows that UAE and selective MIP-based extraction are effective techniques for sample pretreatment and isolating coumarins from citrus, allowing their subsequent quantification using the HPLC-FLD method. The proposed method is green and has potential for analyzing other citrus samples.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Research and Development Agency under the Contract no. SK-SRB-23-0015 and project VEGA (1/0332/24). A. Špačková thank for financial support from the call for doctoral students and young researchers of Slovak University of Technology in Bratislava to start a research career (Grant 23-04-01-A).

REFERENCES

Andrade, M. A., Barbosa, C. H., Shah, M.A., Ahmad, N., Vilarinho, F., Khwaldia, K., Silva, A. S., Ramos, F. (2023) Citrus By-Products: Valuable Source of Bioactive Compounds for Food Applications. *Antioxidants*, 12 (1), 38.

Anticona, M., Lopez-Malo, D., Frigola, A., Esteve, M. J., Blesa, J. (2022). Comprehensive Analysis of Polyphenols from Hybrid Mandarin Peels by SPE and HPLC-UV. LWT, 165, 113770.

Bartolo, N. S., Gallo, L. L., Szyrner, K., Buhagiar, P. I., & Szijj, J. V. (2024). Greenness Assessment of Analytical Methods for Determination of Cannabinoids in Oils Using NEMI, Analytical Eco-Scale, AGREE and GAPI. Analytical Methods, Advance article, https://doi.org/10.1039/D4AY01083C

Dosoky, N.S., Satyal, P., Setzer, W.N. (2022) Authentication of Citrus spp. Cold-Pressed Essential Oils by Their Oxygenated Heterocyclic Components. Molecules, 27 (19), 6277.

Dugrand, A., Olry, A., Duval, T., Hehn, A., Froelicher, Y., Bourgaud, F.J. (2013) Coumarin and Furanocoumarin Quantitation in Citrus Peel via Ultraperformance Liquid Chromatography Coupled with Mass Spectrometry (UPLC-MS). Journal of Agricultural and Food Chemistry, 61 (45), 10677-10684.

García-Martín, J. F., Feng, C. H., Domínguez-Fernández, N. M., Álvarez-Mateos, P. (2023) Microwave-Assisted Extraction of Polyphenols from Bitter Orange Industrial Waste and Identification of the Main Compounds. Life, 13 (9), 1864.

Goh, R. M. V., Pua, A., Luro, F., Ee, K. H., Huang, Y., Marchi, E., Yu, B. (2022). Distinguishing Citrus Varieties Based on Genetic and Compositional Analyses. PLoS One, 17 (4), e0267007.

Gómez-Mejía, E., Rosales-Conrado, N., León-González, M. E., & Madrid, Y. (2019). Citrus Peels Waste as a Source of Value-added Compounds: Extraction and Quantification of Bioactive Polyphenols. Food Chemistry, 295, 289-299.

Hroboňová, K., Jablonský, M., Májek, P. (2021) Optimization and Application of Green Solvent Extraction of Natural Bioactive Coumarins from Lamiaceae and Asteraceae Herbal Plants. Journal of Molecular Liquids, 338, 116691.

Hroboňová, K., Špačková, A. (2020) Application of Selective Polymeric Sorbents for Simple Coumarins Extraction from Deodorant Samples. Acta Chimca Slovaca, 13 (1), 56-62.

Chen, L., Wang, X., Lu, W., Wu, X., & Li, J. (2016). Molecular Imprinting: Perspectives and Applications. Chemical society reviews, 45 (8), 2137-2211.

Jungen, M., Lotz, P., Patz, C. D., Steingass, C. B., & Schweiggert, R. (2021). Coumarins, Psoralens, and Quantitative 1H-NMR Spectroscopy for Authentication of Lemon (Citrus limon [L.] Burm. f.) and Persian Lime (Citrus× latifolia [Yu. Tanaka] Tanaka) Juices. Food Chem., 359, 129804.

Kumar, K., Srivastav, S., Sharanagat, V. S. (2021) Ultrasound Assisted Extraction (UAE) of Bioactive Compounds from Fruit and Vegetable Processing by-Products: A review. Ultrasonic Sonochemistry, 70, 105325.

Porras-Dávila, L. S., Zamilpa, A., Jiménez-Ferrer, E., Jiménez-Aparicio, A., Santillan-Urquiza, A. M, Díaz-Patricio, F., Herrera-Ruiz, M. (2023) Anti-Inflammatory and Neuroprotective Effects of Standardized Fractions in Herniarin and Daphnoretin from Distictis buccinatoria. Chemistry & Biodiversity, 20 (5), e202200969.

Lončar, M., Jakovljevic, M., Šubaric, D., Pavlic, M., Buzjak-Služek, V., Cindric, I., Molnar, M. (2020) Coumarins in Food and Methods of Their Determination. Foods, 9 (5), 645.

Machyňáková, A., Hroboňová, K. (2017) Preparation and Application of Magnetic Molecularly Imprinted Polymers for the Selective Extraction of Coumarins from Food and Plant Samples. Analytical Methods, 9, 2168-2176.

Saini, R. K., Ranjit, A., Sharma, K., Prasad, P., Shang, X., Gowda, K. G. M., Keum, Y. S. (2022) Bioactive Compounds of Citrus Fruits: A Review of Composition and Health Benefits of Carotenoids, Flavonoids, Limonoids, and Terpenes. Antioxidants (Basel), 11 (2), 239.

PATHOGENICITY OF Fusarium spp. ON POTATO TUBERS

Dobrila Radić*, Milica Meseldžija, Teodora Mihaljfi, Mila Grahovac, Jozef Gašparovski, Vera Stojšin, Dragana Budakov

Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: dobrila,kovacevic@polj.edu.rs

ABSTRACT

Dry rot caused by several species from genus *Fusarium* is a devastating fungal disease affecting potatoes worldwide. Infected tubers and seed pieces rot after harvest or planting. Losses from dry rot during storage are estimated to range from 6 % to 25 %, and occasionally rise up to 60 % in extreme cases. Symptoms of *Fusarium* dry rot first appear on tubers at wound sites as shallow, small brown lesions after about a month of storage. Cavities below the brown, dry decay areas are usually lined with mycelium. Infections of potato tubers by certain *Fusarium* species are usually accompanied by the production of mycotoxin contamination. The task of this work is to check the pathogenicity and determine the difference in virulence of twenty isolates originating from potato tubers with symptoms of tuber dry rot, which were identified as *Fusarium* spp. based on morphological and cultural characteristics. The sample consisted of randomly selected tubers, with typical symptoms of dry rot. Pathogens were isolated from the infected tubers using standard phytopathological methods on Potato Dextrose Agar (PDA). The pathogenicity test for each isolate was performed by wounding and artificially inoculating potato tubers of the Orchestra variety. The inoculated tubers were placed in plastic boxes and incubated in humid conditions at room temperature for 21 days. After the incubation period, the horizontal and vertical diameters of the necrosis were measured. Subsequently, the pathogen was re-isolated from the infected tuber. Data analysis was done using ANOVA (Analysis of variance) followed by Tukey's HSD test. Significant differences ($p \leq 0.05$) were observed in the diameter of necrosis caused by different *Fusarium* spp. isolates. The highest virulence was detected in isolates FK47 and FK39, both from Zobnatica, whereas isolate FK48 (Pivnice) had the lowest degree of virulence. The remaining isolates were within the group with medium virulence. This research is part of a study on potato dry rot in Serbia, a significant issue due to the substantial quantitative losses and qualitative damages it causes.
***Keywords**: Fusarium spp, potato, dry rot*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the main non-cereal food in terms of consumption. In Serbia, potato production was over 520.000 tons in 2022 with an average yield of 21.6 tons/ha (FAOSTAT 2022).

Among the biotic stresses, fungal pathogens are the major limiting factor in the potato production system that can cause economic losses in the field as well as during transportation and storage (Tiwari et al., 2020a). *Fusarium* is one of the most important genera of phytopathogenic fungi, which causes wilting of potatoes in the field and dry rot of potato tubers during storage (Azil et al., 2021).

According to Cullen et al., (2005), 13 species of *Fusarium* are considered causative agents of *Fusarium* dry rot in potatoes worldwide, but species composition depends on geographic location and season. Due to differences in potato cultivars and climatic conditions, different *Fusarium* species have been isolated and identified from *Fusarium* dry rot of potatoes in different countries and regions. Among them, *F. sambucinum* is considered in Europe, China and North America as the most aggressive fungal species that causes dry rot in tubers (Du et al., 2012; Secor and Salas, 2001). In general, *Fusarium* spp. can infect potato tubers through superficial wounds or natural tuber openings during, before, or after harvest.

Typical symptoms on the skin of potato tubers infected with *Fusarium* spp. generally include a wrinkled brown appearance and sunken tissue with a dry and leathery appearance. Initial symptoms were observed to be shallow, small brown spots at tuber wound sites after

approximately 30 days of storage. After that, the infected tissue begins to enlarge in all directions, and the periderm of the tuber gradually sags and decays. Finally, concentric rings are observed on the enlarged lesions, and the dead tissue begins to dry out (Liu et al., 2022; Zhang et al., 2023). Cotton-white, purple, yellow, pink or brick-colored spores and mycelia of *Fusarium* spp. they are observed in the cavity under the rotten lesion (Vatankhah et al., 2019). In addition to economic losses, *Fusarium* spp. they are known to produce mycotoxins that contaminate potato tubers and are toxic to humans and animals (Senter et al., 1991; Bennett and Klich, 2003*)*. Most *Fusarium* species are able to produce one or more mycotoxins with varying degrees of toxicity (Bottalico and Perrone, 2002), often classified as trichothecene and non-trichothecene mycotoxins (Mills, 1990). The main types of mycotoxins produced by *Fusarium* spp. include bovericin, moniliformin, fumonisins, trichothecenes and zearalenone, the last two being considered the most important due to their frequent occurrence. Trichothecene products of *F. culmorum*, *F. graminearum* and *F. sambucinum* (Stępień *and* Waśkiewicz*, 2013*). An experiment conducted by Xue et al., 2014 found that trichothecenes accumulate not only in the lesion, but also in the adjacent asymptomatic tissue of potato tubers, which is important from a consumer perspective.
Control strategies for *Fusarium* dry rot include cultural practices such as crop rotation, use of disease-free seed, and avoidance of tuber injury during harvest. Biological control agents, ultraviolet radiation, and chemical control are also used to control the disease (Heltoft et al., 2015).
The goals of this research were to: (i) form a collection of *Fusarium* spp. isolates from potato tubers with typical symptoms of dry rot (ii) to assess their pathogenicity on potato tubers in laboratory conditions.

MATERIAL AND METHODS

Sampling and isolation of pure fungal cultures - Potato tubers with symptoms of dry rot were collected in 2022 from warehouses in the locations: Čačak, Kušić, Zobnatica, Čurug and Pivnice. The sample consisted of randomly selected tubers, with typical symptoms of dry rot. Pathogens were isolated from the infected tubers using standard phytopathological methods on Potato Dextrose Agar (PDA). Potato tubers were washed with tap water to remove the soil, then they were cut in the middle of the necrosis with a sterile scalpel, and tissue fragments were taken at the cross section between healthy and diseased tissue. Tissue fragments were placed on PDA and incubated in the dark at 25 ± 1 °C for seven days. After incubation, different types of fungal colonies were observed on the PDA medium, but only typical colonies with *Fusarium* characteristics were selected. Mycelia were transferred to Petri dishes with sterile PDA to obtain pure cultures. The formation of monohyphal isolates was performed using the method described by Leslie and Summerell (2006).
Pathogenicity test - The pathogenicity test for each of the 20 collected isolates was performed by wounding and artificially inoculating potato tubers of the Orchestra variety. The tubers were washed with tap water, surface sterilized by immersion in a solution of 1% sodium hypochlorite for 3 minutes, then they were washed with distilled water and left to dry on sterile blotter paper overnight. Isolates were grown for seven days on PDA medium at 25 ± 1 °C. The apical end of each tuber was wounded with a sterile metal punch leaving a wound 3 mm deep and 4 mm wide. The tubers were inoculated with round fragments of mycelia (3 mm in diameter) so that the mycelia came into direct contact with the damaged tissue of the tuber. Two tubers were prepared for each isolate. The inoculated tubers were incubated in plastic boxes, at room temperature and under natural conditions of day and night shifts for 21 days while maintaining high humidity inside the boxes. After the incubation period, the necrosis diameter was measured. The pathogen was then re-isolated from the tubers in the same way as the initial pathogen isolation
Statistical analysis - For the results of the pathogenicity test, the size of the diameter of the lesion of each individual tuber was used in the analysis. Statistical data processing was performed using the Statistica 14.0 software package (TIBCO Software, USA) using analysis of variance (ANOVA) and post hoc test (Tukey's HSD) in a 95% confidence interval.

RESULTS AND DISCUSSION

Potato samples with rot symptoms were collected from different warehouses from 5 locations. All sampled tubers showed symptoms of dry rot. During the sample collection period, a large number of isolates of phytopathogenic fungi were obtained. For many of them, based on the appearance of the colony and spores, it could be safely concluded that they do not belong to the genus *Fusarium*, and they were excluded from the collection and further research. A total of 45 isolates of *Fusarium* spp. monohyphal cultures were formed for 20 representative isolates (FK8, FK23, FK25, FK27, FK28, FK30, FK32, FK33, FK34, FK35, FK36, FK37, FK38, FK39, FK40, FK46, FK47, FK48, FK50, FK51). For each of the 20 isolates, pathogenicity was checked and then re-isolation was performed. The symptoms that developed on the tubers of the cultivar Orchestra were identical to the symptoms on the tubers from which the isolates were isolated. The diameter of the developed necrosis was measured after the incubation period of 21 days. Analysis of variance determined the existence of significant differences ($p \leq 0.05$) between isolates in the diameter of the necrosis they caused on artificially inoculated tubers (Table 1).

Table 1. One-factor analysis of the variance of the diameter of necrosis on artificially inoculated potato tubers of the Orchestra variety with isolates of Fusarium spp.

Effect	Univariate Tests of Significance for AUDPC (food tech) Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of (Freedom)	MS	F	p
Intercept	21587.67	1	21587.67	3551.23163	0
IZOLAT	643.15	19	33.85	5.56839	1.5223E-07
Error	364.74	60	6.08		

The largest necrosis diameter was measured in isolate FK47 of 42.25 mm. However, isolates FK8, FK23, FK28, FK39 were at the same level of significance. The smallest necrosis diameter was measured in isolate FK48 and was 10.5 mm. The remaining 14 isolates were at the same level of significance. *Fusarium* spp. were detected on tubers with symptoms of dry rot and could have caused mycotoxin contamination. During the research, *Fusarium* species were among the main fungi isolated from tuber rot based on morphological and cultural characteristics. In potato tuber inoculation tests, lesion sizes varied from 10.5 mm to 42.25 mm. Based on the size of the lesions, the pathogenicity of *Fusarium* spp. isolates were divided into four groups: virulent (over 39 mm), moderately virulent (30 - 39 mm), hypovirulent (11 - 29 mm) and non-virulent (less than 11 mm) (Figure 1).

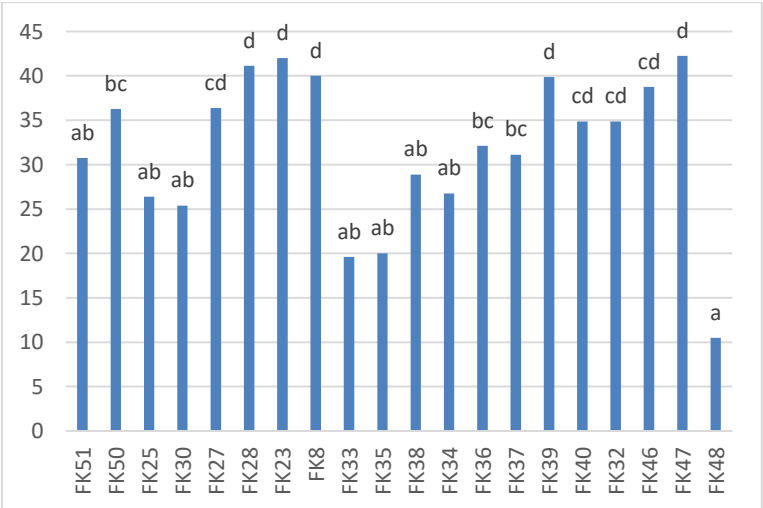


Figure 1. Presentation of isolates and diameter of necrosis
Rank a, b, c, d – According ANOVA and Tukey's HSD test for significance level of 95%

Fusarium species are ubiquitous in soil and may exist as saprophytes or pathogens in plant tissues and debris or as opportunistic pathogens awaiting stress in their host (Palmer and Kommedahl, 1969). Accurate species identification is essential to develop appropriate pathogen control practices.It is important to know the identity of *Fusarium* species that predominate as inoculum in potato stocks as this will allow growers and marketers to determine the risk of tuber dry rot development based on a number of factors (including crop location, cultivar and seed tuber fungicide treatment). For example, *F. sambucinum* has been identified as a particularly important species due to its general aggressiveness and resistance to thiabendazole. The main agents that cause dry rot of potato tubers vary by location, but may also change over time, especially under climate change conditions. Isolates of *Fusarium* species from infected plant tissues vary in their pathogenicity, just as in earlier reports (Ray and Hammerschmidt 1998; El-Hassan et al., 2007). Not all fungi isolated from diseased potato tubers or other plant organs were able to cause dry rot or tuber wilt under laboratory conditions. Such isolates may be saprophytes, secondary colonizers or contaminants, as hypothesized by Stefańczyk et al., (2016). These isolates could be used in biological protection after testing their effect on pathogenic *Fusarium* species, which will be considered in the following period. Data from our research can be used to monitor *Fusarium* spp. in potatoes. Knowing which *Fusarium* spp. are present in potato tubers with dry rot, could help reduce crop losses and mycotoxin contamination by fungicide application, as resistance to fungicides can vary in frequency among isolates of different species (Peters et al. 2008a). Moreover, the obtained data can be helpful for planning the crop rotation, but also the crops near the investigated area. Some of the *Fusarium* spp. that we have isolated from potato tubers known to be important pathogens of cereals and cultivation of such cereal host species before or next to potatoes should be avoided to limit the risk of potato tuber dry rot.

CONCLUSIONS

This study concludes that the main causal agent of dry rot on potato tubers belong to *Fusarium* spp. Collected isolated had significantly different pathogenicity level and therefore implicated that this study should be continued in order to determine species complex involved in potato dry rot in Serbia. *Fusarium* species are important for not only yield losses, but they can also produce mycotoxins that affect human and animal health. The appearance of mycotoxins in potatoes by *Fusarium* species is causing great concern worldwide.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Grant Nos: 451-03-65/2024-03/ 200117, and 451-03-66/2024-03/ 200117).

REFERENCES

Azil, N., Stefańczyk, E., Sobkowiak, S. *et al.* Identification and pathogenicity of *Fusarium* spp. associated with tuber dry rot and wilt of potato in Algeria. *Eur J Plant Pathol* 159, 495–509 (2021). https://doi.org/10.1007/s10658-020-02177-5

Bennett, J. W., & Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16(3), 497–516.

Bottalico, A., & Perrone, G. (2002). Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *European Journal of Plant Pathology, 108*, 611–624.

Cullen, D. W., Toth, I. K., Pitkin, Y., Boonham, N., Walsh, K., Barker, I., & Lees, A. K. (2005). Use of quantitative molecular diagnostic assays to investigate *Fusarium* dry rot in potato stocks and soil. *Phytopathology, 95*, 1462–1471.

El-Hassan, K. I., El-Saman, M. G., Mosa, A. A., & Mostafa, M. H. (2007). Variation among *Fusarium* spp. the causal of potato tuber dry rot in their pathogenicity and mycotoxins production : Egypt. *Journal of Phytopathology, 35*(2), 53–68.

FAOSTAT. (2024). http://www.fao.org/faostat/en/#data/QC. Accessed on 23.08.2024

Heltoft, P., Molteberg, E. L., Nærstad, R., & Hermansen, A. (2015). Effect of maturity level and potato cultivar on development of *Fusarium* dry rot in Norway. *Potato Research, 58*, 205–219. https://doi.org/10.1007/s11540-015-9300-x.

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. Ames: Blackwell Publishing.

Liu, J.; Sun, Z.Q.; Zou, Y.P.; Li, W.H.; He, F.Y.; Huang, X.Y.; Lin, C.L.; Cai, Q.N.; Wisniewski, M.; Wu, X.H. Pre- and postharvest measures used to control decay and mycotoxigenic fungi in potato (*Solanum tuberosum* L.) during storage. *Crit. Rev. Food Sci. Nutr.* 2022, *62*, 415–428.

Mills, J. T. (1990). Mycotoxins and toxigenic fungi on cereal grains in western Canada. Canadian Journal of Physiology and Pharmacology, 68(7), 982–986.

Palmer LT, Kommedahl T (1969). Root-infecting Fusarium species in relation to rootworm infestations in corn. Phytopathology, 59: 1613- 1617.

Peters, J. C., Lees, A. K., Cullen, D. W., Sullivan, L., Stroud, G. P., & Cunnington, A. C. (2008a). Characterisation of *Fusarium* spp. responsible for causing dry rot of potato in Great Britain. *Plant Pathology*, *57*, 262–271.

Ray, H., & Hammerschmidt, R. (1998). Responses of potato tuber to infection by *Fusarium sambucinum*. *Physiological and Molecular Plant Pathology, 53*(2), 81–92.

Secor, G. A., & Salas, B. (2001). *Fusarium* dry rot and *Fusarium* wilt. In W. R. Stevenson, R. Loria, G. D. Franc, & D. P. Weingartner (Eds.), *Compendium of potato diseases* (pp. 23–25). St. Paul: APS Press.

Senter, L. H., Sanson, D. R., Corley, D. G., Tempesta, M. S., Rottinghaus, A. A., & Rottinghaus, G. E. (1991). Cytotoxicity of trichothecenes mycotoxins isolated from Fusarium sporotrichioides (MC- 72083) and Fusarium sambucinum in baby hamster kidney (BHK-21) cells. Mycopathologia, 113, 127–131.

Song, H., Lee, H., Jeong, J., Park, H., & Lee, C. (2008). Diversity in beauvericin and enniatins H, I and MK1688 by *Fusarium oxysporum* isolated from potato. *International Journal of Food Microbiology, 122*, 296–301.

STATISTICA (Data Analysis Software System) (1984-2020). v.14.0.0.15., TIBCO Stat-Soft, Inc., USA (www.statsoft.com)

Stefańczyk, E., Sobkowiak, S., Brylińska, M., & Śliwka, J. (2016). Diversity of *Fusarium* spp. associated with dry rot of potato tubers in Poland. *European Journal of Plant Pathology, 145*, 871–884.

Stępień, Ł., & Waśkiewicz, A. (2013). Sequence divergence of the enniatin synthase gene in relation to production of beauvericin and enniatins in Fusarium species. Toxins, 5(3), 537–555.

Tiwari, R.K.; Kumar, R.; Sharma, S.; Sagar, V.; Aggarwal, R.; Naga, K.C.; Lal, M.K.; Chourasia, K.N.; Kumar, D.; Kumar, M. Potato dry rot disease: Current status, pathogenomics and management. Biotechnology 2020, 10, 503.

Vatankhah, M.; Saberi Riseh, R.; Moradzadeh Eskandari, M.; Sedaghati, E.; Alaie, H.; Afzali, H. Biological control of Fusarium dry rot of potato using some probiotic bacteria. *J. Agric. Sci. Technol.* 2019, *21*, 1301–1312.

Xue, H. L., Bi, Y., Tang, Y. M., Zhao, Y., & Wang, Y. (2014). Effect of cultivars, *Fusarium* strains and storage temperature on trichothecenes production in inoculated potato tubers. *Food Chemistry, 151*, 236–242.

Zhang, X.F.; Wu, C.Y.; Hua, H.H.; Cai, Q.N.; Wu, X.H. Characterization of the first *Alternavirus* identified in *Fusarium avenaceum*, the causal agent of potato dry rot. *Viruses* 2023, *15*, 145.

DIETARY INTAKE OF IRON FROM DIFFERENT MEATS AND LIVERS IN SERBIAN ADULTS

Ivana Branković Lazić*, Sasa Janković, Jasna Đinović-Stojanović

Institute of Meat Hygiene and Technology, Kacanskog 13, 11000 Belgrade, Serbia

*Corresponding author:
E-mail address: ivana.brankovic@inmes.rs

ABSTRACT

Meat and meat products are an important part of the human diet in Serbia. They are a convenient source of high-value proteins, essential vitamins, and important minerals needed for good health throughout life. Iron (Fe) is found especially in red meat, and it is indicated that vegetarians may be at risk of iron deficiencies. The aim of this study was to analyse beef, lamb, pork, equine, chicken, and turkey meats, as well as livers from the same animal species, except turkey liver, regarding the iron content. Samples were gathered from different meat processing facilities in Serbia during 2023. The level of iron was determined by inductively coupled plasma mass spectrometry (ICP-MS). The estimated daily dietary intake (EDI) of Fe was calculated using data of Fe levels obtained in this study as well as data of dietary intake of estimated meats and livers from the European Food Safety Authority (EFSA) database. The following mean levels of Fe were found (μg g^{-1}) in meat: beef 20.2, lamb 17.8, pork 7.90, equine 38.8, chicken 4.43, and turkey 6.74. The highest Fe mean level was obtained in equine liver (330.00 μg g^{-1}) while the lowest level was obtained in beef liver (57.7 μg g^{-1}). The results for EDI are expressed as percent of the Recommended Dietary Allowance (RDA) for adults (male: 8 μg g^{-1}; female: 18 μg g^{-1}). The analysed meats and livers provide in total 16.2% and 7.20% for male and female respectively of the RDA for Fe. The obtained results showed that the estimated meats and livers can be considered as important dietary sources of Fe, but other food types are clearly necessary to provide adequate dietary levels of Fe for the Serbian adult population.
***Keywords**: iron, meats, livers, Serbian adults, intake*

INTRODUCTION

Meat and meat products are significant components of the human diet, offering essential nutrients such as omega-3 fatty acids, B-vitamins (especially niacin and riboflavin), and bioavailable forms of iron and zinc. Moreover, it contains notable amounts of macronutrients (proteins and fat). Meat contains significant amounts of macronutrients, such as proteins and fat and essential nutrients, such as omega-3 fatty acids, B-vitamins (especially niacin and riboflavin), bioavailable forms of iron and zinc, and other different elements and that depends of its composition (Mann, 2013; Lopez-Alonso at al., 2016). Essential elements such as iron (Fe), copper (Cu), zinc (Zn), selenium (Se) and manganese (Mn) are required in appropriate amounts to maintain a range of physiological functions in humans (Noël et al., 2012 (Dawczynski et al., 2022).

From ancient times, man has recognized the special role of iron in human health and its deficiency can cause some disease (Beard et al., 1997). Iron is component of a number of proteins including hemoglobin, myoglobin, cytochromes and enzymes involved in redox potential, and is biologically essential component of every living organism (Aisen et al., 2001). Almost two-thirds of the iron in body is found in hemoglobin in circulating erythrocytes, which is important for transport of oxygen to tissues throughout the body.

Wholegrain cereals, meats, fish and poultry are the major contributors to iron intake, while the iron from plant sources is less bioavailable. The form in which iron is consumed will affect dietary intake requirements as not all dietary iron is equally available to the body. The factors that determine the proportion of iron absorbed from food are complex. They include the iron status of an individual, as well as the iron content and composition of a meal. Normal absorption may vary from 50% in breast milk to 10% or less in infant cereals. Dietary Iron occurs in two general forms – as heme or non-heme iron (Hurrell and Egli, 2010). The primary source of heme iron are hemoglobin and myoglobin from consumption of meat,

poultry and fish, while nonheme iron is obtained from cereals, legumes, fruits and vegetables (FAO/WHO, 2001). Heme iron is highly bioavailable (15%-35%) and dietary factors have little effect on its absorption, whereas nonheme iron absorption is much lower (2%-20%) and strongly influenced by the presence of other food components (Hurrell and Egli, 2010).
According to the data of Nutrient Reference Values for Australia and New Zealand to achieve iron balance, adult men need to absorb about 1 mg/day and adult menstruating women about 1.5 mg/day, although this is highly variable. Requirements are higher during periods of rapid growth in early childhood and adolescence. Inadequate iron intake can lead to varying degrees of deficiency, from low iron stores (as indicated by low serum ferritin and a decrease in iron-binding capacity); to early iron deficiency (decreased serum transferrin saturation; increased erythrocyte protoporphyrin concentration and increased serum transferrin receptor) to iron-deficiency anemia (low hemoglobin and hematocrit as well as reduced mean corpuscular hemoglobin and volume). These biochemical measures are used as the key indicators in setting the iron requirements. Iron and the other essential elements in meat and meat products have been intensively analysed in past decade, because these elements improve quality of meat and meat products and affect at consumer health (Djinovic-Stojanovic et al., 2017).
Meat and meat products are significant portion of daily consumption in Serbian adult population. The average annual consumption of meat in Serbia in 2013 was 60.8 kg/per capita (the EU average is 78 kg/per capita) of which pork was the most commonly consumed meat (27.3 kg), followed by poultry (17.2 kg) and beef (14.4 kg) (USDA Foreign Agricultural Service, 2013). There is very little scientific data available on the content of Fe in meat and meat products from Serbia. These data are necessary for future studies on the total dietary intake of Fe by the Serbian adult population. Therefore, the aim of the present study was to determine the Fe levels in meats and livers from different species of animals from Serbia as well as assess Fe intake through consumption of meat and livers. The established Fe levels in commonly consumed meats were also compared in order to suggest to consumers which of analysed meats are better sources of Fe.

MATERIAL AND METHODS

In total 219 meat and liver samples were collected: 148 red meat samples (11 turkey, 19 chicken, 12 lamb, 60 pork, 35 beef, 11 equine meats) and 71 liver samples (17 chicken, 12 lamb, 17 pork, 14 beef, 11 equine liver). Meat and liver samples were collected from different meat facilities in Serbia during 2023. After collection samples were homogenized, labeled and stored in polyethylene bags and frozen at -18 °C prior to analysis.

Sample preparation and ICP-MS analysis

Frozen samples were thawed at +4 ºC for a day before analysis and then homogenized. An amount, approximately 0.5 g, of each thawed, homogenized tissue, was transferred into a teflon vessel with 5 mL nitric acid (67% Trace Metal Grade, Fisher Scientific, Bishop, UK), for microwave digestion. The microwave (Mars 6, CEM Corporation, Matthews, NC, USA) program consisted of three steps: 5 min from room temperature to 180°C, 10 min hold at 180°C, 20 min vent. After cooling, the digested sample solutions were quantitatively transferred into disposable flasks and diluted to 100 mL with deionized water produced by a water purification system (Purelab DV35, ELGA, Buckinghamshire, UK).
Analysis of the iron (Fe) was performed by inductively coupled plasma mass spectrometry (ICP-MS), (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). The most abundant isotope ^{57}Fe was used for quantification.
Torch position, ion optics and detector settings were re-adjusted daily using tuning solution (Tune B, Thermo Scientific), in order to optimize mechanical and electrical parameters and minimize possible interference. Basic operating conditions of the instrument were: RF power (1550 W); cooling gas flow (14 L/min); nebulizer flow (1 L/min); collision gas flow (1 mL/min); operating mode (Kinetic Energy Discrimination-KED); dwell time (100 ms).

Standard stock solution containing 1000 mg/L of Fe was purchased from CPAchem (Bogomilovo, Bulgaria). This solution was used to prepare standards for five-point calibration curves (including zero). Multielement internal standard (^{6}Li, ^{45}Sc, ^{71}Ga, ^{89}Y, ^{209}Bi) was introduced online by an additional line through the peristaltic pump. All solutions (standards, internal standards and samples) were prepared in 2% nitric acid.

Quality assurance

The quality of the analytical process was verified by analysis of the certified reference material NIST 1577c (bovine liver, Gaithersburg, MD, USA). Reference material was prepared as samples using microwave digestion. Measured Fe concentrations were corrected for response factors of internal standards using the interpolation method and were within the range of the certified value (Table 1).

Table 1. Range of the certified value of Fe

Element	Certified value*	Analysed value**	Recovery
Fe	μg kg^{-1}	μg kg^{-1}	%
	197.94 ± 0.65	197.43 ± 5.21	99.7

* Certified value as given by the manufacturer.
** The data are presented as means ± standard deviation.

Statistical analysis

Statistical analysis of experimental data was performed using software Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance - ANOVA and Tukey's HSD test for comparison of means were used to analyse variations of the Fe levels in meats and livers different species of animals.

RESULTS AND DISCUSSION

The measured contents of the Fe in the meats and livers of different animal species are shown in Tables 2 and 3.

Table 2. Fe contents in analysed meat samples

Type of sample	**Number of samples**	**Fe content*** ***μg g^{-1}***
Chicken meat	19	4.43 ± 2.07 ^{a}
Turkey meat	11	6.74 ± 3.04 ^{a}
Pork meat	60	7.90 ± 4.71 ^{a}
Lamb meat	12	17.83 ± 6.68 ^{b}
Beef meat	35	20.25 ± 8.54 ^{b}
Equine meat	11	38.8 ± 11.07 ^{c}

* results presented as mean ± standard deviation
$^{a–c}$ Different superscripts within the column indicate significantly different means according to Tukey's HSD test; ($p < 0.05$)

The concentrations of Fe in pork, turkey and chicken meat were not significantly different ($p > 0.05$), while the Fe content in equine meat was significantly higher compared with other meats. The mean Fe levels in beef and lamb meat were significantly higher than levels in pork, turkey and chicken meat. Fe level in beef meat from present study was in line with data available in Danish Food database (22.00 μg g^{-1}) (FRIDA, 2024). Also, according to the same database equine and lamb meat had content of Fe (35.0 μg g^{-1} and 15.0 μg g^{-1}, respectively) similar to our results. Bilandžić et al (2021) analyzed Fe content in meats and livers from the Croatian market in chicken (4.2 μg g^{-1}), pork (11 μg g^{-1}) and beef meat (20 μg g^{-1}) and they were in line with results from this study. However, the Fe concentrations in this study were higher than Fe concentrations presented in meat and poultry obtained from the Lebanese TDS study (Nasreddine et al, 2010).

Table 3. Fe contents in analysed liver samples

Type of sample	Number of samples	Fe content* $\mu g\ g^{-1}$
Beef liver	14	57.68 ± 24.81 [a]
Lamb liver	12	76.48 ± 31.48 [a]
Chicken liver	17	125.27 ± 36.34 [b]
Pork liver	17	220.95 ± 50.42 [c]
Equine liver	11	330.00 ± 12.34 [d]

* results presented as mean ± standard deviation
[a–d] Different superscripts within the column indicate significantly different means according to Tukey's HSD test; ($p < 0.05$)

The average Fe contents in beef and lamb liver were not significantly different ($p > 0.05$) and were lower than Fe in liver of other species. Equine livers had the highest mean Fe content and it was significantly different than Fe levels in all other analyzed livers. The Fe contents for chicken (91.5 μg g^{-1}) and pork livers (134 μg g^{-1}) reported by FRIDA database (FRIDA, 2024) were lower than levels in present study. The Fe content in beef liver in the current study was lower than the Fe content in beef liver (73±69 μg g^{-1}) from Croatian market (Bilandžić et al, 2021), but in line with respective FRIDA database (FRIDA, 2024).

Intake assessment

The dietary intake of Fe was expresed as the daily intake (DI) and calculated using the equation: DI = C x DC where C is the Fe content (μg g^{-1}) and DC is daily consumption of meats and livers (g per day). DC data for meats and livers for Serbian adult population were used from the food consumption data which was published as part of the EFSA Comprehensive European Food Consumption Database (EFSA, 2022). Mean values of meats and livers consumption, expressed as grams per day (g day^{-1}) are shown in Table 4.

Table 4. Daily meats and livers intake

Type of product	Intake (g/day)
Pork liver	0.63
Pork meat	41.05
Chicken liver	0.69
Chicken meat	50.98
Beef meat	23.84
Lamb meat	2.51

Data for intake of equine meat and beef, lamb and equine livers are not available in EFSA database for Serbia probably because it's insignificant.

Calculated daily intake of Fe from different meats and livers as well as their contribution in total intake are shown in Table 5.

Table 5. Daily intake of Fe

Type of product	Fe intake (μg/day)	Contribution (%)
Lamb meat	44.82	3.44
Chicken liver	85.90	6.59
Pork liver	139.22	10.69
Chicken meat	225.82	17.33
Pork meat	324.29	24.89
Beef meat	482.47	37.03
Total	**1302.78**	**100**

Our results shown that highest Fe daily intake was by beef meat (37.03%), followed by pork meat (24.89%), while the lowest intake was through lamb meat consumption (3.4%).

Intake recommendations for Fe provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (formerly National Academy of Sciences) (Institute of Medicine, 2001). According of this source recommended daily intake of Fe in adult is 8 mg for male and 18 mg for female. Total Fe intake by examination food items (1302.78 µg/day) represents 16.25% and 7.2% of recommended daily intake for male and female, respectively.
The obtained results regarding the iron content in liver indicate a possible path of increasing Fe intake through increased consumption of this iron-rich foodstuff. Having in mind relatively low current consumption of just 0.63 g/day (pork liver), increase in consumption to e.g 2 g/day would result in 23.3% of increase in total iron intake through meat consumption, i.e. one large pork liver meal (250 g) would cover recommended weekly intake for males and three day intake for females.

CONCLUSIONS

This study presents the Fe content in meats and livers from different animal species originating from meat processing facilities in Serbia during 2023. The highest mean contents of Fe were measured in equine and beef meat, equine and pork livers, while the lowest Fe content were established in chicken and turkey meats. Total Fe intake by meat and liver consumption of different animal species represents 16.25% and 7.2% of recommended daily intake for male and female, respectively. The obtained results showed that the estimated meats and livers can be considered as important dietary sources of Fe, hence other food types are clearly necessary to provide adequate dietary intake of Fe for the Serbian adult population.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science, Technological Development and Innovation, Republic of Serbia, Grant No. 451-03-66/2024-03/200050 from 05.02.2024.

REFERENCES

Aisen, P., Enns, C., Wessling-Resnick, M. (2001). Chemistry and biology of eukarotyc iron metabolism. *Int j Biochem Cell Biol.* 33, 940-55.

Beard, J.L., Dawson, H.D., Iron, In: O'Dell, B.L., Sunde, R.A. (1997). *Handbook of Nutritionally Essential Mineral Elements* (p. 275-334). New York. CRC Press.

Bilandžić, N., Sedak, M., Čalopek, B., Dokić, M., Varenina, I., Solomun Kolanović, B., Božić Luburić, Đ., Varga, I., Hruškar, M. (2021). Dietary exposure of the adult Croatian population to meat, liver and meat products from the Croatian market: Health risk assessment. *Journal of Food Composition and Analysis*, 95, 103672.

Dawczynski, C., Weidauer, T., Richert, C., Schlattmann, P., Dawczynski, K., Kiehntoph, M. (2022). Nutrient intake and nutrition status in vegetarians and vegans in comparasion to omnivores- the nutritional evaluation (NuEva) Sudy. *Front. Nutr*.

Djinovic-Stojanovic, J.M., Nikolic, D.M., Vranic, D.V., Babic, J.A., Milijasevic M.P., Pezo L.L., Jankovic S.D. (2017). Zinc and magnesium in different types of meat and meat produsts from Serbian market. *J. Food Compos*. *Anal*. 59, 50-54.

EFSA (2022) Compressive European Food Consumption Database, Chronic Food Consumption Survey on adults in Serbia

FAO/WHO (2001). *Food based approachesto meeting vitamin mineral needs. In:human vitamin and mineral requerements* . Rome. P7-25

FRIDA (2024). Food data (frida.fooddata.dk), version 5.2. National Food Institute, Technical University of Denmark.

Hurrell, R., Egli, I. (2010). Iron bioavailability and dietary reference values. *Am J Clin Nutr.* 91: 1461-7S.

Institute of Medicine (2001). Food and Nutrition Board . Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc : a Report of the Panel on Micronutrientsexternal link disclaimer. Washington, DC: National Academy Press.

Lopez-Alonso, M., Miranda, M., Bonedito, J.I., Pereira, V., Garcia-Vaquero, M. (2016). Essential and toxic trace element concentracion in different commercial veal cuts in Spai. *Meat Science*, 121, 47-52.

Mann, N (2013). Human evolution and diet: a modern conundrum of health versus meat consumption, or is it? *Anim. Prod. Sci.*, 53 (11), 1135-1142.
Mann, N. (2007). Meat in human diet: An anthropological perspective. *J. Nutr. Diet,* 64 (4), s102-s107.
Nasreddine, L., Nachalian, O., Naja, F., Itani, L., Parent-Massian, D., Nabhani-Zeidan, M., Hwalla, N. (2010). Dietary exposure to essential and toxic trace elements from a Total diet study in an adult Lebanese urban population. *Food Chemistry and Toxicology*, 48, 1262-1269.
National Food Institute, Technical University of Denmark (2024) https://frida.fooddata.dk/links?lang=en
Noel, L., Chekri, R., Millour, S., Vastel, C., Kadar, A., Sirot, V., Leblanc, J.C., Guerin, T. (2012). Li, Cr, Mn, Co, Ni, Cu, Zn, Se and Mo levels in foodstufs from the 2th french TDS. *Food Chem.* 132, 1502-1513.
Shone, F., Ibel, A., Larkowski, S., Ihling, M., Ramminger, S., Kirmse, R., Sporl, K., Kieβling, G., Glei, M. (2023). Composition of pork and german meat products with a focus on iron, selenium and iodine. *Journal of food Composition and analysis*, 19, 105246.
USDA Foreign Agricultural Service. (2013).

APPLICATION OF ATOMIC ABSORPTION SPECTROMETRY USING THE DIRECT SAMPLING OF SOLID AND LIQUID SAMPLES AND PCA ANALYSIS IN THE DETERMINATION OF SELECTED ELEMENTS IN PROTEIN POWDER SAMPLES

Jakub Masác*, František Čacho, Jana Sádecká, Andrej Bednár, Andrej Kudry

Faculty of Chemical and Food Technology STU in Bratislava, Radlinského 9, 81237 Bratislava, Slovenská republika

*Corresponding author:
E-mail address: jakub.masac@stuba.sk

ABSTRACT

This work deals with elemental analysis of protein powders of animal and plant origin which are among popular food supplements. In total, 40 samples of protein powders of different types were analyzed, in which 15 elements were determined. The preparation of the samples consisted in its mineralization using microwave digestion where nitric acid was used as a decomposition agent. Flame and electrothermal atomic absorption spectrometry methods techniques (FAAS and ETAAS) were used to determine the elements. The conditions were optimized for the determination of the elements Mg, Cr and Pb. FAAS was used to determine Mg, using burner height of 10 mm and an acetylene-air fuel flow rate of 80 $L.h^{-1}$. ETAAS was used to determine Cr where the pyrolysis temperature was 1650 °C and the atomization temperature was 2700 °C. In the case of Cr, the LOD, LOQ and repeatability were 0.61 $\mu g.L^{-1}$, 2.05 $\mu g.L^{-1}$, and 4.6%, respectively with the linear range of 1-100 $\mu g.L^{-1}$. For the determination of Pb, the possibility of direct sampling was used, which provided a satisfactory repeatability of 7.6%. The pyrolysis temperature in this determination was 1100°C and the atomization temperature 1800°C. Based on the principal component analysis, it was found that whey proteins show very similar characteristics with respect to the element contents, thereby forming one group. Another group was formed by rice proteins, with very similar characteristics. The third group was made of mixed proteins which contain soy that also have a specific elemental composition and a high degree of similarity. In contrast, the pumpkin protein was markedly different from all other groups.
Keywords*: protein powder, ETAAS, FAAS, PCA*

INTRODUCTION

Due to their easy availability, nutritional supplements are more and more used by people all over the world. Vitamins, various minerals and protein powders are undoubtedly among the most used nutritional supplements. Protein powders dominate mainly among athletes, because they help to increase performance, regeneration and growth of muscle mass.
Due to the huge demand, protein powders made from different types of food have been developed. Based on the protein source, proteins are divided into several types. The oldest and still the most widespread source is milk, from which several milk proteins, whether whey or casein, are produced using various technologies. In terms of protein content, whey proteins can be divided into concentrates with a protein content in the range of 30-80%, isolates that range from 90 to 99% and hydrolysates, which are produced by splitting proteins into peptides using enzymes. Therefore, the fractions can range from whole proteins, through peptides, to individual amino acids. Casein proteins differ in amino acid content and show slower sorption in the human body. Others include egg proteins, which are rich in leucine and are suitable for people with lactose intolerance, as they are made from egg whites. Among the last animal proteins are beef proteins. The production process produces a hydrolyzed isolate with a high protein content, decreased amount of fats and carbohydrates with good digestibility (Wanasundara et al., 2017). Proteins produced from vegetable protein sources, such as, soy, peas, rice and many others are becoming more and more popular (Deeeth & Bansal, 2019).
With increasing consumption of these proteins, it is also necessary to control their quality by analyzing the various elements. The most frequently analyzed elements include macro

essential elements such as sulfur, or elements of the 1st and 2nd groups, micro essential elements such as Cr, Fe or Mn, but also non-essential and toxic elements, which can include e.g. Cd or Pb. The most frequently used methods for the elemental analysis of samples of this type include ICP-MS (Pinto et al., 2020; Guefai et al., 2022; Lofaso, 2021), ICP-OES (Bethencourt-Barbuzano et al., 2023; Neveen et al., 2023; Elgammal et al., 2019). The use of the AAS method was less represented in the publications. In the case of Pb determination, ETAAS was used (Elgammal et al., 2019) and FAAS was used in the work (Grdeń & Sołowiej, 2022) for multi-element analysis.
The aim of this work was to perform an elemental analysis of protein powders of different origins using HR-CS AAS and to determine the difference in element content between animal and vegetable protein powders. Determination of 15 selected elements using both electrothermal and flame atomization technique was performed. Optimization of conditions for determination was performed for few selected elements. Results of determination were used in PCA analysis to compare content of elements between protein powders of different origin. Furthermore, direct sampling of such samples was explored for future analyses.

MATERIAL AND METHODS

In the field of elemental analysis, microwave digestion is most often used for preparation of complex organic samples. In this case, a small amount of the sample is weighed into a teflon container, and strong oxidizing acids are added, such as hydrochloric acid, nitric acid, hydrofluoric acid, sulfuric acid or hydrogen peroxide. The container closed in this way is exposed to increased pressure and microwave radiation, which heats the sample in its entire volume. In our case, the Multiwave GO microwave digestion system from Anton Paar was used.
A high-resolution atomic absorption spectrometer with a continuum source of radiation (HR-CS AAS) from Analytik Jena, which has both an electrothermal and a flame atomizer, was used for the analysis of the samples. Unlike routine AAS, the device is equipped with a high-pressure xenon discharge lamp, of which the radiation intensity is 100 times higher in the entire spectral range compared to hollow cathode lamps. High resolution of wavelengths is achieved by means of two monochromators, which are in the Littrow arrangement. A linear CCD detector is used as a detector, the advantage of which is that it has extremely low noise compared to the photomultiplier used in classical AAS and is able to detect the intensity not only on the analytical line but also in its close spectral vicinity, i.e. approximately 0.2 - 1 nm around the selected wavelength.
Elements determined by ETAAS were Cr, Cd, Pb, Sn, Ni, Se and elements determined by FAAS were S, Na, K, Mg, Ca, Fe, Zn, Mn, Cu.
Software used for PCA and all statistical analysis was STATISTICA version 12 (StatSoft, USA).
Deionized water purified by the NANOpure system from Wilhelm Werner GmBh was used to prepare all the solutions. All chemicals used were of analytical grade.
40 samples of various protein powders of animal and plant origin, both from Slovak and foreign manufacturers were analyzed:

15x whey concentrate (WC)
10x whey mixture (WM)
3x mixture of soy, whey and casein protein (M)
1x mixture of whey and egg protein (WE)
2x casein protein (C)
3x rice protein (R)
2x pea protein (P)
1x egg protein (E)
1x pumpkin protein (PU)
1x vegan (not further defined) protein (VG)
1x coconut protein (CO)

RESULTS AND DISCUSSION

Microwave digestion

Before the actual analysis, all samples were modified by the method of microwave digestion. From each protein powder sample 0.3 g was weighed into collapsible teflon containers and 12 ml of HNO_3 was added. Each sample was digested simultaneously in 4 containers with an optimized temperature program. The containers were heated for 20 minutes to a temperature of 190 °C, then this temperature was maintained for 17 minutes, followed by cooling. The decomposed samples were quantitatively transferred into open teflon bowls, placed under an IR lamp where a significant part of the solvent was allowed to evaporate in order to achieve a sufficient preconcentration of the sample. The concentrated samples were quantitatively transferred into 25 ml volumetric flasks.

Optimization of conditions for FAAS and ETAAS

The FAAS and ETAAS methods were used for elemental analysis. For both methods, one element was chosen for which the determination conditions were optimized. For FAAS it was magnesium and for ETAAS it was chromium.

To optimize the conditions for magnesium determination, a standard solution with a concentration of 200 $\mu g.L^{-1}$ was used. The height of the burner was optimized in the interval from 4 to 12 mm. A height of 10 mm was chosen as the optimal value when the highest absorbance signals were recorded. Another optimized parameter was the acetylene/air fuel flow, which was continually increasing from 40 $l.h^{-1}$ to 100 $l.h^{-1}$. The highest absorbance value was recorded at a flow rate of 80 $l.h^{-1}$.

To optimize the parameters of chromium determination, a standard solution with a concentration of 10 $\mu g.L^{-1}$ was used.

The pyrolysis temperature was optimized in the interval from 800 to 2000 °C with a step of 100 °C. In temperatures higher than 1650 °C, a decrease in the signal caused by the loss of the analyte in the pyrolysis step was observed.

The atomization temperature was optimized in the temperature range from 2000 to 2800 °C. The absorbance signal grew up to 2800 °C, due to the extension of the life of the carbon cuvette, 2700 °C was chosen as the optimal temperature.

The accuracy of the determination of magnesium and chromium was verified by selecting two random samples of protein powders and analyzing them by the standard addition method. The results of these determinations were compared by the statistical T-test, where it was shown that there are no statistically significant differences between the concentrations. The determined metal contents are listed in Table 1.

Table 1. Comparison of Mg and Cr concentrations determined by different methods to verify accuracy

Element	Sample code	Determined by calibration curve method ($mg.kg^{-1}$)	Determination by standard addition method ($mg.kg^{-1}$)
Mg	WC4	1207±54	1246±37
	P1	6907±94	6933±62
		($\mu g.kg^{-1}$)	($mg.kg^{-1}$)?
Cr	R1	1014±71	996±33
	P1	201.6±8.8	192.4±5.7

Direct sampling

In order to minimize errors arising during sample preparation, saving time and chemicals, the possibility of direct sampling was investigated. Thanks to highly effective background correction of HR-CS AAS, it can suppress interferences from complex organic matrices, even in the case of non-mineralized protein powder samples. When sampling solid samples, the repeatability was insufficient, as it was not possible to manually weigh the same amount of sample repeatedly and sample it to the same place in the platform. For the reasons

mentioned, the analysis was started using direct sampling of liquid samples. These were prepared by weighing 1.0000 g of the sample in 99 g of demineralized water with a magnetic stirrer that continuously homogenized the sample. Subsequently, 25 µL of such a sample and 5 µL of the mixed modifier $Pd(NO_3)_2$ and $Mg(NO_3)_2$ were sampled into the platform using micropipette.
Optimization of the conditions of direct sampling of liquid samples was carried out for the determination of Pb. In the first step, the pyrolysis temperature was optimized in the temperature range from 600 °C to 1400 °C. Based on the absorbance values, a temperature of 1100 °C was chosen as optimal. Due to the sampling of a large amount of organic content, the pyrolysis time was also optimized in the interval from 5 s to 40 s. It turned out that from 20 s the absorbance signal decreased significantly, 10 s was chosen as the optimal time when the highest signals were obtained.
The atomization temperature was optimized in the interval from 1500 °C to 2500 °C, and 1800 °C was chosen as the optimal temperature, at which the highest absorbance signals were measured.
Repeatability was calculated from 10 repeated measurements after manual sampling of the sample solution and calculated as RSD = 7.6%.
The content of Pb was determined in three random samples using direct sampling. In order to verify the accuracy of the determination, Pb was determined in these samples by the standard addition method and subsequently also in the original digested samples. The results of these determinations were compared by a statistical T test for two paired samples, showing that there are no statistically significant differences between them. The results are shown in Table 2.

Table 2. Pb concentrations determined by different methods

Sample code	Determined by calibration curve method ($\mu g.kg^{-1}$)	Determination by standard addition method ($\mu g.kg^{-1}$)	Determination in original digested samples ($\mu g.kg^{-1}$)
VG1	57,4±2,4	58,8±2,3	60,2±3,0
WC14	100,8±4,4	101,4±3,2	97,0±3,2
R2	287±14	292,7±8,2	302±14

Results of analysis by FAAS and ETAAS methods
A total of 15 elements were determined in all 40 protein powder samples. Sulfur was the most abundant of the individual elements, which was found up to tens of grams per kilogram in the samples. This result is expected in the case of such samples, since sulfur is a natural part of some amino acids. The elements Na and K were present in smaller amounts, followed by Mg and Ca. Fe and Zn were determined in concentrations ranging from a few mg up to hundreds of mg per kilogram. Mn was determined in the range from tens of µg to hundreds of mg per kilogram and Cu in the range of several tenths to tens of µg per kilogram.
Using the ETAAS method, the highest concentration was recorded for the element Se, which is beneficial to health, and its content in protein powders can also be an advantage. It was followed by Sn and Ni, which were represented in the range of tens to thousands of µg per kilogram. Cr was represented in tens to thousands of µg per kilogram, as well. Since Cr naturally occurs in two oxidation states, a speciation analysis would be necessary to assess the benefit or toxicity of the contained Cr. Pb was almost the least represented in the range of tens to hundreds of µg per kilogram. The least represented was Cd, whose concentrations were below the LOQ up to a few tens of µg per kilogram.
To assess differences between individual protein powders, a PCA analysis was performed, which identified 6 principal components that explained 85% of the data variability. As a representative example of PCA analysis results, the graph of component 2 (28%) vs. component 3 (15%), was chosen and is shown in Figure 1. Based on the data, it can be concluded that some types of protein powders are significantly different from others, but not all proteins can be reliably divided into different groups based on PCA analysis alone.

Proteins from the group of whey concentrates and whey mixtures (sample code SK and SZ) show similar characteristics and cannot be reliably separated based on any components. All these proteins are located near the central cross and form a large cluster of points on the graph. This division is relatively easy to explain, since all these proteins, both concentrates and whey mixtures, come from the same source. The slight dispersion of these points in the graph can be mainly responsible for the geographical origin of the whey and the production technology.The rice proteins are all located very close to each other in the lower right quadrant, which means that their properties in terms of the content of the determined elements are very similar. These elements are especially dominant in the Cr content, as they contain the highest amounts of all analyzed proteins. In addition, they are characterized by a high content of Pb and a relatively high content of Cd. Close to this group is also a protein marked as vegan, which is made from a mixture of rice, peas and hemp. This protein is also characterized by a high content of Cr and Pb.In the upper right quadrant, there are proteins from the mixed group, i.e. Z1 - Z3, but they are not as close as in the case of rice proteins. These mixed proteins are made from a mixture of whey, casein and soy. We assume that the similarity of these proteins is mainly influenced by the presence of soy. These proteins have very similar concentrations of almost all elements and differ from the others mainly in the content of Na, Zn and Fe. This group of proteins has the highest Na content among all proteins.Casein proteins are in the upper half of the graph, but each in a different quadrant. Proteins differ mainly in the content of Na, Se, Mn, and Fe. Compared to other proteins, they have relatively low concentrations of Cr and, conversely, high concentrations of Sn. Another important fact is that they are not very significantly different from whey proteins, which results from the same protein source.What is interesting is the difference between the pea proteins, which are also very different from each other, as they are located on opposite sides of the graph. They differ greatly mainly in the content of Na, Cu, Ca and Mn, but also differ in the content of other elements. The difference can be caused firstly by the different geographical origin of the raw material.An interesting finding is the similarity of coconut protein to whey proteins. This protein is located at the bottom of the cluster of points and shows the same similarity mainly based on Cr, Mn and Na content. It differs from whey proteins in the content of S, Cu, Mg and K. Since we only had one sample of coconut protein, it is not possible to reliably describe the similarity, or the difference of this protein from others.Egg protein is slightly different from the others and has the greatest similarity with mixed proteins that contain soy.Pumpkin protein is quite different from the others and is on the far left of the graph. It is also true that a more representative sample of this group would be needed for a more reliable assessment of the different characteristics.

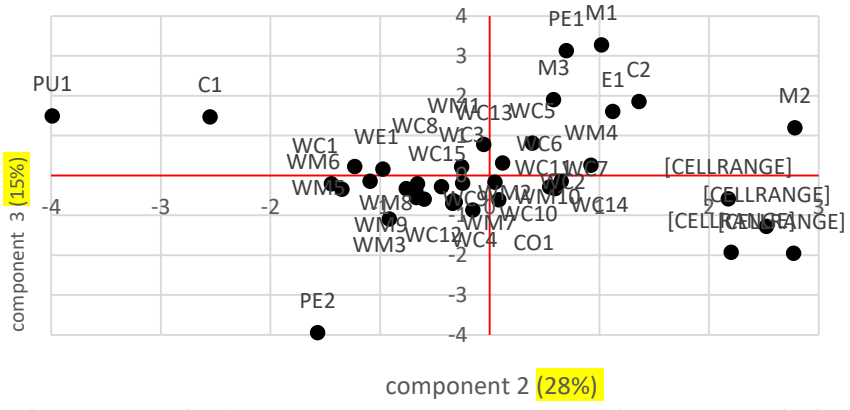


Figure 1. Graph of component 2 vs. components 3 from PCA analysis

Analysis using direct sampling is very efficient because no sample treatment is required, saving a great deal of chemicals and time. In addition, the increased occurrence of various errors during sample treatment will be prevented.

CONCLUSIONS

A total of 15 elements were determined in 40 protein powder samples by FAA and ETAAS methods. Based on the chemometric analysis, it was found that in terms of the element content, whey proteins, show very similar characteristics hence form one group. Another separate group is formed by rice proteins, which are also very similar to each other. Mixed proteins that contain soy also have a specific elemental composition and show a degree of similarity. Pumpkin protein was on the far-left side of the graph, so the very different elemental composition can be assumed.
Regarding direct sampling, using non-mineralized liquid samples turned out to be the most appropriate, providing sufficient repeatability of the results. Another subject of research in this area could be the elemental analysis of more types of proteins, or analysis of different groups with a representative number of samples.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Research and Development Agency under the Contract no. SK-SRB-23-0015, APVV-23-0066 and VEGA - Scientific Grant Agency of the Ministry of Education under the project number 1/0017/23.

REFERENCES

Bethencourt-Barbuzano, E., Gonzáles-Weller, D., Paz-Montelongo, O., Gutiérrez-Fernández, J.A., Hardisson, A., Carrascosa, C., Cámara, M., Rubio-Armandáriz, C. (2023). Whey protein dietary supplements: Metal exposure assessment and risk characterization. *Nutrients*, 15(16), 3543.

Deeth, H., Bansal, N. (2019). *Whey proteins: From milk to medicine*. London: Academic Press, (Chapter 16).

Elgammal, S., Khorshed, A.M., Ismail, H.E. (2019). Determination of heavy metal content in whey protein samples from markets in Giza, Egypt, using inductively coupled plasma optical emission spectrometry and graphite furnace atomic absorption spectrometry: A probabilistic risk assessment study. *Journal of Food Composition and Analysis*, 84, 103300.

Grdeń, A.S. A Sołowiej, B.G. (2022). Macronutrients, amino and fatty acid composition, elements, and toxins in high-protein powders of crickets, Arthrospira, single cell protein, potato, and rice as potential ingredients in fermented food products. *Applied Sciences*, 12(24), 12831.

Guefai, F.Z., Martínez-Rodríguez, A., Grindlay, G., Mora, J., Gras. (2022). Elemental bioavailability in whey protein supplements. *Journal of Food Composition and Analysis*, 112, 104696.

Lofaso, M. (2021). Determination of Metals in Whey and Vegan Protein Supplements using Inductively Coupled Plasma Mass Spectrometry. *Honors Theses*. 1649.

Neveen F., Mennatallah, K.S., Samar, M.A-R. (2023) Authentication of whey protein supplements with their labeling. *Alexandria Journal of Food Science and Technology*, 21(1), 25–32. doi:10.21608/ajfs.2023.321144.

Pinto, E., Ferreira, I., Almeida, A. (2020). Essential and non-essential/toxic trace elements in whey protein supplements. *Journal of Food Composition and Analysis*, 86, 103383.

Wanasundara, J.P.D., Scanlin, L. AND Nadathur, S.R. (2017). *Sustainable protein sources*, Amsterdam: Elsevier, (Chapter 1).

THE ROLE OF HPLC IN FOOD ANALYSIS USING COMBINED CHROMATOGRAPHIC AND FLUORESCENCE DATA WITH CHEMOMETRIC TOOLS

Katarína Hroboňová[1]*, Jana Sádecká[1], Sonja Veljović[2]

[1]Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, 81237 Bratislava, Slovakia
[2]Faculty of Hotel Management and Tourism in Vrnjacka Banja, University of Kragujevac, Vojvodjanska bb, 36210 Vrnjacka Banja, Serbia

*Corresponding author:
E-mail address: katarina.hrobonova@stuba.sk

ABSTRACT

The work aimed to point out the role of high-performance liquid chromatography (HPLC) with different types of detectors in the complex evaluation of beverage samples. HPLC is characterised by good selectivity, sensitivity and ability to provide qualitative and quantitative information on a large group of compounds. Spectrometric methods such as fluorescence spectrometry and others often combined with chemometrics allow us to classify products according to geographical origin, variety, and others. Preliminary estimation of groups of chemical compounds related to the observed differences is possible based on the spectral characteristics of the samples. Still, the assignment of individual compounds is more reliable based on the comparison of the spectra profiles with the results of HPLC separation. Other areas of use of HPLC include, i) the clarification of differences in the spectra of samples, ii) a reference method in the development of multivariate calibration models for the determination of individual compounds in food using spectrometric methods. Combining data from fluorescence analysis (untargeted analysis) and chromatographic analysis (target quantitative analysis) with chemometric tools is useful for classifying spirit beverages.
Keywords*: product classification, food quality, target analysis, untargeted analysis*

INTRODUCTION

The importance and scope of the use of analytical chemistry are constantly growing due to the increasing demand for reliable analytical information about the processes taking place in various material objects of different origins and their composition. The effort of analysts is to find suitable solutions to face real problem analysis in practice. To achieve it, the specific characteristics of analyzed samples considered together with the nature and concentration level of target analytes must be recognized. The development of new methodologies (procedures) should be also aware of the crucial criteria such as accuracy, precision, selectivity and detection limit.

The powerful methods for characterization, classification and determining the geographical origin of food products are pattern recognition methods of the data provided by analytical chemical instruments. High-performance liquid chromatographic methods with spectrometric detection (HPLC-UV) are widely used in targeted analysis because they provide reliable qualitative and quantitative information about separated individual compounds. Their advantages are high separation efficiency; determination of almost any type of compound present in a food sample; and depending on which technique is employed, it is possible to obtain very low detection limits for a wide range of analytes. HPLC-UV has been widely used for the determination of phenolic compounds in beverages. Qualitative analysis was made by comparison with standards (Canas et al., 2003; Jakubíková et al., 2022). In the case of liquid samples, it often enables direct analysis. Samples with a complicated matrix or samples with low analyte content, need to be treated using a wide variety of sample preparation techniques such as solid phase extraction (SPE) including microextraction modifications (Pereira et al., 2022).

Recently, attention has focused on the development of non-invasive and non-destructive instrumental techniques such as ultraviolet (UV), visible (Vis), fluorescence (FL), near-

infrared (NIR) and mid-infrared (MIR) spectrometry. Furthermore, fluorescence spectroscopy can determine some properties of solid or liquid food in a green way, i.e. without sample preparation in a very short time compared to wet techniques. The fluorescence spectrometry was recently used for the classification of Slovak and foreign spirit drinks, fruit spirits, plum spirits or wines according to geographical origin using various multivariate data analyses such as parallel factor analysis (PARAFAC), principal component analysis (PCA) and linear discriminant analysis (LDA) (Hroboňová et al. 2020; Pecic et al., 2012; Tomková et al. 2015).
Chemometric tools allow improving the spectral resolution, quantification of the analytes in the presence of interfering compounds, classification of the samples as well as detection of discrepant samples. Chemometrics has also been applied, particularly for reducing the steps in chemical analysis and consuming less energy when using sensor-related research and information/data handling (Jakubíková et al., 2019; Uríčková et al., 2015).
The aim of this work was to point out to role of high-performance liquid chromatography with different types of detectors in the complex evaluation of beverage samples. A combination of data from chromatographic and fluorescence analysis with chemometric tools was used.

MATERIAL AND METHODS

The studies were performed on brandies, plum and juniper-flavored spirit drinks and sweet wine samples produced in Slovakia. Samples were purchased from the local supermarkets and stored in the dark at room temperature (23 ^{o}C).
The HPLC analyses were carried out with an Agilent 1200 HPLC instrument equipped with a diode array and fluorescence detectors. Reversed-phase mode with a C18 type of analytical column maintained at a temperature of 23 °C was used for separation of analytes. Compounds were separated using the gradient elution of the mobile phase consisting of 1% acetic acid (A) and methanol:acetic acid (99:1, v/v; B) pumped at a constant flow rate of 1 mL/min. The injection volume was 20 µL. The chromatograms were recorded at 280 or 320 nm and UV spectra were recorded in wavelength range of 190-400 nm. The fluorescence detector was set at 320 nm (λ_{ex}) and 450 nm (λ_{em}). Fluorescence spectra were recorded in a wavelength range of 340-500 nm. Samples were filtered through a nylon membrane filter (pore size 0.45 µm) before analysis.
UV–Vis absorption measurements were made using a UV 1800 Spectrophotometer equipped with a quartz cell with an optical path of 1 cm, over the range of 200–600 nm with a 1 nm resolution. The scanning speed was 200 nm/min. Software UV PROBE 2.33 was used to process the spectra. UV–Vis spectra were recorded for undiluted and diluted (1:1, 1:20 or 1:100, v/v) samples. Water purified by a Milli-Q system was used for dilution.
Synchronous fluorescence spectra (SFS) were collected using a Perkin-Elmer LS 50 Luminescence spectrometer equipped with a Xenon lamp, a quartz cell with an optical path of 10 mm and FL Data Manager Software (Perkin-Elmer) for spectral acquisition and data processing. Excitation and emission slits were both set at 5 nm. The spectra were depicted as the contour plots of total SFS. The contour plots were constructed in such a way that x-axis shows the λ_{ex} (nm), y-axis represents the $\Delta\lambda$, and z-axis is plotted by linking points of equal fluorescence intensity. SFS were recorded on the bulk or diluted (1:100 or 1:20, v/v) samples.
The raw spectral data was converted to ASCII format and processed with Microsoft Excel 2010 (Microsoft Office, 2010), OriginPro 2018, and STATISTICA version 12 (StatSoft, 2017) software. SFS data of all relevant samples was arranged in the two-dimensional matrices (number of samples × number of λ_{ex}) for particular $\Delta\lambda$ values. Partial least squares (PLS) regression model development, the prediction subsets enabled verification and comparison of the quality of PLS models.

RESULTS AND DISCUSSION

The analysis procedure and clarifying the results for the samples evaluation process was tested for wine spirit and fruit spirit samples. (Sádecká et al. 2023; Tóthová et al. 2008). Traditional spectrometric methods such as UV-VIS absorption and fluorescence (and also infrared and Raman) spectrometry allow products' classification according to geographical origin, variety, and others. Figure 1 shows the UV-Vis spectra of wine spirit samples in the range from 200 to 600 nm. Undiluted samples presented off-scale absorbance values in the UV region from 200 to 300 nm, some of them up to 400 nm. It is observed in spectra of diluted samples a broad band centred at about 280 nm, corresponding to phenolic (also furan) compounds. UV-VIS spectrophotometry is usually used for the evaluation of the total phenolic index (by measuring the absorbance at 280 nm) of spirit samples.

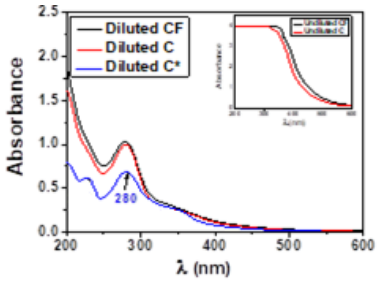


Figure 1. The Mean UV-Vis Spectra of Undiluted (a Small Figure) and Diluted Wine Distillates. (CF- Caramel-Free distillate, C,C - Caramel-Containing Distillates)*

Examples of total synchronous fluorescence spectra of the wine spirit in the form of contour maps are shown in Figure 2. Regarding undiluted samples, the contours were centered at 410−460 nm ($\Delta\lambda$ = 70−90 nm) for right angle geometries. For diluted samples, the maxima of synchronous fluorescence spectra were observed at 290−360 nm ($\Delta\lambda$ = 60−100 nm). Fluorescence was attributed to phenolic acids (260−280 nm, $\Delta\lambda$ = 60−80 nm), coumarins (340 nm, $\Delta\lambda$ = 100 nm), and caramel (340−360 nm, $\Delta\lambda$ = 90−100 nm).
The short-wavelength fluorescence, with excitation at 220 and 270–280 nm and emission maxima located at 320–340 nm, was observed in juniper samples, along with the longer-wavelength fluorescence, with excitation at 290 nm and emission at 406 nm.

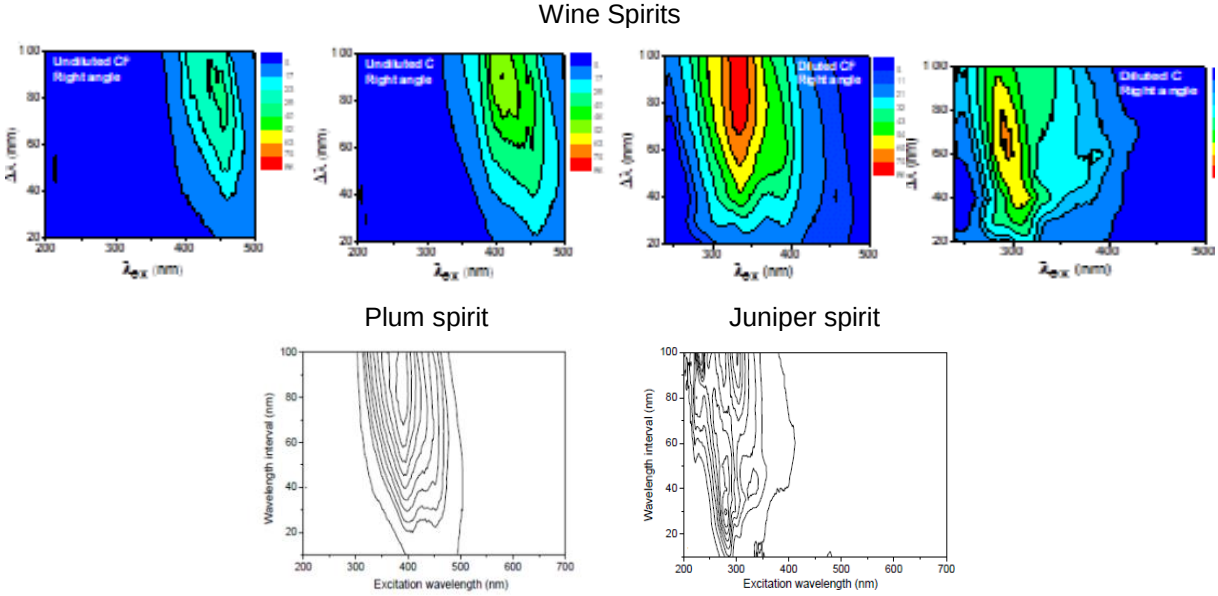


Figure 2. The Representative Total Synchronous Fluorescence Contour Plots of Undiluted and Diluted Wine and Fruit Spirit Samples. (C,C - Caramel-Containing Distillates)*

After classifying the products using spectral data, it was necessary to determine the properties responsible for the differences between the products observed in the spectra. A preliminary assessment of chemical compounds related to the observed differences showed several groups of compounds in tested spirit samples: phenolic acids, aldehydes and coumarins, caramel and as well as caramel and typically found in oak and charred wood extracts in wine brandy. In fruit spirits, phenolic acids, coumarins, phenols, and anisols were identified.

In the next step, the assignment of individual compounds was more reliable based on the comparison of spectra profiles with the results of HPLC separation. HPLC is a significant method of beverage analysis due to its good selectivity, sensitivity and ability to provide information on a large group of compounds including phenolics. The concentrations of selected compounds in tested samples analyzed by HPLC-DAD ranged depending on sample type (0.7 - 28.1 mg/L of 5-hydroxymethylfurfural and 2.3 - 65.9 mg/L of furfural in wine spirit, 5 years ageing, caramel-free; 5.7 - 66.8 mg/L of 5-hydroxymethylfurfural and 0.3 - 14.1 mg/L of furfural in Wine spirit, commercial, caramel-containing). Comparing the content of individual compounds was challenging because, in the case of CF samples, it largely depends on the production technology used. For caramel-containing samples not only on the previously mentioned factors but also on the amount of caramel added. (HMF is the most abundant of the furanic aldehydes in caramel).

Another area of use of HPLC is the clarification of differences in the spectra of samples that are commonly named outliers, i.e. having atypical properties. For example, in some caramel-containing samples (C*) characterized by atypical UV-Vis spectra (Fig. 1), vanillin contents were found at the levels of 24.0 – 54.9 mg/L, but syringaldehyde contents in both samples were below the LOQ value of HPLC-DAD method.

The third area is the use of HPLC as a reference method in the development of multivariate calibration models for the determination of individual compounds in food using spectrometric methods.

As was mentioned in the introduction, many of the spirit samples were analyzed directly or after dilution (e.g. wine spirit samples). The purification of samples from an excess of the matrix as well as the concentration of analytes is sometimes useful before HPLC analysis. Solid phase extraction (SPE) is a popular method for the extraction of food samples as it provides better extraction efficiency and requires less consumption of organic solvents than liquid-liquid extraction (LLE) (Barnes et al. 2022). The current trend in SPE is the application of selective adsorbents such as molecularly imprinted polymers (MIP) prepared for the extraction of selected analyte. Adsorbents are prepared usually by block polymerization or by imprinting on the surface of magnetite (magnetic MIP). This was used in the analysis of coumarins in beverages (Hroboňová et al. 2020). The HPLC with UV spectrophotometric and fluorescence detection combined with MIP (7-hydroxycoumarin as template)-SPE sample treatment was used. The evaluation of MIP properties (adsorption capacity, morphology, kinetics) showed that the adsorbent was selective for the extraction of simple coumarins (group selective adsorbents). In the tested wines, scopoletin and 4-methylumbeliferone were determined at concentration levels ranging from 10 ng/mL to 171 ng/mL. The representative HPLC-FLD chromatogram wine sample extract is shown in Figure 3.

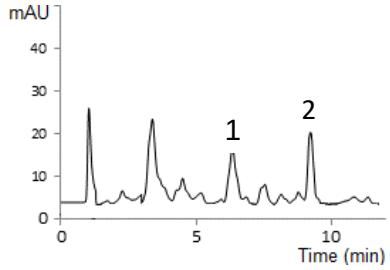


Figure 3. The Representative HPLC-FLD chromatogram of Tokaj wine extract.

Conditions: Kinetex C18 (100 x 4.6 mm, 5 µm) stationary phase, the gradient elution of the mobile phase consisted of 1% acetic acid (A) and methanol: acetic acid (99:1, v/v; B): 0–12 min linear gradient of B from 20 to 45% then to 100% of B over 0.5 min, and held at 100% of B for 2 min. Legend: 1- scopoletin, 2- 4-methylumbeliferone

The results of quantification of targeted analytes (e.g. coumarins in wines, furanic aldehydes in brandy) obtained by HPLC method could be compared with values predicted by the partial least squares (PLS) model on the base data from fluorescence spectrometry. The spectra were used as the independent variable data and analytes concentrations (or sum of analytes content) were used as the dependent variable (Y-block) data in the PLS regression (example of coumarins determination is shown in Figure 4) (Hroboňová et al. 2020). A linear regression between the concentrations predicted by the PLS model versus true values obtained by the HPLC method confirms good agreement between the two methods. Similar conclusions were also reached for caramel in beverages (R^2 more than 0.978) (Sádecká et al. 2023), which demonstrated the ability if this methodology in the spirit samples evaluation.

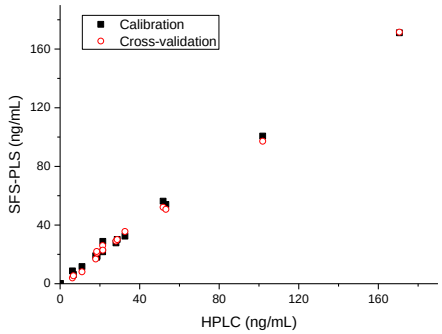


Figure 4. Values of Total Coumarins Calculated Using the Synchronous Fluorescence Spectra Combined with PLS Regression vs. the Values Obtained by the HPLC method

CONCLUSIONS

HPLC has become a useful tool in proposing solutions to various problems in food analysis using spectrometric methods often combined with chemometrics. The usual procedure is: (1) to register the spectra of the samples by the spectrometric method, (2) to determine the target analytes in the samples by the HPLC method, (3) to use the results from the quantitative HPLC analysis as dependent variables and the spectral data as independent variables in multivariate chemometric models. The results obtained using non-targeted methods are necessarily verified by the targeted analysis of the samples using the HPLC with different types of detectors (diode array detector, fluorescence detector as well as mass spectrometry detector), which confirmed not only the results of the classification of the samples but also the content of analyte/s as well as the state of the sample for outlying samples. Non-targeted UV-Vis, fluorescence methods can be used to search for suspect samples, the study of which can be continued by targeted HPLC analysis.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Research and Development Agency under the Contract no. SK-SRB-23-0015 and project VEGA 1/0332/24.
Sonja Veljovic thanks to the project of Serbian Ministry of Science, Technological Development and Innovation, no. 337-00-3/2024-05/145.

REFERENCES

Barnes, Q., Vial, J., Thiébaut, D., De Saint Jores, C., Steyer, D., Contamin, M.-A., Papaiconomou, N., & Fernandez, X. Characterization of flavor compounds in distilled spirits: Developing a versatile analytical method suitable for micro-distilleries. *Foods, 11,* 3358.

Canas, S., Belchior, A. P., Spranger, M. I., & Bruno-de-Sousa, R. (2003). High-performance liquid chromatography method for analysis of phenolic acids, phenolic aldehydes, and furanic derivatives in brandies. Development and validation. *Journal of Separation Scientes, 26*, 496–500.

Hroboňová, K., & Sádecká, J. (2020). Coumarins content in wine: application of HPLC, fluorescence spectrometry, and chemometric approach, *Journal of Food Science and Technology, 57,* 200-202.

Jakubíková, M., Sádecká, J., & Hroboňová, K. (2019). Classification of plum brandies based on phenol and anisole compounds using HPLC. *European Food Research and Technology, 245,* 1709–1717.

Jakubíková, M., Sádecká, J., & Hroboňová, K. (2022). Determination of total phenolic content and selected phenolic compounds in sweet wines by fluorescence spectroscopy and multivariate calibration. *Microchemical Journal, 181*, 107834.

Pecić, S., Veljović, M., Despotović, S., Leskošek-Čukalović, I., Jadranin, M., Tešević, V., Nikšić, M., & Nikićević, N. (2012). Effect of maturation conditions on sensory and antioxidant properties of old Serbian plum brandies, *European Food Research and Technology, 235,* 479-487.

Pereira, J. A. M, Casado, N., Porto-Figueira, P., & Câmara, J. S. (2022). The potential of microextraction techniques for the analysis of bioactive compounds in food. *Frontiers in Nutrition, 189*, 825519.

Sádecká, J., Jakubíková, M., Hroboňová, K., Švorc, Ľ., & Haššo, M. (2023). Non-targeted and targeted analytical approaches for estimating the features of wine spirits. *Microchemical Journal, 195*, 109442.

Tomková, M., Sádecká, J., & Hroboňová, K. (2015). Synchronous fluorescence spectroscopy for rapid classification of fruit spirits. *Food Analytical Methods 8,* 1258–1267.

Tóthová, J., Žiak, Ľ., & Sádecká, J. (2008). Characterization and clasifivation of distilled drinks using total luminescence and synchronous fluorescence spectroscopy. *Acta Chimica Slovaca 1,* 265-275.

Uríčková, V., & Sádecká, J. (2015). Determination of geographical origin of alcoholic beverages using ultraviolet, visible and infrared spectroscopy: A review, *Spectrochimica Acta A, 148,* 131–137.

THE QUALITY OF EGGS FROM DIFFERENT HOUSING SYSTEMS IN BELGRADE SUPERMARKETS, IN EXTREME TEMPERATURE CONDITIONS

Tolimir Nataša[1*], Maslovarić Marijana[1], Škrbić Zdenka[2], Utvić Stefana[1], Pezo Lato[3], Lukić Miloš[2], Nedeljković Trailović Jelena[4]

[1]Institute for Science Application in Agriculture, Bulevar Despota Stefana 68b, 11000, Belgrade, Republic of Serbia
[2]Institute for Animal Husbandry, Autoput 16, 11080 Belgrade-Zemun, Republika Srbija
[3] Institute of General and Physical Chemistry, Studentski trg 12/V, 11158 Belgrade, Serbia
[4]Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobođenja 18, 11000 Beograd, Republic of Serbia

*Corresponding author:
E-mail address: ntolimir@ipn.bg.ac.rs

ABSTRACT

The aim of the research was to examine the supply and the quality of table eggs produced in different housing systems, i.e., cage, floor and free-range, in the conditions of extremely high summer temperatures. The supply was recorded in 10 Belgrade supermarkets and the analysis of the physical egg quality characteristics (external and internal) included grade M eggs, from 9 egg producers, up to 15 days old, i.e., laid between 3rd and 18th of July, during the heat wave in Serbia, with an average maximum air temperature of 34.43°C. According to the research results, it can be concluded the eggs from the cage system are still dominant in Belgrade supermarkets, which can be linked to the slow transition of producers to alternative housing systems. In terms of the external egg quality characteristics, statistically significant differences between housing systems were found for the egg shell color and cleanliness, while for the internal characteristics, statistically significant differences were recorded only for the yolk color. As measured by Haug's units, the quality of eggs was found to be below the valid standards, for all supermarkets, producers, and housing systems. The eggs from the free-range system had the highest Hog's units, with an average value of 60.67. The research's findings indicate that exceptionally high, prolonged summer temperatures had a very negative effect on the quality of eggs in Belgrade supermarkets. In order to improve the quality of eggs in the summer season, more research should be done to determine factors affecting egg handling on farms, transportation, storage in distribution centres and retail stores.
Keywords: *egg quality, housing system, high air temperatures, Belgrade supermarkets*

INTRODUCTION

Eggs are considered one of the most nutritious foods in the human diet (Sakanaka et al. 2000; Rehault-Godbert et al., 2019; Pal & Molnar, 2021) important for the nutrition of all age categories (Tolimir et al., 2021). According to the data from the The Food and Agriculture Organisation (FAO), there was a noticeable increase in egg consumption between 2000 and 2018 and it is estimated that the global consumption of eggs will continue to increase in the following period (FAO, 2018). While the quality of eggs has been studied for decades in the scientific and professional community, consumers' attention has been drawn to this issue in the last ten years due to their growing demand and awareness of the significance of food quality, safety and animal welfare (Savović et al., 2012; Duman et al., 2016; Rondoni et al., 2020). For consumers in Serbia, legislation in the field of veterinary medicine and food safety ensures access to safe, fresh and high-quality eggs. One of the key documents is the Rulebook on the quality of eggs (2019). However, in addition to the regulation, research of the quality of eggs present on the market is also important and it was conducted by a large number of authors (Hidalgo et al., 2008; Moula et al. 2013; Hisasaga et al., 2020).
Examination of the factors that influence the quality of eggs before laying, involves determination of: 1) Genetic factors (Zita et al., 2009; Wolc et al., 2012; Škrbić et al., 2020); 2) Hens' diet (Bouvarel et al., 2011); 3) Housing system and hygiene practice on the farm (Holt et al., 2011; Kucukkoyuncu et al., 2017; Yilmaz-Dikmen et al., 2017); 4) Microclimatic conditions on the farm (Kilic & Simsek, 2013); 6) age (Lee et al., 2016, Perić et al., 2017) and

7) health status of hens (Roberts, 2004). The initial quality of eggs starts to decline as soon as they are laid, while the rate of this process depends how the eggs are managed (Pavlovski et al., 1996). The quality of eggs after laying can be affected by: 1) handling of eggs during packaging and transportation; 2) conditions at sales facilities; and 3) storage conditions (such as egg cooling, light protection, hygienic practices, etc). The factors influencing the quality of eggs from farm to consumer, were studied by a number of authors (Jin et al., 2011; Zaheer, 2015; Jones et al., 2018).

Over the past ten years, research on the relationship between egg quality and housing system has become increasingly important for Serbian producers and consumers. This is because Serbian poultry is currently transitioning from conventional cage production to enriched cage and non-cage systems (Law on Animal Welfare, 2009; Rulebook, 2010). Data on the supply and the quality of eggs from different housing systems on the market of Serbia are quite scarce, i.e. this topic was studied by Tolimir et al. (2017) and Čobanović et al. (2022). Nevertheless, by monitoring the representation of eggs from cage and non-cage systems on the Belgrade market, Tolimir et al. (2024) observed a growing number of egg producers from non-cage systems in comparison to the previous decade.

In addition to the housing system, the impact of high summer temperatures on egg quality requires particular attention. According to the data of the Republic Hydrometeorological Service of Serbia (RHMZ), in Serbia, July 2024 was recorded to be the warmest in the last 73 years (RHMZ, 2024). High temperatures can have adverse influence on egg quality, due to the effect on hens, storage and retail conditions. The effect of heat stress on production performance and egg quality traits was investigated by Mashaly et al. (2004), Yoshida et al. (2011), Fouad et al. (2016). The influence of high temperatures during storage and sale on the quality of eggs, given that they can cause a faster decline in egg quality, was studied by Samli et al. (2005), An et al. (2023), Quan et al. (2021).

The aim of the present research is to determine the supply and quality of eggs from different housing systems in Belgrade supermarkets, in conditions of extremely high summer temperatures, i.e. prolonged heat wave that lasted several weeks. As the first of several studies examining the quality of eggs from housing systems available on the Belgrade market, the research covers only the summer season and therefore has a preliminary character. Apart from contributing to scientific findings in this area, the study is also important for consumers of table eggs, producers in the egg production sector, distributors and retailers.

MATERIAL AND METHODS

The examination of the supply and quality of eggs in supermarkets was carried out in July 2024. The supply of eggs was recorded in 10 super markets (SM1 to SM10), which make up the largest part of the market offer in the city of Belgrade. Within each of the supermarkets, the number of producers and the representation of eggs according to the production system - cage system, floor system, eggs from pasture and eggs from organic production were recorded. The supply of eggs in supermarkets was recorded during one day.

Egg sampling/puchase was done on the same day as supermarket supply was recorded. Ten grade M eggs, up to 15 days old, were purchased for each of the three housing systems - cage, floor, and free range - for each of the represented producers.

The age of the eggs was determined by the sell-by date printed on the package. Examination of the physical characteristics of egg quality (external and internal) was performed the day after the puchase/sampling, on a total of 244 eggs - 144 from the cage system, 50 from the floor system and 50 from the free system. From the moment of purchase until analysis, the eggs were kept in refrigerator, i.e. under the same conditions as in the supermarket.

The egg quality testing was performed for each egg individually, and the following physical characteristics of the egg quality were determined – external: egg weight (measured on a technical scale with an accuracy of 0.01g), shell colour (visually rated from 1 - the lightest to 5 - the darkest), shell cleanliness (visually rated from 1 - the lowest grade to 5 – the best grade), and internal: yolk color (visually graded using the Roche Yolk Color fan), egg white

and yolk height (measured with a tripod micrometer) and shell thickness and yolk diameter (measured with a caliper). The measurements of yolk were taken with the yolk in the natural position when the egg was broken out (Funk, 1948).
Egg shape index (%) was calculated as: (egg diameter/egg height) x 100.
Haugh Unit (HU) score was calculated using the formula as given by Haugh (1937):
HU = 100 × log (H + 7.5 – 1.7W0.37); where: H – albumen height (mm); W – egg weight (g).
Yolk index (YI) was calculated as: YI = (yolk height/yolk diameter) × 100.
During July 2024, Serbia was affected by a heat wave, with an average maximum air temperature of 34.43°C (RHMZ, 2024).
Data were statistically analysed using the STATISTICA 10.0 software package (StatSoft Inc., Tulsa, OK, USA). All analyzes were performed on the basis of three measurements. The obtained results are expressed as mean value ± standard deviation (SD). All quality characteristics were analyzed using a oneway ANOVA and Tukey's HSD post hoc test. Significance was determined at $P<0.05$.

RESULTS AND DISCUSSION

An overview of table egg producers and housing systems in 10 Belgrade supermarkets, which account for the majority of Belgrade market's sales in marketplaces, is given in Table 1. According to the data in table 1, 10 egg producers were represented in Belgrade supermarkets, with 90% of them having a cage system, 40% floor and free-range system, while there was only one producer of organic eggs. In terms of supermarkets' egg supply, 90% of them offered eggs from the cage system and 50% from floor and free-range system. Organic eggs were present in only one supermarket, which was expected, considering that these eggs on the Belgrade market canusually be found in specialized organic food stores. Findings from the present research are consistent with the study conducted in 2023 (Tolimir et al., 2024). Given that both studies provide an outline of the situation for particular, short period, there is a need for continuous egg supply monitoring throughout the year, in order to obtain a more objective picture of the representation of eggs from different housing systems on the Belgrade market.
Regarding the representation of housing systems among producers (Table 1), it can be stated that the number of housing systems per producer ranged from one system (4 producers only produce eggs in the conventional cage system and 1 only organic eggs), to a maximum of 3 systems per producer (2 producers have simultaneous egg production in cage, floor and free-range housing system). The obtained results indicate that a certain number of producers still combine production in the cage system with one or more alternative systems, which was enabled by the extension of the deadline for abolishing the conventional cage system in Serbia. It is expected that the further course of transition to enriched cage and non-cage systems will be affected not only by the legal regulations, but also by the growing awareness of producers about animal welfare, as well as about the potential benefits of using food from alternative rearing systems.

Table 1. Egg supply in Belgrade supermarkets

Total number of supermarkets	**Total number of egg producers**	**Number of cage eggs' producers**	**Number of floor system eggs' producers**	**Number of free-range system eggs' producers**	**Number of organic eggs' producers**
10	**10**	**9**	**4**	**4**	**1**

Supermarket	**Total number of producers per supermarket**	**Number of housing systems per supermarket**	**Number of egg producers**			
			Cage system	Floor system	Free-range system	Organic
SM1	2	3	2	1	1	0
SM2	3	2	1	0	1	0
SM3	3	3	2	1	1	0

SM4	2	2	0	1	0	0
SM5	3	2	1	1	0	0
SM6	1	1	1	0	0	0
SM7	1	1	1	0	0	0
SM8	1	1	1	0	0	0
SM9	1	2	0	1	1	0
SM10	3	3	2	0	1	1

Representation of the housing systems among producers in Belgrade supermarkets

	Cage system	Floor system	Free-range system	Organic
Producer 1	**+**	**+**	**-**	**-**
Producer 2	**+**	**+**	**+**	**-**
Producer 3	**+**	**+**	**-**	**-**
Producer 4	**+**	**-**	**+**	**-**
Producer 5	**+**	**-**	**-**	**-**
Producer 6	**+**	**-**	**-**	**-**
Producer 7	**+**	**+**	**+**	**-**
Producer 8	**+**	**-**	**-**	**-**
Producer 9	**+**	**-**	**-**	**-**
Producer 10	**-**	**-**	**-**	**+**

The values of HU, as one of the most important parameters of internal egg quality and egg freshness, ranged from 54.54 to 60.67, which indicates the low quality of eggs from all examined housing systems. As stated by Malfatti et al. (2021), the lowest acceptable value of HU for table eggs is 60. According to USDA quality standards (USDA, 2000) eggs from the cage and floor system belonged to quality class B (HU=31-59.9), and eggs from the free-range system to quality class A (HU=60-71.9). It is important to point out that the obtained values for HU are significantly lower compared to those from the previous research on egg quality in Belgrade supermarkets. Hence, HU varied from 73.2 to 91.7 in the research by Tolimir et al. (2017) conducted in autumn, and from 92 to 115 in the research by Čobanović et al. (2022). This supports what was previously stated - continuous, extended research on the quality of eggs on the Belgrade market is needed.

Given that all the eggs in this research were up to 15 days old and that they were kept in the supermarkets in refrigerated display cases, the reasons for the low quality of the eggs could be a qonsequence of several factors in the supply chain, which were not the subject of this research. In particular, it should be pointed out that egg quality is highly sensitive to high temperatures during summer, whether within management on the farm or in the retail chain and during storage. The negative impact of elevated temperatures on egg quality during storage and a significant decrease of the HU have been confirmed in several studies (Feddern et al., 2017; Martínez et al., 2021; Quan et al., 2021). According to the findings of Jin et al. (2011), the HU value decreased by 15 units during only two days of storage at a temperature of 29 ºC.

Table 2 summarises physical quality characteristics of eggs from different housing systems, collected in Belgrade supermarkets.

Table 2. Quality of eggs from different housing systems in Belgrade supermarkets

External egg quality characteristics				
Housing system	Egg weight, g	Shape index, %	Shell colour, score (min=1, max=5)	Shell cleanliness, score (min=1, max=5)
Cage	59.00±3.77^{a}	81.47±3.25^{a}	4.08±0.49^{b}	4.73±0.78^{a}
Floor	57.88±1,75^{a}	81.11±2.42^{a}	3.80±0.49^{a}	4.76±1.06^{a}
Free range	58.23±2.39^{a}	82.09±3.10^{a}	4.02±0.55^{ab}	4.91±0.27^{a}

Internal egg quality characteristics					
Housing system	Yolk colour	Albumen height, mm	HU	Yolk index	Shell thickness, mm
Cage	12.49±0.86[b]	3.88±1.43[a]	54.54±17.99[a]	0.342±0.034[a]	39.22±4.01[a]
Floor	12.04±1.13[a]	4.03±0.99[a]	58.83±11.28[a]	0.346±0.029[ab]	39.73±3.62[a]
Free range	11.98±0.86[a]	4.25±1.08[a]	60.67±12.84[a]	0.354±0.023[b]	38.76±4.19[a]

* different superscript letters in the same column represent values of significant differences ($p<0.05$)

Yolk index (YI) is also an important indicator of the internal quality and freshness of eggs (Huang et al., 2012). In this research, YI ranged from 0.342 to 0.354 and a statistically significant difference was recorded between eggs from the cage and free system. According to DSM egg quality manual (2022) eggs with a yolk index in the range of 0.28 to 0.38 are considered fresh, and over 0.38 are considered extra fresh. However, the obtained values are much lower than those found in other research on egg quality, which were over 0.38 for eggs of similar age (Samli et al., 2005; Hidalgo et al., 2008; Imran & Nayak, 2020; Hisasaga et al. 2020)

There were no statistically significant differences found in egg weight, shape index, albumen height, HU and shell thickness between the housing systems. Yolk color is a particularly important quality attribute from the perspective of consumers, who prefer a darker yolk color (Bertechini, 2017; Sass et al., 2018). Eggs from the cage system had the darkest yolk color, which is in agreement with the results of Hidalgo et al. (2008), Đukić-Stojčić et al. (2009) and Kucukkoyuncu et al. (2017), while in the research of Hisasaga et al. (2020) and Gandarillas et al. (2023) eggs from the free-range system had a darker yolk color compared to eggs from the cage system. Since the yolk colour is directly influenced by hens' diet, variations in the color of the yolk can be explained by the different amount of pigments (carotenoids) in the feed (Titcomb et al., 2019). It should be taken into account that hens nutrition of hens in a cage system is more controlled with the possibility of adding pigments to the feed, while the nutrition in alternative systems is less controlled, so the proportion of pigments in the diet is variable, sometimes higher and sometimes lower compared to the cage system.

CONCLUSIONS

The supply of eggs in Belgrade in supermarkets is characterized by a larger assortment compared to the previous decade. Eggs from the cage system are still dominant, but an increased supply of eggs from alternative systems is noticeable, while the offer of organic eggs is insufficient. These changes in the market can be related to the implementation of legal regulations on animal welfare, therefore, in Serbia, an increase in the share of eggs from non-battery systems can be expected in the upcoming period. During the transition period, it is necessary to educate producers, but also consumers who, in addition to the possibility to choose, need to be provided with knowledge about the differences in the production and quality of eggs from various housing systems.

During a multi-week heat wave, in July 2024, the quality of eggs from all three production systems - cage, floor, and free-range was much lower compared to the earlier research, performed during autumn. Given that this research only concerns the quality of eggs in supermarkets, further, more comprehensive research of all participants in the supply chain could provide answers as to whether the low egg quality during summer is a consequence of production management, storage or retail stores' conditions. Such research would provide guidelines for eliminating critical points in the entire supply chain, with the aim of achieving better egg quality and consumer safety, especially in the conditions of increasingly hot summers, with extremely high temperatures and prolonged heat waves. Also, continuous research on the quality of eggs in supermarkets, during the year, would make it possible to

more objectively determine the quality of eggs and their variations depending on the housing systems.

ACKNOWLEDGEMENTS

Acknowledgement This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, contracts: 451-03-66/2024-03/200045 and 451-03-66/2024-03/200022.

REFERENCES

An, J. H., Hwang, Y., Hwang, S., Kwon, H., Gu, H., Park, K & Choi, C. (2023). Comparative evaluation of egg quality in response to temperature variability: from farm to table exposure scenarios. *Food Science of Animal Resources*, 43, 1002-1016.

Bertechini, A. B. (2017). Chapter 21 - Economic and Cultural Aspects of the Table Egg as an Edible Commodity, In Hester, P. Y. (Eds.), Egg Innovations and Strategies for Improvements (pp. 223-232), Academic Press.

Bouvarel, I., Nys, Y. & Lescoat, P. (2011). Hen nutrition for sustained egg quality. In Nys, Y., Bain, M. & Immerseel, F. V. (Eds.), *Improving the Safety and Quality of Eggs and Egg Products* (pp. 261-299). Cambridge: Woodhead Publishing.

Čobanović, N., Todorović, N., Kovandžić, M., Vićić, I., Suvajdžić, B., Grković, N. & Karabasil, N. (2022). Assessment of marketed table egg quality originating from different production systems. *Meat Technology*, 63, 66-76.

DSM (2022). DSM egg quality manual. *DSM Nutritional Products* Ltd, https://www.dsm.com/content/dam/dsm/anh/en/documents/dsm-egg-quality-manual.pdf

Đukić-Stojčić, M., Perić, L., Bjedov, S. & Milošević, N. (2009). The quality of table eggs produced in different housing system. *Biotechnology in Animal Husbandry*, 25, 1103-1108.

Duman, M., Şekeroğlu, A., Yıldırım, A., & Eleroğlu, H. (2016). Relation between egg shape index and egg quality characteristics. *European Poultry Science*, 80, 1-9.

FAO. (2018). Eggs: Harnessing their power for the fight against hunger and malnutrition [Report of activity No154]. FAO Global Forum on Food Security and Nutrition (FSN Forum).

Feddern, V., De Prá, M. C., Mores, R., da Silveira Nicoloso, R., Coldebella, A. & de Abreu, P. G. (2017). Egg quality assessment at different storage conditions, seasons, and laying hen strains. *Ciência e Agrotecnologia,* 41, 322-333.

Fouad, A. M., Chen, W., Ruan, D., Wang, S., Xia, W. G., & Zheng, C.T. (2016). Impact of heat stress on meat, egg quality, immunity and fertility in poultry and nutritional factors that overcome these effects: A review. *International Journal of Poultry Science*, 15, 81-95.

Funk, E. M. (1948). The relation of yolk index determined in natural position to the yolk index as determined after separating the yolk from the albumen. *Poultry Science*, 27, 367.

Gandarillas, M., Olmos, V., Piña, B., Keim, J. P. & Vargas-Bello-Pérez, E. (2023). Physical quality of different industrial versus non-industrial eggs obtained from groceries and markets in southern Chile. Austral journal of veterinary sciences, 55, 87-94.

Haugh, R. R. 1937. The Haugh unit for measuring egg quality. *US Poultry Magazine*, 43, 552–573.

Hidalgo, A., Rossi, M., Clerici, F. & Ratti, S. (2008). A market study on the quality characteristics of eggs from different housing systems. *Food Chemistry,* 106, 1031-1038.

Hisasaga, C., Griffin, S. E. & Tarrant, K. J. (2020). Survey of egg quality in commercially available table eggs. *Poultry Science*, 99, 7202-7206.

Holt, P. S. Davies, R. H., Dewulf, J., Gast, R. K., Huwe, J. K., Jones, D.R., Waltman, D. & Willian, K.R. (2011). The impact of different housing systems on egg safety and quality 1. Poultry Science, 90, 251-262.

Huang, Q., Qiu, N., Ma, M. H., Jin, Y. G, Yang, H., Geng, F. & Sun, S. G. (2012). Estimation of egg freshness using S-ovalbumin as an indicator. *Poultry Science*, 91, 739-43.

Imran, S. K. & Nayak, Y. (2020). Temperature and storage period modulate the egg quality. *International Journal of Research Culture Society*, 4, 192-200.

Jin, Y. H., Lee, K. T., Lee W. I. & Han Y. K. (2011). Effects of storage temperature and time on the quality of eggs from laying hens at peak production. *Asian-Australasian Journal of Animal Sciences*, 24, 279-284.

Jones, D. R., Ward, G. E., Regmi, P. & Karcher, D. M. (2018). Impact of egg handling and conditions during extended storage on egg quality. *Poultry Science*, 97:716–723.

Kilic, I. & Simsek, E. (2013). The effects of heat stress on egg production and quality of laying hens. *Journal of Animal and Veterinary Advances*, 12, 42-47.

Kucukkoyuncu, E., Okur, A. A., Tahtabicen, E., Korkmaz, F. & Samli, H. E. (2017). Comparing quality of free range and battery cage eggs. *European Poultry Science*, 81.

Law on Animal Welfare. 2009. Official Gazette of RS, 41/2009
Lee, M.H., Cho, E.J., Choi, E.S., Bang, M.H., & Sohn, S.H. (2016). The effect of hen age on egg quality in commercial layer. *Korean Journal of Poultry Science*, 43, 253-261.
Malfatti, L. H., Zampar, A., Galvão, A. C., Robazza,W. S. & Boiago, M. M. (2021). Evaluating and predicting egg quality indicators through principal component analysis and artificial neural networks, LWT, 148, 111720.
Martínez, Y., Diaz Soliz, N., Bejarano, M. A., Paz, P. & Valdivie, M. (2021). Effect of storage duration and temperature on daily changes in external and internal egg quality of eggs from Dekalb White® laying hens. *European Poultry Science*, 85.
Mashaly, M. M., Hendricks, G. L., Kalama, M. A., Gehad, A. E, Abbas, A. O. & Patterson, P. H. (2004). Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poultry Science*, 83, 889-94.
Moula, N., Ait-Kaki, A., Leroy, P., & Antoine-Moussiaux, N. (2013). Quality assessment of marketed eggs in bassekabylie (Algeria). *Brazilian Journal of Poultry Science*, 15, 395-399.
Pal, M. & Molnár, J. (2021). The role of eggs as an important source of nutrition in human health. *International Journal of Food Science and Agriculture*, 5, 180-182.
Pavlovski, Z., Cmiljanić, R. & Hopić, S. (1996). Inicijalni kvalitet jaja i njegove promene u uslovima skladištenja i tržišta. *Tehnologija mesa*, 3-4, 87-91.
Perić, L., Đukić Stojčić, M. & Bjedov, S. (2017). The effect of storage and age of hens on the quality of table eggs. *Advanced research in life sciences*, 1, 64-67.
Quan, C., Xi, Q., Shi, X., Han, R., Du, Q., Forghani, F., Xue, C., Zhang, J. & Wang, J. (2021). Development of predictive models for egg freshness and shelf-life under different storage temperatures. *Food Quality and Safety*, 5, 1-7.
Réhault-Godbert, S., Guyot, N. & Nys, Y. (2019). The golden egg: nutritional value, bioactivities, and emerging benefits for human health. *Nutrients*, 11, 684.
RHMZ (2024). Monthly Agrometeorological Bulletin - July 2024. https://www.hidmet.gov.rs/data/klimatologija/latin/Jul.pdf
Roberts, J.R. (2004). Factors affecting egg internal quality and egg shell quality in laying hens. The Journal of Poultry Science, 41, 161-177.
Rondoni, A., Asioli, D. & Millan, E. (2020). Consumer behaviour, perceptions, and preferences towards eggs: a review of the literature and discussion of industry implications. *Trends in Food Science and Technology*, 106, 391-401.
Rulebook 2010. Rulebook on conditions for animal welfare in terms of animal housing, rooms and equipment in facilities where animals for production purposes are kept, reared and placed on the market, the manner of keeping, breeding and trade of certain species and categories of animals, as well as the content and management of animal records' keeping. Official Gazette of the RS, 6/10, 57/14, 152/20, 115/23.
Rulebook on the quality of eggs (2019). Official Gazette of the Republic of Serbia, no. 7/2019, 35/2019 i 78/2019.
Sakanaka, S., Kitahata, K., Mitsuya, T., Gutierrez, M. A., & Juneja, L. R. (2000). Protein quality determination of delipidated egg-yolk. *Journal of Food Composition and Analysis*, 13, 773-781.
Samli, H. E., Agma, A. & Senkoylu, N. (2005). Effects of storage time and temperature on egg quality in old laying hens. *Journal of Applied Poultry Research*, 14, 548-553.
Sass, C. A. B., Kuriya, S., da Silva, G. V., Silva, H. L. A., da Cruz, A. G., Esmerino, E. A. & Freitas, M. Q. (2018). Completion task to uncover consumer's perception: a case study using distinct types of hen's eggs. *Poultry Science*, 97, 2591-2599.
Savović, I., Kokić Arsić, A., Kanjevac Milovanović, K., & Đorđević, A. (2012). Kvalitet i bezbednost hrane iz ugla korisnika. Zbornik radova 39. nacionalne konferencija o kvalitetu "FQ2012 FESTIVAL KVALITETA", Kragujevac, Serbia, B105-B112. https://www.cqm.rs/2012/cd1/pdf/7/14.pdf
Škrbić, Z., Lukić, M., Petričević, V., Bogosavljević-Bošković, S., Rakonjac, S., Dosković, V., & Tolimir, N. (2020). Quality of eggs from pasture rearing layers of different genotypes. *Biotechnology in Animal Husbandry*, 36, 181-190.
Titcomb, T. J, Kaeppler, M. S., Cook, M. E., Simon, P. W. & Tanumihardjo S. A. (2019). Carrot leaves improve color and xanthophyll content of egg yolk in laying hens but are not as effective as commercially available marigold fortificant. *Poultry Science*, 98, 5208-5213.
Tolimir, N., Maslovarić, M., Škrbić, Z., Lukić, M., Milić, D. & Nedeljković Trailović, J. (2024). Situation on the market of eggs from noncage production systems. *Biotechnology in Animal Husbandry*, 40, 29-40.
Tolimir, N., Maslovarić, M., Škrbić, Z., Lukić, M., Rajković, B. & Radišić, R. (2017). Consumer criteria for purchasing eggs and the quality of eggs in the markets of the city of Belgrade. *Biotechnology in Animal Husbandry*, 33, 425-437.
Tolimir, N., Škrbić, Z., Maslovarić, M., Lukić, M. & Milić D. (2021). The importance of eggs in the diet, consumer preferences, the production and market of table eggs in Serbia. Proceedings of 13th international symposium "MODERN TRENDS IN LIVESTOCK PRODUCTION", 6-8 October 2021, Belgrade, Serbia, 126-148.

USDA (United States Department of Agriculture). (2000). United States standards, grades, and weight classes for shell eggs. U.S. Government Printing Office, Washington DC.

Wolc, A., Arango, J., Settar, P., O'Sullivan, N. P., Olori, V.E., White, I. M. S., Hill, W. G. & Dekkers, J. C. M. (2012). Genetic parameters of egg defects and egg quality in layer chickens. *Poultry Science*, 91, Issue 6, 1292-1298.

Yilmaz-Dikmen, B. Y., İpek, A., Şahan, Ü., Sözcü, A. & Baycan, S. C. (2017). Impact of different housing systems and age of layers on egg quality characteristics. *Turkish Journal of Veterinary & Animal Sciences*, 41, Article 12.

Yoshida, N., Fujita, M., Nakahara, M., Kuwahara, T., Kawakami, S. & Bungo, T. (2011). Effect of high temperature on egg production, serum lipoproteins and follicle steroid hormones in laying hens. *The Journal of Poultry Science*, 48, 207–211.

Zaheer, K. (2015). An updated review on chicken eggs: production, consumption, management aspects and nutritional benefits to human health. *Food and Nutrition Sciences*, 06, 1208-1220.

Zita, L., Tůmová, E., & Štolc, L. (2009). Effects of genotype, age and their interaction on egg quality in brown-egg laying hens. *Acta Veterinaria Brno*, 78, 85-91.

THE INFLUENCE OF BLENDING AND LAMINATION OF DIFFERENT OILSEED CAKES ON THE PROPERTIES OF COMPOSITE BIOPOLYMER FILM

Danijela Šuput[1]*, Nedeljka Spasevski[2], Slađana Rakita[2], Nevena Hromiš[1], Senka Popović[1], Jovana Pantić[1]

[1]Faculty of Technology Novi Sad, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: suput.danijela@gmail.com

ABSTRACT

The aim of this paper was to produce biopolymer films on the basis of two oilseed cakes – pumpkin oil cake (PuOC) and Camelina Sativa oil cake (CSoC). The films were produced by blending and laminating film-forming suspensions obtained from both oilseed cakes in various ratios (25%-75%, 50%-50%, and 75%-25%). Plain biopolymer films served as controls. The aim was to characterize the obtained 8 film samples regarding mechanical, physico-chemical, and barrier properties and to check if, and how, each blended/laminated sample differs from controls. The obtained results pointed to better mechanical properties of the films obtained from PuOC and all samples with a higher ratio of this oil cake in its composition. On the other hand, CSoC transferred better barrier properties against water vapor to the samples with a higher ratio of applied CSoC. Both mechanisms – lamination and blending are effective methods for improving the mechanical and barrier properties of biopolymer films. Still, lamination proved to be a more efficient method than blending, as films with improved characteristics were obtained by this method.
***Keywords**: oilseed cakes, blending, lamination, biopolymer films, properties*

INTRODUCTION

The production of biopolymer packaging generated a lot of interest due to the possibility of utilization of food industry byproducts and residues. By-product recovery raises the notion of sustainability and encourages recycling and reuse, increasing food waste value while lowering expenses and environmental hazards associated with environmental disposal (Hamed et al., 2022). Annually, approximately one-third of the produced food is lost or wasted (Blakeney, 2019), so it is imperative to explore ways to minimize losses through their valuation, such as biopolymer materials production (Esparza et al., 2020). Oilseeds are grains used to produce vegetable oil. In 2023, 659 million metric tons of oilseeds were produced worldwide (USDA, 2023). Cake or meal are important byproducts left behind after oil is extracted. The potential uses of the oilseed cakes are as a source of energy and animal feed, or they can be extracted to produce bioactive chemicals and value-added food products or biopolymers packaging materials (Petraru & Amarei, 2020; Gupta et al., 2019; Petraru & Amarei, 2020). Using complete oilcakes, which are inherently complex mixtures of lipids, polysaccharides, and proteins (up to 50% recyclable proteins), is an intriguing strategy which is why they present a promising raw material for edible, environmentally friendly biopolymers that may find application in packaging. Additionally, the defatted seed wastes usually have significant polyphenol contents, so derived edible films and coatings, may possibly have antibacterial and antioxidant properties positively expressed on the packaged content. As a result, the packaged products are shielded against deterioration (oxidation, rancidity, and microbial spoiling). The total polyphenols ingested by the consumer along with biopolymer films provides an additional nutritional contribution to these films (Toma et al., 2015; Kadam et al., 2018).
Pumpkin oilseed cake (PuOC), a by-product obtained after the extraction of oil from pumpkin seeds (Curcubita pepo L.) by cold pressing, is rich in proteins (up to 63%), carbohydrates, oils, crude fibers, and other components. Examination of the composition, properties, characterization and modification of properties, as well as the application of PuOC cake and

its proteins, in various spheres of interest, such as packaging materials, filmogenic coatings in the encapsulation process, natural emulsifiers, bioactive peptides, has been studied in recent years (Popović et al., 2011; Vaštag et al., 2011; Bučko et al. 2016; Čakarević et al., 2020). The possibility of obtaining biopolymer films based on PuOC was examined in the work of Popović et al. (2011). The obtained results showed that the biopolymer film based on PuOC, obtained at a temperature of 90 °C and pH 12, had the best mechanical properties, as well as the best barrier to gases. In addition, films based on PuOC also showed antioxidant activity, particularly those films obtained at pH 10 and a heating temperature 60 °C. After the complete characterization of the obtained films based on PuOC was carried out, further research was focused on the possibility of forming packaging, i.e. PuOC based bags (Bulut et al., 2017), as well as the possibility of maintaining a modified atmosphere in bags based on composite laminar biopolymer materials (Bulut et al., 2016).

Camelina Sativa oilseed cake (CSoC), a by-product obtained after the extraction of oil from wild flax seeds (Camelina Sativa), consists of approximately 10% fat, 45% crude protein, 13% fiber, 5% minerals. The addition of camelina oilseed cake, in combination with other bioplastics, can be the basis for obtaining material with beneficial properties used in food packaging (Sydor et al., 2022). In the Šuput et al. study (2023a), the effect of cake granulation and the presence of mucilage on film properties was investigated, and it was concluded that biopolymer films based on camelina with the lowest cake granulation and with the presence of naturally occurring mucilage exhibited optimal film properties. Further study aimed to synthesize, characterize and optimize the development of biopolymer film based on CSoC. By studying physico-chemical, mechanical, barrier, structural, antioxidant and antimicrobial of the obtained films, and by applying appropriate mathematical models, optimal process parameters were selected: pH=10, temperature=100°C, and concentration=5% (Šuput et al., 2024a). The consequential study aimed to confirm the effects of the CSoC-based biopolymer coating as a packaging layer on the quality and sustainability of the osmotically dried beetroots (Šuput et al., 2024b).

In this work, the optimal process parameters for both cakes were applied and blended biopolymer films with different cake ratios were produced to examine how each of the oilseed cakes would affect film properties. Additionally, laminated film samples were produced in the same ratio as blended to examine whether the film-making process affect the obtained film properties.

MATERIAL AND METHODS

Materials

Ground pumpkin oilseed cake (PuOC) was obtained from the company Linum doo (Čonoplja, Serbia), while Camelina Sativa seeds were kindly supplied by the Institute of Field and Vegetable Crops (Novi Sad, Serbia). The fraction of cold-pressed camelina seed cake (<180 μm) was used for biopolymer film preparation. PuOC contained 63.52% proteins, 8.66% oils, and 4.5% cellulose, while CSoC fraction contained 39.4 % protein, 19.4 % oil, and 5.9 % cellulose. Glycerol (92,1 g/mol) was obtained from Lach-Ner (Croatia), deionized water was obtained from Alfapanon (Serbia) and granulated NaOH was obtained from NPK- Inžinjering (Serbia).

Methods

The glycerol (30% calculated on the weight of the cake) was added to both aqueous solutions of the cakes (10% for PuOC and 5% for CSoC). The pH of the suspension was adjusted to 12 for PuOC and 10 for CSoC and then heated to 90°C for PuOC and 100°C for CSoC for 20 minutes. Both film-forming suspensions were filtered and ready for further use. The first group of samples were 2 controls obtained when 50g each of PuOC and CSoC suspensions were poured onto Petri plates. The second group of samples was obtained by mixing film-forming suspensions into one suspension in the ratio of 25%PuOC (12.5g) - 75%CSoC (37.5g), 50%PuOC (25g) - 50%CSoC (25g), and 75%PuOC (37.5g) - 25%CSoC (12.5g) and subsequently poured onto Petri plates (50g of each mixture). The third group of

film samples was obtained by lamination: PuOC suspension parts (25%, 50% and 75%, which corresponds to 12.5g, 25g and 37.5g) were poured on Petri plates and when they were dried under room conditions the remaining amount of CSoC suspension (75%, 50% and 25%, respectively; which corresponds to 37.5g, 25g and 12.5g, respectively) was poured (laminated) over it and eventually dried. Consequently, this procedure resulted in 8 film samples labeled as 1-8 (Figure 1), where labels correspond to the percentages of each oilseed cake in film-forming suspension as follows:

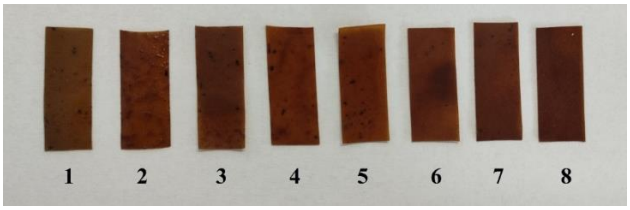


Figure 1. Samples: 1 - 100 PuOC; 2 - 75PuOC-25CSoC Bl; 3 - 75PuOC-25CSoC Lam; 4 - 50PuOC-50CSoC Bl; 5 - 50PuOC-50CSoC Lam; 6 - 25PuOC-75CSoC Bl; 7 - 25PuOC-75CSoC Lam; 8 - 100 CSoC.

Bl - blended, Lam - laminated.

Mechanical properties - tensile strength (TS) and elongation at break (EB) were measured following the standard method EN ISO 527-3:2018 (clamps distance 50 mm; test speed 50 mm/min).
Water vapor permeability was determined according to the standard method ISO 2528:2017 (temperature 23°C, relative humidity 50%).
Moisture content (MC) was determined as the percentage of weight reduction after film drying until constant weight, expressed on the total weight of the film (Šuput et al., 2024a).
Film solubility was evaluated when dried film samples (m1) were immersed in 30 mL of deionized water and for 24h at room conditions after which films were dried again until constant mass (m2). The total solubility of the films (%) was calculated according to equation:
S (%) = (m1-m2)/m1 * 100
Film thickness, tensile strength, and elongation at break were expressed as mean values of five repetitions ± SD while moisture content, film solubility, and water vapor permeability were expressed as mean values of three repetitions ± SD.

RESULTS AND DISCUSSION

The mechanical properties of biopolymer packaging depend on the nature and composition of the film-forming solution (the structure and coherence of the polymer matrix chains, and the way the film is produced (Petraru & Amariei, 2023). In this case, PuOC-based films proved to have superior mechanical properties over films based on CSoC (Figure 2), which correlates with previous literature findings (Bulut, 2021; Šuput et al., 2024a). When combining these two materials in different ratios PuOC has transferred its good mechanical properties to the blended/laminated films with its positive influence being greater in films with a higher PuOC ratio. This effect was more notable when examining elongation at break. Tensile strength values were in the range of 0.7-0.97 MPa, which is comparable with other biopolymer films obtained from oilseed cakes (Mirpoor et al., 2024; Jang et al., 2011; Bulut, 2021). On the other hand, elongation at break values we significantly different when applying PuOC (95.23%) and SCoC (7.90%) as raw materials for biopolymer film preparation. Further, laminated film samples proved to have favorable mechanical properties over blended films which is most noticeable in samples 4 and 5, where 50% of each oilseed cake participated in film-forming. Elongation at break in laminated sample 5 was 54.31% while blended film 4 displayed 13.04%.
The obtained results are in agreement with other studies that also reported that incorporating zein into chitosan film could result in a rougher, more elastic, and softer film structure

compared with a single chitosan film (Escamilla-Garcia et al., 2013); shellac addition, applied as blend and lamination, improved zein film properties (Šuput et al., 2023b) and that gelatin addition into starch biopolymer films, applied as blend and lamination, improved starch film properties (Šuput et al., 2022). The most pronounced differences between laminated and blend films have been observed by monitoring the mechanical characteristics so lamination is suggested as the optimal method of film preparation (Šuput et al., 2022).

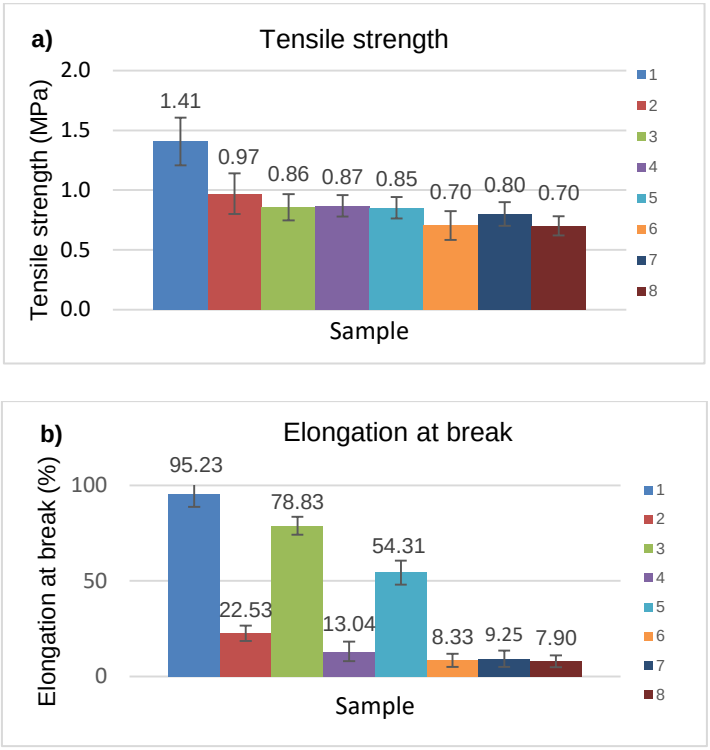


Figure 2.a. Tensile strength (MPa), b. Elongation at break (%) of blended and laminated PuOC-SCoC-based films

Product water content, humidity, and temperature all greatly impact biopolymer-based film properties. As a result, if these factors change, the film's physical characteristics might also change, which restricts their application. Water sensitivity depends on film moisture content in a given environment, its ability to absorb water, and its solubility in water. Because it affects how films are used in technical applications, water solubility is very important (Petraru & Amariei, 2023).

Table 1. Moisture content (%) and solubility (%) of blended and laminated PuOC-SCoC-based films

	1	2	3	4	5	6	7	8
MC (%)	19.78 ±0.76	19.62 ±0.59	19.70 ±0.74	17.52 ±0.11	16.37 ±0.13	16.49 ±0.43	15.12 ±0.68	14.81 ±0.68
S (%)	34.11 ±0.33	33.31 ±0.42	35.63 ±0.16	33.11 ±0.36	33.08 ±0.58	36.25 ±0.54	35.44 ±0.60	35.65 ±0.83

Moisture content values were 19.78 % for PuOC-based film and 14.81 % for CSoC-based film. Since it is favorable if the moisture content is lower, this means that CSoC has a positive effect on blend/laminated film properties. This is more pronounced when lamination was applied as it can be seen from samples couples 4-5 and 6-7. Solubility values were in the range of 33.08-36.25 % indicating no significant effect of the film-making process on the examined property.

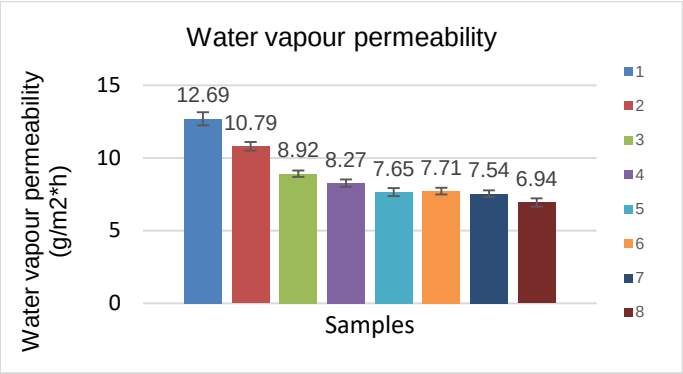


Figure 3. Water vapor permeability of blended and laminated PuOC-SCoC-based films

The value of water vapor permeability of films based on PuOC was 12.69 g/m2h, while the permeability of films based on CSoC was 6.94 g/m2h. In all other film samples, without exception, where the percentage of PuOC was higher, higher water permeability values were obtained (10.79 and 8.92 g/m2h) compared to the samples with lower PuOC share in the film-forming matrix (7.71and 7.54 g/m2h). It can be concluded that the addition of CSoC in a larger amount contributed to the improvement of the barrier properties of the obtained films. A difference in the value of water vapor permeability was observed for all pairs of samples (at the ratio of 75-25, 50-50, and 25-75) depending on whether the films were obtained by blending or lamination. In all tested samples, lamination gave better results since lower values of water vapor permeability are considered desirable, which is in agreement with Phan The et al. (2008) and Šuput et al. (2023b).
Both mechanisms are effective methods for improving the mechanical and barrier properties of biopolymers. As for blending films with monolayer structures, added components are probably evenly distributed in the biopolymer matrix. However, their water resistance ability is much lower than the laminated films. In case of laminated films, the biopolymer matrix offers smooth surfaces and structural-mechanical support, making it easier to apply the second layer. This is why laminated films have higher mechanical and barrier efficiency (Šuput et al., 2023b).

CONCLUSIONS

The use of food processing industry waste to obtain new biopolymer materials has been on the rise in recent years. This work aimed to point out how different ratios of oilseed cakes in film-forming suspension affect the obtained film properties. The obtained results indicated that all samples having a larger ratio of PuOC in their composition had superior mechanical capabilities. Conversely, samples with a larger ratio of applied CSoC had better barrier qualities against water vapor. The mechanical and barrier qualities of biopolymer films can be effectively enhanced by either lamination or blending process. Nevertheless, lamination turned out to be a more effective technique if strong mechanical strength and good barrier qualities were essential. Blended films may be the better option in applications where biodegradability and ease of processing are more crucial. A further step would be the application of the resulting films as packaging materials for various food products, where film`s advantages will be highlighted through different application.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant number 451-03-66/2024-03/200134.

REFERENCES

Blakeney, M. (2019). Food Loss and Food Waste: Causes and Solutions. Edward Elgar Publishing: Cheltenham, UK, ISBN 9781788975391.

Bučko, S., Katona, J., Popović, Lj., Petrović, L., Milinković, J. (2016). Influence of enzymatic hydrolysis on solubility, interfacial and emulsifying properties of pumpkin (Cucurbita pepo) seed protein isolate. *Food Hydrocolloids, 60*, 271-278.

Bulut, S. (2021). Research of obtaining, characterization and optimization of properties of active, biodegradable, packaging material based on pumpkin oil cake. Doctoral Thesis, University of Novi Sad, Faculty of Technology Novi Sad, Serbia.

Bulut, S., Lazić, V., Popović, S., Hromiš, N., Šuput, D. (2017). Influence of storage period on properties of biopolymer packaging materials and pouches. *Acta Periodica Technologica, 48*, 53–62.

Bulut, S., Lazić, V., Popović, S., Šuput, D., Hromiš, N., Popović, L. (2016). Possibility to maintain modified atmosphere in pouches made from biopolymer materials. Proceedings of 3rd International congress "Food Technology, Quality and Safety" & 17th International symposium "Feed Technology" (FoodTech 2016), 23-25 October, Novi Sad, Serbia, 122–127.

Čakarević, J., Šeregelj, V., Tumbas Šaponjac, V., Ćetković, G., Čanadanović Brunet, G., Popović, S., Hadnađev Kostić, M., Popović, Lj. (2020). Encapsulation of beetroot juice: a study on the application of pumpkin oil cake protein as new carrier agent. *Journal of Microencapsulation, 37*, 121-133.

Escamilla-Garcia, M., Calderon-Dominguez, G., Chanona-Perez, J.J., Farrera-Rebollo, R.R., Andraca-Adame, J.A., Arzate-Vazquez, I., Mendez-Mendez, J.V., Moreno-Ruiz, L.A. (2013). Physical and structural characterization of zein and chitosan edible films using nanotechnology tools. *International Journal of Biological Macromolecules, 61*, 196–203.

Esparza, I., Jiménez-Moreno, N., Bimbela, F., Ancín-Azpilicueta, C., Gandía, L.M. (2020). Fruit and Vegetable Waste Management: Conventional and Emerging Approaches. *Journal of Environmental Management, 265*, 110510.

Gupta, A., Sharma, R., Sharma, S., Singh, B. (2019). Oilseed as Potential Food Ingredient. In Trends and Prospects in Foods Technology, Processing and Preservation (pp.191-215). Delhi, India: Today & Tomorrow's Printers and Publishers.

Hamed, I., Jakobsen, A.N., Lerfall, J. (2022). Sustainable Edible Packaging Systems Based on Active Compounds from Food Processing Byproducts. *Comprehensive Reviews in Food Science and Food Safety, 21*, 198–226.

Jang, S.-A., Lim, G.-O. Song, K.B. (2011). Preparation and Mechanical Properties of Edible Rapeseed Protein Films. *Journal of Food Science, 76*, C218-C223.

Kadam, D., Shah, N., Palamthodi, S., Lele, S.S. (2018). An investigation on the effect of polyphenolic extracts of N. sativa seedcake on physicochemical properties of chitosan-based films. *Carbohydrate Polymers, 15*, 347–355.

Mirpoor, S.F., Zannini, D., Santagata, G., Giosafatto, C.V.L. (2024). Cardoon seed oil cake proteins as substrate for microbial transglutaminase: Their application as matrix for bio-based packaging to extend the shelf-life of peanuts. *Food Hydrocolloids, 147*, 109339.

Petraru, A., Amariei, S. (2020). Oil Press-Cakes and Meals Valorization through Circular Economy Approaches: A Review. *Applied Sciences, 10*, 7432.

Petraru, A., Amariei, S. (2022). Recovery of Bioactive Compounds From Oilcakes—A Review. *Food and Environment Safety Journal, 21*, 364–381.

Petraru, A., Amariei, S.A. (2023). Novel Approach about Edible Packaging Materials Based on Oilcakes—A Review. *Polymers, 15*, 3431.

Phan The, D., Debeaufort, F., Luu, D., Voilley, A. (2008). Moisture barrier, wetting and mechanical properties of shellac/agar or shellac/cassava starch bilayer bio-membrane for food applications. *Journal of Membrane Science, 325*, 277-283.

Popović, S., Peričin, D., Vaštag, Ž., Popović, L., Lazić, V. (2011). Evaluation of edible film-forming ability of pumpkin oil cake; effect of pH and temperature. *Food Hydrocolloids, 25*, 470–476.

Šuput, D., Pezo, L., Rakita, S., Spasevski, N., Tomičić, R., Hromiš, N., Popović, S. (2024a). Camelina sativa Oilseed Cake as a Potential Source of Biopolymer Films: A Chemometric Approach to Synthesis, Characterization, and Optimization. *Coatings, 14*, 95.

Šuput, D., Popović, S., Hromiš, N., Pantić, J., Lončar, B., Pezo, L. (2023b). Shellac resin effect on the properties of zein film. *Journal on Processing and Energy in Agriculture, 27*, 53-57.

Šuput, D., Popović, S., Hromiš, N., Rakita, S., Spasevski, N., Lončar, B., Erceg, T., Knežević, V. (2023a). The influence of oil cake granulation and ultrasonic pretreatment on the properties of biopolymer films based on Camelina sativa oilseed cake. *Food and Feed Research, 50*, 61-75.

Šuput, D., Popović, S., Ugarković, J., Hromiš, N,. (2022). Influence of synthesis on composite/laminated starch-gelatine based biopolymer film properties. *Analecta Technica Szegedinensia, 16*, 50-55.

Šuput, D., Rakita, S., Spasevski, N., Tomičić, R., Dragojlović, D., Popović, S., Hromiš, N. (2024b). Dried Beetroots: Optimization of the Osmotic Dehydration Process and Storage Stability. *Foods, 13*, 1494.

Sydor, M., Kurasiak-Popowska, D., Stuper-Szablewska, K., Rogozinski, T. (2022). Camelina sativa. Status quo and future perspectives. *Industrial Crops and Products, 187*, 115531.

Toma, C.C., Olah, N.K., Vlase, L., Mogosan, C., Mocan, A. (2015). Comparative studies on polyphenolic composition, antioxidant and diuretic effects of Nigella sativa L. (Black Cumin) and Nigella damascena L. (Lady-in-a-Mist) seeds. *Moleculles, 26*, 9560–9574.

USDA (2023). Oilseeds: World Markets and Trade. United States Department of Agriculture Foreign Agricultural Service. Available online: https://www.fas.usda.gov/data/oilseeds-world-markets-and-trade

Vaštag, Ž., Popović, Lj., Popović, S., Krimer, V., Peričin, D. (2011). Production of enzymatic hydrolysates with antioxidant and angiotensin- I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. Food Chemistry, 124, 1316–1321.

THE USE OF ESSENTIAL OILS AND ESSENTIAL OIL NANOEMULSIONS IN THE DEVELOPMENT OF EDIBLE PECTIN-BASED COATINGS

Jasna Halambek[1]*, Sandra Zavadlav[1], Danijela Bursać Kovačević[2]

[1]Karlovac University of Applied Sciences, Trg J.J. Strossmayera 9, 47000 Karlovac, Croatia
[2]Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia

*Corresponding author:
E-mail address: jasna.halambek@vuka.hr

ABSTRACT

The increased demand for food that is nutritionally rich and safe for consumption is influencing the use of different methods and materials to extend its shelf life. The increased demand for food that is nutritionally rich and safe for consumption affects the use of different methods and materials to extend its shelf life. Today's use of plastic and similar packaging material results in large amounts of waste that later creates environmental problems. Therefore, the increasing use of natural and biodegradable coatings and films is being examined today extensively. The edible coatings have long been used to provide natural casing materials for meat products, to delay respiration rate and moisture loss of fruits and vegetables, and to prevent diffusion of moisture and oxygen into nuts. These coatings can be enriched with other additives, which affect the improvement of aroma, colour, taste, smell and appearance of food. Moreover, they have more functional properties than classic packaging because they can contain different bioactive substances, so they can have antimicrobial and antioxidant properties, as well as probiotic activity. An important source of biologically active components are essential oils, which are increasingly used in the food industry, especially because of their antimicrobial effects, and as a natural preservative. Pectin is a natural thickener, stabilizer and gelling agent, and due to its extremely good water solubility and gelling ability, it belongs to the most commonly used hydrocolloid in the production of edible coatings. Pectin coatings have good barrier properties for gases, are environmentally friendly, biodegradable, available and cheap. Incorporation of essential oils into the pectin-based coatings, can create active packaging systems, which provide more efficient preservation and shelf life of fresh and processed food items. The aim of this paper is the review of the possible use of essential oils in development of pectin-based edible coatings. Moreover, the paper will also refer to the application of essential oil nanoemulsions as a new field of study for the development of edible coatings and films which can extend the shelf life of food.
Keywords: edible coatings, essential oils, food preservation, pectin.

INTRODUCTION

The main benefit of any packaging material is protecting food from environmental factors such as heat, moisture, oxygen, enzymes, unpleasant odour components, as well as from attack of micro and macro-organisms (Vujković et al., 2007). Various polymer materials, especially those obtained by synthetic means, are most widely used in food packaging, but the majority of polymer materials are obtained from non-renewable resources, and they are not biodegradable or compostable, representing a global environmental problem. For these reasons, the food industry is trying to apply not only biodegradable and environmentally friendly packaging, but packaging in the form of edible coatings. In simple terms, edible coatings represent a thin layer of edible material which can be applied directly to a surface of a food product. The edible coatings have long been used to provide natural casing materials for meat products such as sausages, to delay respiration rate and moisture loss of fruits and vegetables, and to prevent diffusion of moisture and oxygen into nuts (Yemenicioglu et al., 2019). In addition to good barrier properties against water vapor and gases on food products, edible coatings improve the quality of food, extend the shelf life and reduce the consumption of packaging material. Pectin is a natural thickener, stabilizer and gelling agent, and due to its good water solubility and gelling ability, it belongs to the most commonly used hydrocolloid in the production of edible coatings and films. Pectin-based coatings show good

barrier properties for gases, they are easily available, cheap and environmentally friendly (Nisar et al., 2018). Moreover, pectin-based edible coatings can be carriers of bioactive and active compounds, including antioxidants, flavorings and antimicrobial compounds, among others. Very often, these coatings have been used as carriers of plant essential oils (EOs) which are natural flavoring substances with GRAS status (Generally Recognized As Safe by FDA). EOs antimicrobial effects, including antibacterial and antifungal activity, as well as pharmaceutical and therapeutic potentials, have been well documented (Burt, 2004).
However, the required amounts of EOs to impart effective antimicrobial and other properties generally exceed the organoleptic acceptance levels. For that reason, studies have proposed nanoemulsions as a new delivery system for encapsulating and releasing EOs from edible coatings into food products. The use of EOs in nanoemulsion coating stabilizes the bioactivity, provides antimicrobial properties and maintains the effect on flavor, aroma and taste of coated foodstuffs (Pandey et al., 2022). The use of edible coatings in the protection of foods such as fresh fruit and vegetables, but also meat products are increasing today. A lot of research is focused on the possible application of various EOs and their individual components as bioactive ingredients in films and coatings that affect the extended shelf life of packaged food. For this reason, the main goal of this paper is the review of the possible use of EOs in the development of pectin-based edible coatings. Moreover, the paper will also refer to the application of EO nanoemulsions as a new field of study for the development of edible coatings and films which can extend the shelf life of food.

PECTIN-BASED COATINGS WITH ESSENTIAL OILS

The selection of the most appropriate edible materials for coatings in food preservation depends on different factors, such as cost, availability, mechanical properties, transparency, brightness, gas barrier effects and resistance to water and microorganisms (Yemenicioglu et al., 2019) Pectin is a multifunctional polysaccharide widely used in the food industry as a thickening and gelling agent, as well as in edible films or coatings as matrix structuring agents. Pectin-based coatings are excellent barriers of gas exchange such as O_2, CO_2, ethylene, but showed rather poor water barrier properties which can be improved by the addition of hydrophobic additives (e.g., lipids) (Lazaridou and Biliaderis, 2020). Moreover, incorporation of active ingredients (antimicrobials, antioxidants, etc.) into the pectin-based coatings creates an ‘active’ packaging system. The addition of different EOs to such coatings has proven to be desirable, especially if their antimicrobial properties are taken into account (Nisar et al., 2018).
Table 1. shows the most frequently tested EOs or their major compounds that were added to pectin-based edible coatings, as well as the observed effect on packaged food. Table 1. shows that pectin-based edible coatings with the addition of various EOs have been tested the most in the protection of fruits and vegetables, especially fruits such as peaches, strawberries, raspberries, grapes, tomatoes etc. One part also refers to coatings that have been tried to be applied in extending the shelf life of seafood and various fish fillets (Alvarez et al., 2014; Tabatabaei Moradi et al., 2015). Many studies have confirmed that pectin-based edible coatings with EOs can become an alternative to plastic packaging material mostly due to their antimicrobial effects, which influence on prolonged shelf life of coated food (Perez-Vazquez et al., 2023).
However, sometimes the required amounts of EOs to impart effective antimicrobial properties generally exceed the organoleptic acceptance levels (Abdi and Bakshi, 2019). As expected, for this reason, research is increasingly focused on the application of EO nanoemulsions.

Table 1. The effects of essential oils or their major compounds incorporated in pectin-based edible coatings on coated food properties.

EO or its major compound	Pectin solution	Coated food	Effect observed	References
Cinnamon leaf EO	Pectin (3%) + glycerol	Peach (Prunus persica)	Antimicrobial, antioxidant activity. Odour acceptability up to 10 days (5°C)	Ayala-Zavala et al., 2013.
Lemon EO	Orange peel pectin (1.5%)	Table grapes	Prolonged the grapes' shelf life by 35 days. Prevented fungal decay and moisture loss without affecting the grape composition.	Breceda-Hernandez et al., 2020.
Lemon and orange peel EOs	Pectin (1%)	Strawberry Fruit	Increased strawberry shelf life for 12 days compared to the control sample.	Abdi et al., 2017.
Lemon and thyme EOs	Pectin (2%)	Black raspberry	Coating fruits with lemon EO delayed degradation of anthocyanin and vitamin C. In the case of thyme EO, it was concluded that a milder essential oil should be used due to the strong smell of thyme, which affects the final taste of the product	Abdi and Bakshi, 2019.
Clove EO	Citrus pectin (3.5%)	Bream (*Megalobrama ambycephala*)	Maintain the quality of bream fillets and eventually prolong the shelf life of the product up to 15 days. Texture, odour, colour and overall acceptability of coated samples were much better than those of untreated samples.	Nisar et al., 2019.
Oregano EO	Citrus pectin (3%)	Fresh shrimps Cucumber	Pectin-OEO mixtures showed antibacterial effect against food pathogenic and spoilage microorganisms.	Alvarez et al., 2014.
	Pectin (2.25%)	Fresh-cut papaya	Maintain the physicochemical and microbiological quality of the fresh-cut papaya more than the uncoated sample throughout 12 days of storage	Mohd Ridzuan et al., 2022.
	Citrus pectin (3%)	Tomatoes	Antifungal effect, increasing total phenol content and antioxidant activity.	Rodriguez-Garcia et al., 2015.
Lemon and peppermint EO	Pectin (2%)	Rainbow Trout Fillets	Multilayered edible coating with an antimicrobial compound can properly delay the growth of spoilage microorganisms and prolong the shelf life of meat products.	Tabatabaei Moradi et al., 2015.
Citral and eugenol	Pectin (1-2%)	Raspberry fresh fruit	Fresh raspberry can be stored for at least 7 days at 4°C, when edible coatings of PE are enriched with citral and eugenol at concentrations (0.1 and 0.15%, respectively).	Guerreiroa et al., 2015.
Garlic EO	Pectin + gelatin (3%) 50:50 (v/v)	Red Chili	Inhibition of weight loss for 14 days, extend red chili storage life up to the 14 days at 29°C.	Heristika et al., 2023.

PECTIN-BASED COATINGS WITH ESSENTIAL OIL NANOEMULSIONS

The increased consumer demand for natural food preservation has influenced the development of mild preservation technology which will improve the food quality and safety without causing nutritional and sensory loss (Abdi et al., 2017). One of the ways in which the aforementioned properties of food can be influenced is the use of EO nanoemulsions in the production of pectin-based edible coatings. Equally, the use of EOs nanoemulsions instead of synthetic food preservatives such as potassium sorbate, sulphites, or nitrites is in line with the “clean label” trend. Moreover, the utilization of EOs nanoemulsions in edible films has opened up a new field of study for the development of edible coatings, especially while the most EOs added to edible coatings have negative effects on organoleptic properties of coated foodstuff (Baghi et al., 2023). Because their low stability and intense flavour, EOS use is sometimes restricted. The encapsulating EOs in nanoemulsions helps to overcome these problems by increasing their stability and reducing excessive flavour. Nanoemulsions are oil-in-water or water-in-oil emulsions that aid in improving the physicochemical properties of edible coatings applied on different foodstuffs. For example, using orange peel EO nanoemulsion in a pectin-based edible coating on fresh-cut orange slices improves the quality and sensory characteristics of the product (Radi et al., 2017), or cinammon oil on fresh cut apple (Naqash et al., 2021). Table 2. shows some EOs that were added to pectin-based edible coatings in the form of a nanoemulsion and their observed effects on coated foodstuffs.

Table 2. The effects of some essential oil nanoemulsions incorporated in pectin-based edible coatings on foodstuff properties.

Type of EO nanoemulsions	Type of food	Effect observed	References
Curcumin with garlic EO	Chicken fillet	Increased the shelf life up to 12 days.	Abdou, E.S. et al., 2018.
Curcumin with cinnamon EO	Chicken fillet	Improved sensory characteristics of chicken fillet.	Abdou, E.S. et al., 2018.
Cinnamon oil	Fresh-cut apple	Improved safety and quality. More effective in preventing the proliferation of pathogenic as well as general microflora and enhancing its safety.	Naqash et al., 2021.
Orange peel EO	Fresh-cut orange	Reduced the quality loss of the orange slices and improved the sensory scores during storage. Extended the shelf life at 4°C for 17 days without any undesirable impacts on sensory attributes.	Radi et al., 2017.
Oregano essential oil and resveratrol	Pork loin	Prolonged the shelf-life of pork by minimizing the pH and colour change, retarding lipid and protein oxidation, maintaining meat tenderness, and inhibiting microbial growth.	Xiong et al., 2020.
Cinnamaldehyde	Ground beef	Antimicrobial activities, reduction in yeast populations and preservation of meat color.	Baghi, et al., 2023.

EO nanoemulsions can be added to both edible coatings and films, but previous published scientific papers suggest that they have been studied more as additives in pectin-based edible films than in edible coatings. So far, very few studies have been published that include the use of EO nanoemulsions in pectin-based edible films (Norcino et al., 2020). Besides affecting the reduction of taste and smell of EOs in pectin-based edible coatings, some other advantages of nanoemulsions in edible coatings is that they can help limit the wide

transmission of active components, prevent interactions with other food matrix elements, improve the antibacterial and antioxidant activity, as well as uniformity of edible coatings (Pandey et al., 2022). Also, it has been noted that the transformation of EOS into nanoemulsion increases their antimicrobial effect (Baghi, et al., 2023). Edible coatings with EO nanoemulsions would represent an effective approach to placing active ingredients from EOs on the surface of foods.

CONCLUSIONS

The food industry is forced to replace or at least reduce the use of synthetic polymer materials not only because of their non-degradability and ecological unacceptability, but also because of the increasing demands of consumers focusing on the use of natural substances. One of the ways to achieve this is the use of edible coatings, especially in preserving the sensorial and nutritional characteristics of foodstuffs, and prolonging their shelf life, mainly due to reduced microbiological activity. The application of edible coatings based on pectin with the addition of EOs already has its application in the protection of fruits and vegetables, and various EOs are widely tested as additives to coatings to preserve the durability of meat products, seafood and fish. The main disadvantage of adding EOs to edible coatings is their strong smell and taste, which impair the organoleptic properties of the coated food. To avoid this, research is focused on the application of EO nanoemulsions. EO nanoemulsions in edible coatings can prevent interactions with other elements of the food matrix, improve antibacterial and antioxidant activity, as well as the uniformity of edible coatings, but also increase the antimicrobial effect of EOs. The use of EOs in edible coatings is becoming more and more popular and researchers are targeting new methods of application of edible coatings, especially pectin-based edible coatings with the addition of EO nanoemulsions.

REFERENCES

Abdi, S., Bakhshi, D. (2019). Pectin edible coatings enriched with essential oils of lemon and thyme to improve storage of black raspberry. Journal of Postharvest Technology, 7(3), 18-29.

Abdi, S., Roein, Z., Erfanimoghadam, J., Aziznia, S. (2017). Application of Pectin Coating Containing Essential Oil for Increasing Quality of Strawberry Fruit. Journal of Postharvest Technology, 5(4), 83-94.

Abdou, E.S., Galhoum, G.F., Mohamed, E.N. (2018). Curcumin loaded nanoemulsions/pectin coatings for refrigerated chicken fillets. Food Hydrocolloids, 83, 445-453.

Alvarez, M. V., Ortega-Ramirez, L. A., Gutierrez-Pacheco, M. M., Bernal-Mercado, A.T., Rodriguez-Garcia, I., Gonzalez-Aguilar, G.A., Ponce, A., Moreira M., Roura, S.I., Ayala-Zavala, J.F. (2014). Oregano essential oil-pectin edible films as anti-quorum sensing and food antimicrobial agents. Frontiers in microbiology, 5, 699.

Ayala-Zavala, J.F., Silva-Espinoza, B.A., Cruz-Valenzuela, M.R., Leyva, J.M., Ortega-Ramírez, L.A., Carrazco-Lugo, D.K., Pérez-Carlón, J.J., Melgarejo-Flores, B.G., González-Aguilar, G.A., Miranda, M.R.A. (2013). Pectin–cinnamon leaf oil coatings add antioxidant and antibacterial properties to fresh-cut peach. Flavour and Fragrance Journal, 28, 39–45.

Baghi, F., Ghnimi, S., Dumas, E., Chihib, N.E., Gharsallaoui, A. (2023). Nanoemulsion-Based Multilayer Films for Ground Beef Preservation: Antimicrobial Activity and Physicochemical Properties, Molecules, 28, 4274.

Breceda-Hernandez, T. G., Martínez-Ruiz, N. R., Serna-Guerra, L., Hernández-Carrillo, J. G. (2020). Effect of a pectin edible coating obtained from orange peels with lemon essential oil on the shelf life of table grapes (Vitis vinifera L. var. Red Globe). International Food Research Journal, 27(3), 585 – 596.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. International Journal of Food Microbiology, 94, 223 – 253.

Guerreiroa, A.C., Gagoa, C.M.L., Faleiro, M. L., Miguel, M.G.C., Antunes, M.D.C. (2015). Raspberry fresh fruit quality as affected by pectin- and alginate-based edible coatings enriched with essential oils. Scientia Horticulturae 194, 138–146.

Heristika, W., Ningrum, A., Heli Siti, S., Munawaroh, H., Show, P.L. (2023). Development of Composite Edible Coating from Gelatin-Pectin Incorporated Garlic Essential Oil on Physicochemical Characteristics of Red Chili (Capsicum annnum L.). Gels, 9, 49.

Lazaridou, A. and Biliaderis, C. (2020). Edible Films and Coatings with Pectin. In: Pectin: Technological and Physiological Properties (Kontogiorgos, V., Ed.) 10.1007/978-3-030-53421-9_6.

Mohd Ridzuan, D., Abdullah, N., Muhammad, N. (2022). Effect of Pectin Coating Enriched with Oregano Essential Oil on the Fresh-Cut Papaya Quality. Enhanced Knowledge in Sciences and Technology, 2 (1), 69-78.

Naqash, F., Masoodi, F.A., Ayob, O., Parvez, S. (2021). Effect of active pectin edible coatings on the safety and quality of fresh-cut apple. International Journal of Food Science and Technology, doi:10.1111/ijfs.15059.

Nisar, T., Wang, Z.C., Yang, X., Tian, Y., Iqbal, M., Guo, Y. (2018). Characterization of citrus pectin films integrated with clove bud essential oil: Physical, thermal, barrier, antioxidant and antibacterial properties. International Journal of Biological Macromolecules, 106, 670-680.

Nisar, T., Yang, X., Alim, A., Iqbal, M., Wang, Z.C., Guo, Y. (2019). Physicochemical responses and microbiological changes of bream (Megalobrama ambycephala) to pectin based coatings enriched with clove essential oil during refrigeration. International Journal of Biological Macromolecules, 124, 1156–1166.

Norcino, L.B., Mendes, J.F., Natarelli, C.V.L., Manrich, A., Oliveira, J.E., Mattoso, L.H.C. (2020). Pectin films loaded with copaiba oil nanoemulsions for potential use as bio-based active packaging. Food Hydrocolloids 106, 105862.

Pandey, V. K., Islamb, R. U., Shams, R., Dar, A. H. (2022). Comprehensive review on the application of essential oils as bioactive compounds in Nano-emulsion based edible coatings of fruits and vegetables. Applied Food research, 2, 100042.

Perez-Vazquez, A., Barciela, P., Carpena, M., Prieto, M.A. (2023). Edible Coatings as Alternative to Traditional Food Packaging: Enhancing Fruits and Vegetables Shelf-Life, doi: 10.20944/preprints202309.0554.v1.

Radi, M., Akhavan-Darabi, S., Akhavan, H.R., Amir, S. (2017). The use of orange peel essential oil microemulsion and nanoemulsion in pectin-based coating to extend the shelf life of fresh-cut orange. Journal of Food Processing and Preservation, e13441.

Rodriguez-Garcia, I., Reynaldo Cruz-Valenzuela, M., Silva-Espinoza, B.A., Gonzalez-Aguilar, G.F., Moctezuma, G., Gutierrez-Pacheco, M.M., Tapia-Rodriguez, M.R., Ortega-Ramirez, J.A., Ayala-Zavala, J.F. (2016). Oregano (Lippia graveolens) essential oil added within pectin edible coatings prevents fungal decay and increases the antioxidant capacity of treated tomatoes, Journal of the Science of Food and Agriculture, 96, 3772–3778.

Tabatabaei Moradi, L., Sharifan, A., Larijani, K. (2015). Antimicrobial Activity of Lemon and Peppermint Essential oil in Edible Coating Containing Chitosan and Pectin on Rainbow Trout (Oncorhynchus mykiss) Fillets. Journal of Medical Microbiology and Infectious Diseases, 3 (1-2), 38-43.

Vujković, I., Galić, K., Vereš, M. (2007). Ambalaža za pakiranje namirnica. Tectus, Zagreb, ISBN 978-953-97026-9-2.

Xiong, Y., Li, S., Warner, R.D., Fang, Z. (2020). Effect of oregano essential oil and resveratrol nanoemulsion loaded pectin edible coating on the preservation of pork loin in modified atmosphere packaging. Food Control 114, 107226.

Yemenicioglu, A., Farris, S., Turkyilmaz, M., Gulec, S. (2019). A review of current and future food applications of natural hydrocolloids. International Journal of Food Science and Technology, doi:10.1111/ijfs.14363.

COMPARATIVE ANALYSIS OF MECHANICAL PROPERTIES OF CHITOSAN AND COMPOSITE CHITOSAN-STARCH FILMS WITH ANTHOCYANIN EXTRACTS

Milica Arizanova*, Katerina Stoilova, Vaska Zarkovska, Mishela Temkov, Elena Velickova

Department of Food Technology and Biotechnology, Faculty of Technology and Metallurgy, Ss Cyril and Methodius University in Skopje, Rugjer Boskovic 16, 1000 Skopje, North Macedonia

*Corresponding author:
E-mail address: milica@tmf.ukim.edu.mk

ABSTRACT

The mechanical properties of pure and modified chitosan films are the main focus of this study. Four groups of chitosan films were prepared: control (CH-C), chitosan-starch (CH-ST-C) anthocyanin incorporated chitosan (CH-E), and anthocyanin incorporated chitosan-starch (CH-ST-E). The anthocyanins were obtained from purple cabbage, using solvent extraction. The puncture test and tensile test were performed in order to evaluate Young's modulus, tensile strength, breaking strength, deformation, and elasticity using texture analyser (MicroStable systems). The results indicated that CH-C films had the highest breaking strength (2.405 MPa) and deformation (9.840%), demonstrating superior resistance to applied force compared to other films. On the other hand, CH-ST-E films exhibited the lowest breaking strength (0.199 MPa) and deformation of 8.355%, suggesting significant weakening as the extract was added. CH-E films had a slight improvement in tensile strength (11.094 kPa) over CH-C films (10.082 kPa), whereas CH-ST-E films had the lowest tensile strength (1.226 kPa). Elasticity was highest in CH-ST-E films (14.222%), indicating enhanced flexibility with the addition of anthocyanin, while CH-C films had the highest Young's modulus (148.225 kPa), reflecting their stiffness. The incorporation of anthocyanin extract generally reduced the breaking strength and deformation, but improved the elasticity, especially in chitosan-starch films. These results are significant because they highlight the potential use of these films in flexible packaging systems. The inclusion of anthocyanin is a promising alternative for various packaging applications, as visual freshness or spoilage indicators of food products.
Keywords: *chitosan, chitosan-starch, anthocyanin, mechanical properties*

INTRODUCTION

Food spoilage is a perpetual problem that affects public health, as well as global economy, trade and tourism. Despite the excessive regulations and technological developments, foodborne diseases still remain a problem for consumers. Due to the constant demands of consumers for healthy food, minimally processed food and environmental protection, the development of efficient food packaging systems that will minimize the dangers of spoilage is becoming increasingly important. In this regard, intelligent packaging systems begin to grow (Ma et al., 2022; Khan et al., 2024). Intelligent packaging is a system capable of converting the changes in food products into visual or readable forms. These types of packaging systems can monitor the actual situation of the food and give information about its freshness or degree of spoilage during transportation and storage (Azeredo & Correa, 2021).

Anthocyanins are natural, water-soluble polyphenolic pigments that are present in numerous fruits and vegetables, such as berries, purple cabbage, black rice, purple carrot, eggplant. They have a potential to be used in intelligent packaging systems, due to their ability to change color according to the pH of the environment. Under acidic pH conditions, anthocyanins show red hues and the highest stability. As the pH rises, the color of anthocyanins solutions changes from red to pink, purple, blue, green and yellow in most alkaline pH conditions (Oliviera Filho et al., 2021).

Plastics are dominant in the food packaging market because of their flexibility, low price and recyclability. However, efforts are being made to develop alternative environmentally friendly packaging that will replace conventional plastic packaging (Suzihague & Zamroni, 2019). Biopolymers, such as starch, chitosan, gelatin, alginate etc. are successfully used in intelligent packaging systems, thanks to their biodegradability, edibility, film-forming abilities,

and compatibility with functional additives. This study focuses on chitosan and composite chitosan-starch biofilms and their mechanical properties. The mechanical properties are important because it depends on them whether the film can protect the food product from physical damage and maintain its integrity during storage and transportation.

MATERIAL AND METHODS

The scheme of preparation of CH-E (a) and CH-ST-E (b) is shown in Fig.1.

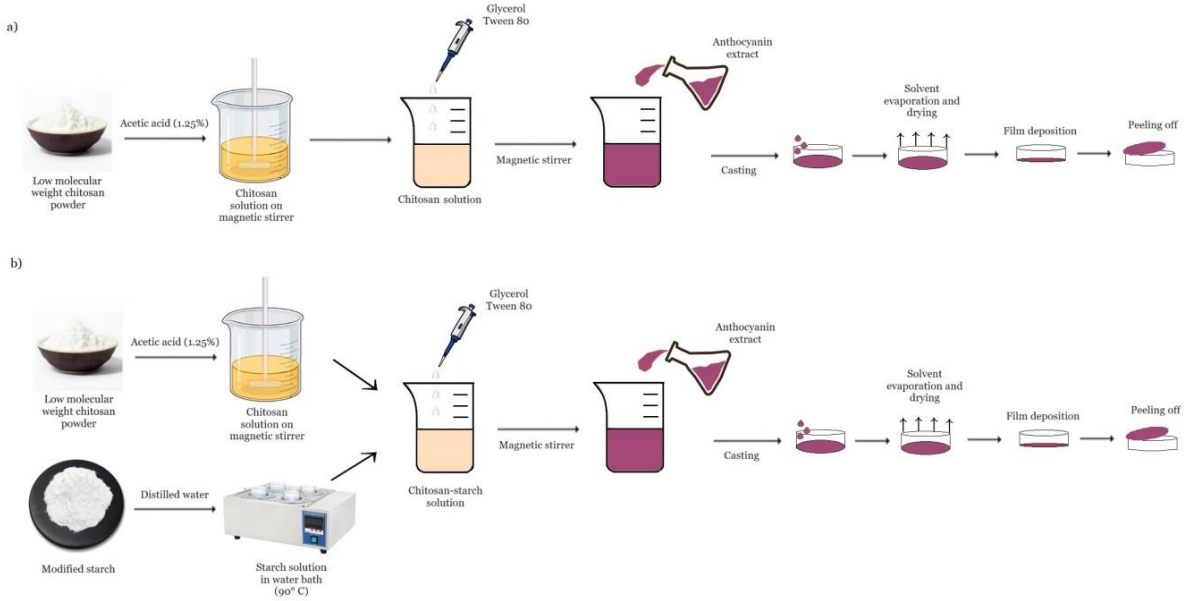


Figure 1. Representative scheme of preparation of CH-E (a) and CH-ST-E (b) biofilms

For preparation of CH-C and CH-E films, 1% chitosan solution was prepared in 1.25% acetic acid. The solution was stirred on a magnetic stirrer until complete dissolution of the chitosan. Then, glycerol and Tween 80 were added at 24% (w/w) in both biofilms. After the addition of the plasticizers, the CH-C solution was casted onto a plastic petri dish. The CH-E solution was made after mixing the CH-C solution with the anthocyanin extract in 4:1 ratio. Both types of films were obtained using the casting method.

The chitosan-starch films were made in such a way that, previously, the starch and chitosan solutions were separately prepared and then combined into one solution. Modified starch powder (1.25% w/v) was mixed with distilled water and incubated for 20 minutes in a water bath at 85°C. The chitosan solution (1%) was prepared in 1.25% acetic acid. For the preparation of CH-ST-C films, chitosan and starch solutions were mixed in 1:1 ratio, with the addition of glycerol (30% w/w) and Tween 80 (30% w/w). The CH-ST-E films were made by combining the chitosan solution, starch solution and anthocyanin extract in 5:3:2 ratio. The plasticizers were also added at 30% (w/w). Casting method was also used for obtaining the CH-ST-C and CH-ST-E films.

Glycerol was added to biofilms to improve their flexibility and elasticity, preventing brittleness and improving handling properties. Tween 80 was used as an emulsifier to enhance the mechanical strength of the biofilms (Yoshida et al., 2008).

The anthocyanin extract from purple cabbage was obtained by solvent extraction, based on previously published procedure with 1% of citric acid in water, with some modifications (Lee & Wrolstad, 2004). The plant material was mixed with 1% citric acid and they were ground in a blender. The mixture was stored on 4°C for 3 hours for continuous extraction, and then it was filtered through filter paper under vacuum.

Samples were cut from each film sample and measured at 10 points for thickness, using Extol Premium Digital Caliper.

In order to determine the breaking strength and deformation, films were cut into five samples with dimensions of 2x2 cm. The penetration was performed with a probe with 2mm diameter, using texture analyser (MicroStable systems). Tensile test was performed using tensile grips, on five samples with dimensions of 7x2 cm to determine the Young's modulus, elasticity and tensile strength of the films. The sample is gripped at either end and stretched until it breaks using tensile grips and test speed of 2 mm/s, according to ASTM D638 test method (ATSM D638, 2014). The performed puncture test (a) and tensile test (b) are shown in Fig.2.

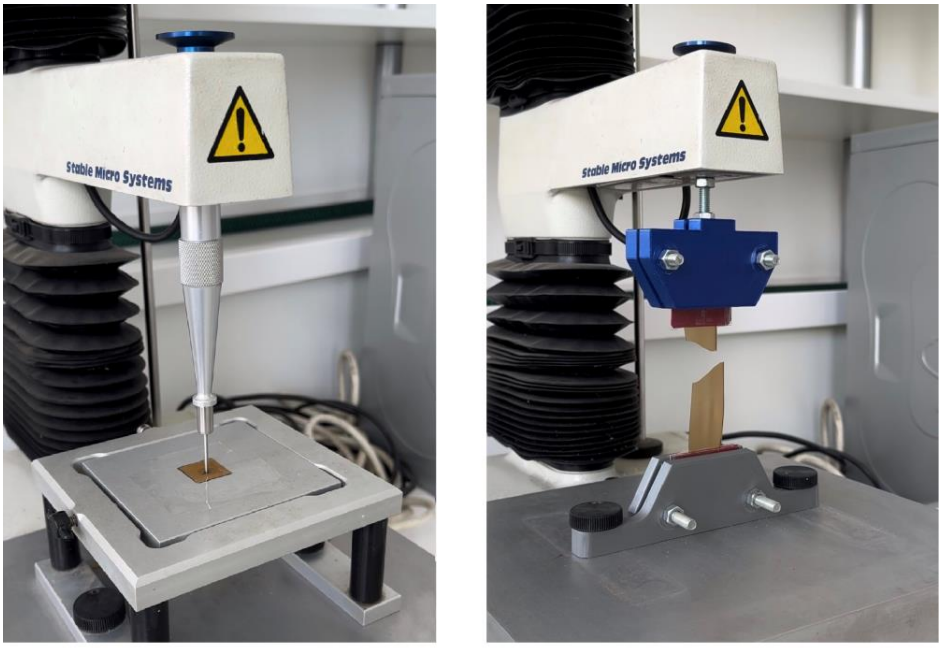


Figure 2. Puncture test (a) and tensile test (b) performed on films

RESULTS AND DISCUSSION

The thickness and the mechanical properties of the four different biofilm samples are shown in Table 1. CH-C and CH-E films had similar thicknesses of 0.056 mm and 0.055 mm, respectively, suggesting that the addition of anthocyanins did not significantly alter the film thickness. The thicker composite films, however, indicate that the addition of starch – more specifically, the combination of starch and anthocyanins – increases the thickness of the film. CH-E films had a slight improvement in tensile strength over CH-C films, whereas CH-ST-E films had the lowest tensile strength. Elasticity was highest in CH-ST-E films, indicating improved flexibility with the addition of anthocyanins. CH-E exhibited the lowest deformation compared to the other biofilms.

Table 1. Thickness, tensile strength, elasticity and deformation of chitosan and composite chitosan-starch biofilms with and without anthocyanins

	CH-C	**CH-E**	**CH-ST-C**	**CH-ST-E**
Thickness (mm)	0.056 ± 0.04	0.055 ± 0.02	0.066 ± 0.03	0.099 ± 0.06
Tensile strength (kPa)	10.082 ± 4.905	11.094 ± 9.810	4.496 ± 3.270	1.226 ± 0.817
Elasticity (%)	7.194 ± 5.333	8.476 ± 10.667	12.875 ± 8.666	14.222 ± 17.833
Deformation (%)	9.840 ± 2.716	2.755 ± 2.357	9.763 ± 5.202	8.355 ± 7.240

Furthermore, the breaking strength (a) and the Young modulus (b) were determined and the results are shown in Fig.3.

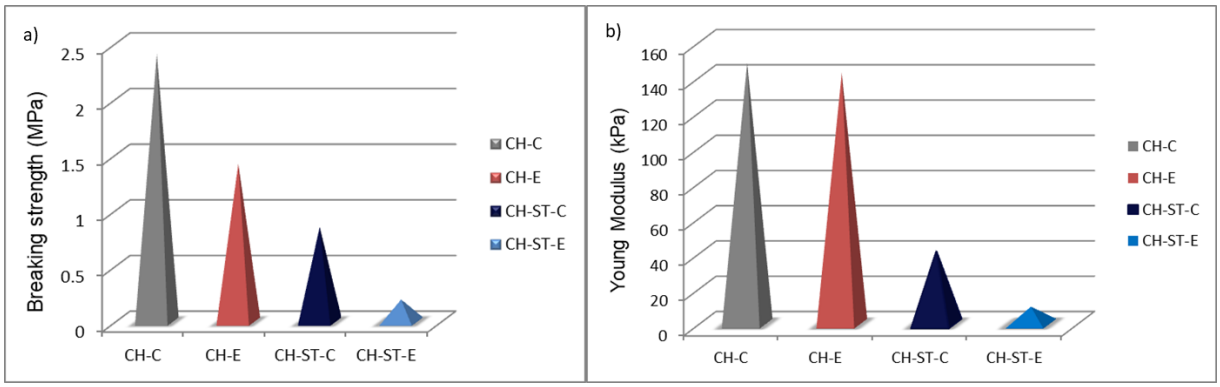


Figure 3. Breaking strength (a) and Young modulus (b)

The chart on the left (a) illustrates that CH-C has the highest breaking strength, followed by the chitosan film containing anthocyanins. The addition of starch significantly reduces the breaking strength, and therefore, it is found that the CH-ST-C exhibits a noticeable decrease. The composite film including both starch and anthocyanins (CH-ST-E) had the lowest breaking strength, which suggests that the presence of anthocyanins contributes to a significant decrease in mechanical resistance.
The chart on the right (b) illustrates the Young's modulus, which measures the rigidity of a material, indicating how much it will deform under a given stress. The lowest Young's modulus in the composite CH-ST-E film indicates that it is more flexible compared to the other films, which is a result of the addition of starch and anthocyanins to the chitosan matrix (Ren et al., 2017). Flexibility in biofilms can be desirable or undesirable, depending on the product that should be packed. For example, biofilms with increased flexibility can be used for packaging of irregularly shaped products. On the other hand, extreme flexibility may compromise the structural integrity and mechanical strength of the packaging, making it less suitable for heavier products during transportation. Therefore, an optimal balance between flexibility and strength is crucial for a successful application (Peelman et al., 2013).

CONCLUSIONS

This research focuses on understanding the changes in mechanical properties due to the film composition, i.e. the addition of anthocyanin extract into biopolymer matrixes. The films of pure chitosan (CH-C) and chitosan with anthocyanins (CH-E) had similar thicknesses, indicating that anthocyanins do not significantly affect the film thickness. However, the addition of starch, especially in combination with anthocyanins, significantly increased the thickness of the films.
Regarding the mechanical properties, CH-E films showed little improvement in tensile strength compared to CH-C, while CH-ST-E had the lowest tensile strength. On the contrary, the highest flexibility was determined in CH-ST-E films, which proves that the addition of starch and anthocyanins increased the flexibility of the films, and this was confirmed with the lowest Young's modulus of the CH-ST-E films.
The lowest deformation was observed for CH-E films. In addition, the breaking strength was highest in CH-C films, while the addition of starch significantly decreased the breaking strength, especially in CH-ST-E films.
Overall, the results show that the addition of starch and anthocyanins to the chitosan matrix increased the thickness and flexibility of the films, but decreased their mechanical resistance. Proper optimization of the film's composition is very important for a successful use in food packaging systems. Combination with other materials or the addition of some additives can improve the mechanical properties of biofilms, which can lead to the production of quality, biodegradable, non-toxic and environmentally friendly packaging systems.

For a practical application of these films in food packaging, future research should focus on optimizing the combination of chitosan, starch, and anthocyanins to achieve a balance between flexibility and mechanical resistance. Furthermore, research into the biodegradability and cost-effectiveness would encourage the transition from conventional to environmentally friendly packaging alternatives.

REFERENCES

American Society for Testing and Materials D638-14 (2014) Standard Test Method for Tensile Properties of Plastics. ASTM International, West Conshohocken.

Azeredo, H.M.C., Correa, D.S. (2021) Smat choices: Mechanisms of intelligent food packaging. *Food Science, 4,* 932-936

Khan, S., Monteiro, J.K., Prasad, A., Filipe, C.D.M., Li, Y., Didar, T.F. (2024). Material Breakthroughs in Smart Food Monitoring: Intelligent Packaging and On-Site Testing Technologies for Spoilage and Contamination Detection. *Advanced Materials, 36*, 2300875

Lee, J., & Wrolstad, R. E. (2004). Extraction of anthocyanins and polyphenolics from blueberry processing waste. *JFS C:Food Chemistry and Toxicology, 69(7)*, C564–C573

Ma, Y., Yang, W., Xia, Y., Xue, W., Wu, H., Li, Z., Zhang, F., Qiu,B., Fu, C. (2022). Properties and Applications of Intelligent Packaging Indicators for Food Spoilage. *Membranes*, *12*, 477.

Oliviera Filho, J.G., Braga, A.R.C., Ribiero de Oliviera, B., Gomes, F.P., Moreira, V.L., Pereira, V.A.C., Egea, M.B. (2021). The potential of anthocyanins in smart, active, and bioactive eco-friendly polymer-based films: A review. *Food Research International, 142*, 110202

Peelman, N., Ragaert, P., De Meulenaer, B., Adons, D., Peeters, R., Cardon, L., Van Impe, F., Devlieghere, F. (2013). Application of bioplastics for food packaging. *Trends in Food Science & Technology*, 32(2), 128-141.

Ren, L., Yan, X., Zhou, J., Tong, J., Su, X. (2017). Influence of chitosan concentration on mechanical and barrier properties of corn starch/chitosan films. *International Journal of Biological Macromolecules, 105,* 1636-1643

Suzihaque, M.U.H, & Zamroni, N.F. (2019). Development of Intelligent Food Packaging fromRed Cabbage Anthocyanin Pigment. *International Journal of Recent Technology and Engineering, 8(4),* 6798-6802

Yoshida, C.M.P., Oliveira Junior, E.N., Franco, T.T. (2008). Chitosan tailor-made films: the effects of additives on barrier and mechanical properties. *Packaging Technology and Science, 22(3),* 161-170

COMPARISON OF PROPERTIES BETWEEN STARCH-GELATIN FILMS WITH AND WITHOUT ANTHOCYANIN EXTRACT DERIVED FROM PURPLE CABBAGE

Milica Arizanova*, Marija Gligorova, Mishela Temkov, Elena Velickova

Department of Food Technology and Biotechnology, Faculty of Technology and Metallurgy, Ss Cyril and Methodius University in Skopje, Rugjer Boskovic 16, 1000 Skopje, North Macedonia

*Corresponding author:
E-mail address: milica@tmf.ukim.edu.mk

ABSTRACT

This study is focused on characterization of composite starch-gelatin films with and without the addition of anthocyanin derived from purple cabbage, aiming at developing intelligent packaging system that can be used to monitor food quality. The comparison is made between two films: control composite starch-gelatin films (ST-GEL-C) and anthocyanin incorporated composite starch-gelatin films (ST-GEL-E). A comprehensive analysis was conducted to determine the thickness of the films, moisture content, optical properties, mechanical properties, degree of swelling, solubility and WVP.
The results demonstrated that the incorporation of anthocyanins significantly affected the properties of the starch-gelatin films. The inclusion of anthocyanin extract resulted in altered transparency, absorbance and transmission spectra, which highlights the potential use of these films as visual spoilage indicators. The changed color of the films at different pH (2-12) was also monitored and photographed and it showed that this film can be successfully used as intelligent packaging. Additionally, the mechanical properties and water vapor permeability of the films were assessed to determine their suitability for food packaging applications. ST-GEL-E films displayed higher tensile strength and elasticity, as well as thickness. The findings suggest that anthocyanin-enriched films not only provide enhanced functional properties but also offer a promising approach for intelligent packaging solutions.
Keywords*: starch-gelatin, intelligent packaging, anthocyanin*

INTRODUCTION

The rapid spoilage of food is a major concern in the food industry. It leads to significant economic losses and health risks associated with foodborne pathogens. The food packaging industry is moving towards creating new packaging systems that will be able to monitor the state of the food at any point in time (Khan et al., 2024).
The aim of this research is to develop and characterize a new packaging system that is based on starch and gelatin. Starch and gelatin are biodegradable, edible, film forming biopolymers that have the potential to be combined with anthocyanin extracts to create an intelligent packaging. Intelligent packaging systems are capable of converting the changes in food products into visual or readable forms and providing the consumer information about the freshness of the food. Anthocyanins are water soluble pigments that can change their color according to the pH of the environment. They can be present in various fruits and vegetables, such as berries, pomegranate, purple cabbage, cherries etc. Incorporated in a biopolymer film, anthocyanins have a promising potential to be used as pH indicators, which can notify the consumer that the spoilage of food products has begun (Rawdkuen et al., 2020). This can be beneficial in more ways. First, if the product isn't stored properly it will start deteriorating faster, and this change can be caught by the indicator. On the other hand, the indicator can show that the food is safe for consumption even after the expiration date and this can contribute to minimizing the food waste.
Purple cabbage is a rich source of anthocyanins and because of its abundance and low cost it has received a lot of attention from researchers (Abedi-Firoozjah et al, 2022). Anthocyanin extract derived from purple cabbage has been used in combination with plenty of biopolymers, such as starch/polyvinyl alcohol, chitosan/polyvinyl alcohol, gelatin, chitosan/corn starch etc (Zhang et al., 2020, Dang & Chen, 2019, Musso et al., 2019, Silva-

Pereira et al., 2015). It has a lot of potential to be used in intelligent packaging systems due to its distinct color change according to pH of the environment.
This study aims to develop a new composite film by combining starch, gelatin and anthocyanin extract from purple cabbage. By comparing the control starch-gelatin films (ST-GEL-C) with anthocyanin-incorporated films (ST-GEL-E), this research aims to explore the impact of anthocyanins on the films' physical, optical, and mechanical properties, and their ability to function as a spoilage indicator.

MATERIALS AND METHODS

Preparation of the extract and biofilms

The anthocyanin extract from purple cabbage was obtained by solvent extraction. The plant material was mixed with distilled water acidified with 2% citric acid and they were ground in a blender. The mixture was stored on 4°C for 3 hours for continuous extraction, and then it was filtered through filter paper under vacuum.
Starch powder (3% w/v) and gelatin powder (3% w/v) were separately mixed with distilled water and incubated for 10 minutes in a water bath at 85°C. Before merging the two solutions in 1:1 ratio, they were stirred continuously on a magnetic stirrer for 15 minutes. The control sample was made by mixing the gelatin and starch solutions on a magnetic stirrer for 2 hours, with the addition of glycerol as plasticizer. The sample with incorporated extract was made by mixing the two solutions, with the addition of extract (14% v/v) and glycerol. The plasticizer was added at 6.67% (w/w) in both biofilms. The films were obtained using the casting method.

Characterization of the extract and biofilms

The color changes of the extract were photographed at different pH values (2-12). The pH of the sample was adjusted using 1M HCl and 1M NaOH. The total anthocyanin content in the extract was expressed as cyanidin-3-glucoside equivalents as in Equation 1 (Le et al., 2019).

$$\text{Anthocyanin pigment}\left(\frac{\text{mg}}{\text{L}}\right) = \frac{\text{A}\times\text{MW}\times\text{DF}\times\text{V}\times 1000}{\text{a}\times\text{l}\times\text{m}} \quad [1]$$

where *A* is the absorbance, MW is the molecular weight of cyanidin-3-glucosode (449.2 g/mol), DF is the dilution factor, V is the solvent volume (mL), *a* is the molar absorptivity (26,900 $L\cdot mol^{-1}\cdot cm^{-1}$), and l is the cell path length (1 cm).
The pH-sensitivity of the anthocyanins-rich films were determined by immersing the films in different buffer solutions (2-12) and the changes of the color were photographed. Samples were cut from each film type and measured at 10 points for thickness, using Extol Premium Digital Caliper. Moisture content was measured using the AOAC 934.01 method on five samples cut into 2x2 cm (AOAC, 2000). The films' moisture content was calculated using Equation 2.

$$\text{Moisture content}\,(\%) = \frac{(\text{W}_\text{i}-\text{W}_\text{f})}{\text{W}_\text{i}} \times 100 \quad [2]$$

where w_i – initial weight of the films, w_f – weight of the dried film.
Water vapor permeability (WVP) measurements were done in accordance with a modified ASTM E96-80 method with 3 samples for each type of film (ASTM, 1989). The films were sealed onto a glass cell with 6 cm diameter and 1 cm depth containing distilled water. The cell was placed in an environmental chamber with 50% relative humidity at 30°C and it was measured every hour over a 24 hour period. Both temperature and relative humidity were also measured every hour. The degree of swelling (DS) and water solubility (WS) of the films were determined by soaking a previously weighted film in distilled water for 1 hour. The weight of the wet film was determined by removing the excess water from the film using

filtered paper and measuring the weight immediately. The films were then dried for 24 hours at 100°C until constant temperature was reached. Equations 3 and 4 were used for calculation of DS (%) and WS (%), respectively.

$$\%DS = \frac{w_e - w_0}{w_0} \times 100 \quad [3]$$

$$\%SW = \frac{w_0 - w_f}{w_0} \times 100 \quad [4]$$

where w_e is the weight of the films at absorbing equilibrium, w_o is the initial dry weight of the films and w_f is the dry weight of the films after the drying process.
Optical properties of the films were determined using UV-VIS spectroscopy. Three samples with dimensions of 2x1 cm were used to determine absorbance at 600 nm and transmission from 200-800 nm. The transparency of the films was calculated using Equation 5 (Rodrigues et al., 2014):

$$\%T = \frac{A_{600}}{\partial} \quad [5]$$

where ∂ is thickness of the film (mm), A_{600} is the absorbance measured at 600 nm.
Films were cut into five samples with dimensions of 2x2 cm to determine the breaking strength and deformation. The penetration was performed with a probe with 2mm diameter and 1 mm/s test speed, using texture analyser (MicroStable systems). Tensile test was performed on five samples from each formulation with dimensions of 7x2 cm and average thickness of 0.66 mm for control samples and 0.105 mm for anthocyanin incorporated samples to determine the Young's modulus, elasticity and tensile strength of the films. The sample is gripped at either end and stretched until it breaks using tensile grips and test speed of 2 mm/s, according to ASTM D638 test method (ATSM D638, 2014).

RESULTS AND DISCUSSION

The color change of the obtained extract is shown in Figure 1.

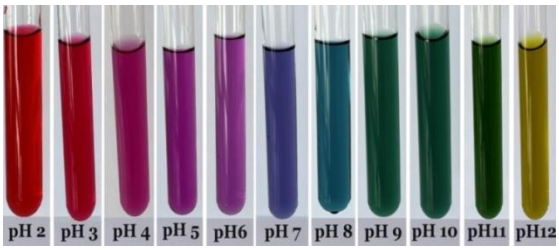


Figure 1. Color change of the anthocyanin extract under different pH

The color change of the anthocyanins-rich films according to the pH is shown in Figure 2. The noticeable color change highlights the potential of these films as intelligent food packaging systems, because the spoilage of food products such as meat, fish, etc. can be detected by the color change of the films.

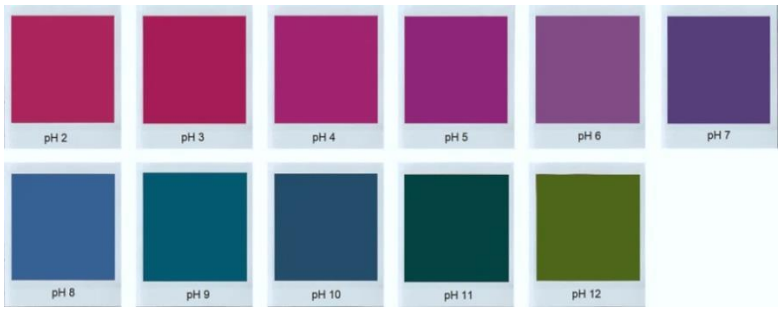


Figure 2. Color changes of the St-Gel-E films under different pH (2-12)

The physical properties, such as moisture content (MC), degree of swelling (DS), water solubility (WS), thickness (*∂*) and water vapor permeability (WVP) of the control and anthocyanin – incorporated films are shown in Table 1.

Table 1. Physical properties of St-Gel-C and St-Gel-E films

Physical properties	**Biopolymer films**	
	ST-GEL-C	**ST-GEL-E**
MC (%)	6.22 ± 2.86	9.32 ± 3.33
DS (%)	-	-
WS (%)	-	-
∂ (mm)	0.066 ± 0.03	0.105 ± 0.06
WVP(10−11 mol·m/m^2·s·Pa)	1.57 ± 0.88	3.23 ± 0.72

The ST-GEL-E film has a higher moisture content compared to the ST-GEL-C film, due to the hydrophilic nature of the anthocyanins, which retain more moisture within the film. The determination of DS and WS wasn't possible as both control and anthocyanin – incorporated films dissolved in the water after 1 hour of immersion. Having this into consideration, the anthocyanin-incorporated films can be used for packaging of solid foods, such as meat, fish etc. As meat or fish begins to spoil, various volatile compounds are released, such as ammonia, hydrogen sulfide, and other amines. These compounds, driven by vapor pressure, come into contact with the packaging film which reacts to the volatile compounds and changes color, indicating that the food has spoiled.

The ST-GEL-E film has a higher WVP, meaning it is more permeable to water vapor compared to the ST-GEL-C film. The increased permeability could be due to the structural changes or the hygroscopic nature of the anthocyanins. The addition of anthocyanin extract also alters the thickness of the film, making the ST-GEL-E thicker compared to the control films.

ST-GEL-E films showed higher transparency (2.98%) compared to ST-GEL-C films (1.75%). Furthermore, the comparison between films' transmission is shown in Figure 3. The addition of anthocyanins in ST-GEL-E decreases the transmission by absorbing more light. This results in the ST-GEL-E film having lower light transmission compared to the control film.

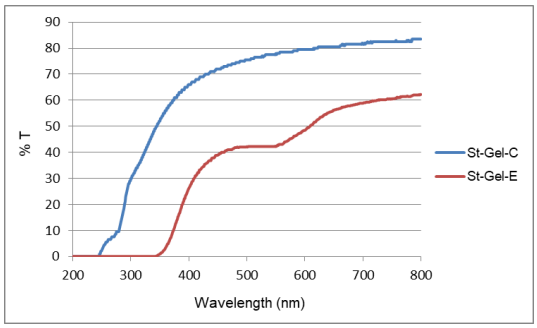


Figure 3. Transmission of the films

The mechanical properties, such as tensile strength, elasticity, deformation, breaking strength and Young's modulus are shown in Table 2.

Table 2. Mechanical properties of St-Gel-C and St-Gel-E films

Mechanical properties	**Biopolymer films**	
	ST-GEL-C	**ST-GEL-E**
Deformation (%)	0.816 ± 1.623	2.222 ± 3.453
Tensile strength (kPa)	5.177 ± 13.897	17.576 ± 18.802
Elasticity (%)	2.444 ± 3.833	5.750 ± 2.833
Breaking strength (MPa)	0.843 ± 0.772	3.159 ± 5.867
Young's modulus (kPa)	266.735 ± 409.378	335.576 ± 528.733

The higher breaking strength in the ST-GEL-E film suggests that the anthocyanins contribute to a stronger and more resilient film structure. However, the addition of the anthocyanins results in a higher Young modulus, which shows that St-Gel-E is less flexible and prone to deformations. The higher tensile strength in the anthocyanin – incorporated film suggests that ST-GEL-E is stronger and more resistant to breaking under tension. Despite having a higher Young's modulus, the ST-GEL-E film shows higher deformation percentage, meaning it can stretch more before breaking. This might indicate that even though ST-GEL-E film is stiffer, it can still undergo more stretching before breaking, possibly due to a combination of factors provided by the presence of the anthocyanins. The elasticity in ST-GEL-E film is higher, which shows that this film can recover its shape better than ST-GEL-C.

CONCLUSIONS

This research presents the potential of anthocyanin incorporated films to be used as intelligent packaging systems. Because the films are made with biodegradable materials, they can replace the traditional plastic packaging while also reducing plastic waste. In addition, the anthocyanin extract is obtained from purple cabbage, which makes this packaging safer and more environmentally friendly. The purple cabbage extract is a food-grade material, meaning it is safe for use in applications that come into direct contact with food. This is particularly important in packaging, where safety is a top priority. Using this packaging helps consumers become more conscious of food safety and sustainability in addition to its practical uses. Customers are more likely to support eco-friendly packaging when these films are included into common food products, such as meat, fish etc.

ST-GEL-E films showed increased tensile strength and thickness, which meet the practical requirements for food packaging. Furthermore, ST-GEL-E films showed altered optical properties, such as changes in transparency, absorbance, and transmission spectra, all of which are essential for their role as visual indicators of food spoilage. The observed pH-sensitive color changes further confirm the potential of these films to be used as effective spoilage indicators, providing real-time information about food quality.

REFERENCES

Abedi-Firoozjah, R., Yousefi, S., Heydari, M., Seyedfatehi, F., Jafarzadeh, S., Mohammadi, R., Rouhi, M., Garavand, F. (2022) Application of Red Cabbage Anthocyanins as pH-Sensitive Pigments in Smart Food Packaging and Sensors. *Polymers, 14,* 1629

American Society for Testing and Materials D638-14 (2014) Standard Test Method for Tensile Properties of Plastics. ASTM International, West Conshohocken.

Association of Official Agricultural Chemists (AOAC) (2000). *Official Methods of Analysis of AOAC International* (17th ed.). Gaithersburg, MD: AOAC International

Dang, T.-H., Chen, B.-H. (2019) Synthesis of intelligent pH indicative films from chitosan/poly(vinyl alcohol)/anthocyanin extracted from red cabbage. *Polymers, 11,* 1088.

Khan, S., Monteiro, J.K., Prasad, A., Filipe, C.D.M., Li, Y., Didar, T.F. (2024). Material Breakthroughs in Smart Food Monitoring: Intelligent Packaging and On-Site Testing Technologies for Spoilage and Contamination Detection. *Advanced Materials, 36*, 2300875

Le, T., Huynh, M.P., Tri Nhut, T., Van T., Quoc Toan, T., Bach, L.G., Trung, N. (2019). Optimization of Total Anthocyanin Content, Stability and Antioxidant Evaluation of the Anthocyanin Extract from Vietnamese Carissa Carandas L. Fruits. *Processes. 7.* 468.

Musso, Y.S., Salgado, P.R., Mauri, A.N. (2019) Smart gelatin films prepared using red cabbage (Brassica oleracea L.) extracts as solvent. *Food Hydrocolloids, 89*, 674–681.

Rawdkuen, S., Faseha, A., Benjakul, S., Kaewprachu, P. (2020). Application of anthocyanin as a color indicator in gelatin films. *Food Bioscience, 36,* 100603

Rodríguez, J., Madera-Santana, T., Sánchez-Machado, D., Lopez-Cervantes, J., Valdez, H. (2014). Chitosan/Hydrophilic Plasticizer-Based Films: Preparation, Physicochemical and Antimicrobial Properties. *Journal of Polymers and the Environment. 22.*

Silva-Pereira, M.C., Teixeira, J.A., Pereira-Júnior, V.A., Stefani, R. (2015) Chitosan/corn starch blend films with extract from Brassica oleraceae (red cabbage) as a visual indicator of fish deterioration. *LWT - Food Science and Technology, 61,* 258–262.

Zhang, K., Huang, T.-S., Yan, H., Hu, X., Ren, T. (2020) Novel pH-sensitive films based on starch/polyvinyl alcohol and food anthocyanins as a visual indicator of shrimp deterioration. *International Journal of Biological Macromolecules*, 145, 768–776.

PRESERVING TRADITIONAL CULTIVAR ”DUGA BELA” PEPPER: THE EFFICACY OF HOT WATER DIPPING

Renata Kovač[1]*, Žarko Kevrešan[1], Zoran Ilić[2], Lidija Milenković[2], Ljiljana Tubić[3], Dragana Ubiparip Samek[1], Marina Đerić[1]

[1]Institute of Food Technology in Novi Sad, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia
[2]Faculty of Agriculture Priština in Lešak, University of Priština in Kosovska Mitrovica, 38219 Lešak, Serbia
[3]Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”- National Institute of the Republic of Serbia, University of Belgrade, Bulevar Despota Stefana 142, 11108 Belgrade, Serbia

*Corresponding author:
E-mail address: renata.kovac@fins.uns.ac.rs

ABSTRACT

The "Duga bela" pepper, a traditional Serbian cultivar, is highly valued for its unique flavor and cultural significance, but faces challenges in maintaining quality during postharvest storage. This study investigates the efficacy of hot water dipping (HWD) as a postharvest treatment to enhance the shelf life and preserve the quality of "Duga bela" peppers. Fuits were subjected to three different storage conditions: recommended storage at 10 ºC, cold storage at 4 ºC, and HWD at 55 ºC for 1 minute followed by cold storage at 4 ºC. The combination of HWD and 4 ºC storage, significantly reduced chilling injury and decay, maintained better color intensity (ASTA units), and preserved quinic and succinic acid content compared to the other treatments. Notably, HWD-treated peppers displayed the best overall quality and marketability after 21 days of storage and 3 days at shelf life. The findings underscored the potential of HWD as an effective postharvest treatment for traditional pepper cultivars, supporting both quality preservation and the extension of marketability. This approach may offer a practical solution for the postharvest management of culturally significant agricultural products.
Keywords*: Capsicum annuum, quality*, *postharvest treatments, shelf life*

INTRODUCTION

Peppers (*Capsicum annuum* L.) are a crucial ingredient of Balkan culinary heritage, prized for their nutritional value, flavor, and cultural significance. Fresh peppers are particularly susceptible to postharvest losses due to water loss, chilling injury, and fungal diseases (Ilić et al., 2013). Traditional cultivars, often characterized by unique flavor profiles and cultural importance, face additional challenges in meeting modern market demands for uniformity and extended shelf life (Ilić et al., 2013). These local populations and traditional cultivars, grown in yards and small local gardens are highly heterogeneous, differing in shape, size, aroma, and heat intensity. The "Duga bela" pepper, a distinctive Serbian cultivar, is renowned for its aromatic flavor and versatility in traditional cuisine. However, this pepper suffers from postharvest quality deterioration when subjected to standard storage conditions. Rapid cooling of peppers and maintaining them at optimal storage conditions of 7-10 °C and 95-98 % humidity can effectively preserve their quality (Kader, 2002). However, in small-scale productions of traditional varieties, optimal conditions are not always accessible. Hot water dipping (HWD) has emerged as a promising postharvest treatment for enhancing pepper shelf life (Fallik & Ilić, 2017). By reducing chilling injury and microbial growth, HWD can potentially preserve the quality attributes of traditional cultivars like "Duga bela" pepper (Fallik & Ilić, 2017).
This study investigated the efficacy of HWD in maintaining the quality of "Duga bela" peppers during storage. By comparing recommended storage condition (at 10 °C) and storage at low temperature (at 4 °C) with and without HWD, we aimed to optimize postharvest management practices for this valuable traditional crop.

MATERIAL AND METHODS

Plant Material and Cultivation

Traditional "Duga bela" pepper plants were cultivated in the Aleksinac region (South Serbia) using standard agricultural practices. Seedlings were prepared in a greenhouse and transplanted to the field on June 8, 2023. Crop management included irrigation, fertilization, pest control, and disease prevention. Morphologically, the “Duga Bela” variety was characterized by a plant height ranging from 50 to 60 cm and the production of 8 to 12 fruits per plant. The fruits weighed between 140 and 160 grams, with an average length of 20.7 cm and a width of 5.5 cm. The fruits were harvested at the stage of physiological maturity.

Experimental Design

Harvested peppers were randomly assigned to three experimental groups: a) fruits stored at recommended temperature (10 ± 2 °C); b) cold storage at 4 °C; and c) hot water dipping (HWD) at 55 °C for 1 minute, and stored at 4 °C. Approximately 25 kg of peppers per group were placed in wooden crates and stored for 21 days in a pilot facility for fruit and vegetable storage. Subsequently, samples were transferred to shelf life conditions (24 ± 2 °C) for an additional 3 days.

Fruit Loss

Fruit loss was assessed two times a week by measuring weight loss (g) for each treatment after the harvest, cold storage and shelf life using a technical balance (Kern 572-35, Kern & Sohn, GmbH, Balingen, Germany). Additionally, peppers were visually inspected for damage, using a 5-point scale (5-excellent; 3-good; 1-poor; with score below 3 considered unmarketable) to rate the fruit overall appearance after 21 days. Damaged fruits were removed every 7 days.

Respiration Rate

Approximately 550 g of peppers (4-5 fruits) were placed in a 700 mL container and sealed with multilayer foil at 24 ± 2 °C. CO_2 concentration was measured using an OXYBABY 6.0 (WIT-Gasetechnik GmbH & Co KG, Witten, Germany) after the harvest, postharvest treatment, and shelf life. CO_2 production (μL g^{-1} h^{-1}) was calculated by comparing CO_2 levels before sealing and after 4 hours, factoring in the fruit weight, volume, container volume, and the exact time from sealing to sampling.

Analysis of fruit anatomy

After the harvest, the segments 2.5 x 2 cm were cut from the middle part of each fruit and fixed in 50% ethanol. Cross-sections were made using a Reichert sliding microtome. The temporary slides were stained with a general reagent according to Tucakov (Kundaković et al., 2017). The sections for permanent slides were stained with safranin (1%, w/v, in 50% ethanol) and alcian blue (1%, w/v, aqueous). All permanent slides were mounted in Canada balsam after dehydration (Lakušić et al., 2010). Anatomical sections of fruits were analyzed on the light microscope (LM) Olympus BX41 with camera Olympus SC30 (Olympus Europa SE & Co. KG, Hamburg, Germany).

Color

Fruit skin color was determined using a chroma meter in CIELAB space using Konica-Minolta CR-400 Chroma Meter (Osaka, Japan). Measurements were taken at two points on each of 10 fruits after harvest, cold storage, and shelf life.

Chemical Analysis

For chemical analysis, quarters from 10 peppers (excluding seeds, calyx, and pedicel) were homogenized, transferred to Ziplock bags, and immediately frozen on dry ice for storage until analysis. Extractable color was determined after the harvest, according to a modified method by Ilić et al. (2017) and expressed as ASTA units. Quinic and succinic acids were quantified

using HPLC (Agilent 1200 series, Santa Clara, CA, USA) following extraction protocol by Milenković et al. (2020). The separation of target acids was performed on a NUCLEOGEL SUGAR 810 H column (Macherey-Nagel, Dueren, Germany) and detection was carried out using a diode array detector.

Statistical Analysis

Differences between means were determined by using factorial ANOVA, while on selected leaf parameters principal component analysis (PCA) was performed. For both analyses TIBCO Software Inc. (2020) Data Science Workbench, version 14. (http://tibco.com) was used.

RESULTS AND DISCUSSION

Losses

Weight loss increased over time for all treatments, with storage at 10 °C consistently resulting in the greatest loss (Table 1). After 21 day chilling injury notably increased at 4 °C (6%) and was also observed at 4 °C + HWD (1%). No chilling injury was observed at 10 °C. Chilling injury became more pronounced over time at 4 °C, especially without HWD treatment. Decay was a prominent issue after 21 days, especially at 10 °C, while HWD treatment at 4 °C effectively helped minimize the decay. Fruits stored at 4 °C + HWD consistently had the best appearance, suggesting this combination effectively preserves marketability, while 10 °C storage resulted in shriveled and less marketable fruits (Table 1). According to Lim et al. (2007), a temperature drop below 7°C, caused chilling injury within a few days, accompanied by significant fruit damage, including pitting, weight loss, and the development of decay.

Table 1. Losses during storage of "Duga bela" pepper fruit on 14th and 21st day

	Treatment		
	10 ºC	4 ºC	4 ºC + HWD
14 days			
Weight loss (%)	8.4	4.33	4.19
Chilling injury (%)	-	1.5	-
Decay Incidence (%)**	0.9	-	-
21 days			
Weight loss (%)	10.6	9.4	9.28
Chilling injury (%)	-	6.0	1.0
Decay Incidence (%)**	17.2	6.5	2.1
Overall appearance***	1.9*	3.1	3.3

* All fruit were shrivelled
(**) Decay – Percentage of decayed fruit from total number of fruits.
(***) General appearance: 5-excellent; 3-good, 1-poor (<3 unmarketable fruits)

Respiration

The respiration rates of fresh pepper fruits during the first couple of days (0-5) were relatively stable and moderate (Figure 1A). However, after 21 days of cold storage, an increase was recorded in all treatments, with the highest increase in respiration observed in the 4 °C (Figure 1B). This suggested a higher metabolic activity due to possible stress from low temperatures (Brizzolara et al., 2020). Contrary to fruits stored at low temperatures, respiration rate of fruits stored at 10 ºC was at similar level as in fruit after 5 days harvest. Our findings align with previous research by Hameed et al. (2015), which observed the highest respiration rates in chili peppers stored at 0 °C and subjected to shelf life conditions. Furthermore, Falik et al. (1999) identified a difference in respiration rates between untreated and HWD-treated pepper fruits.

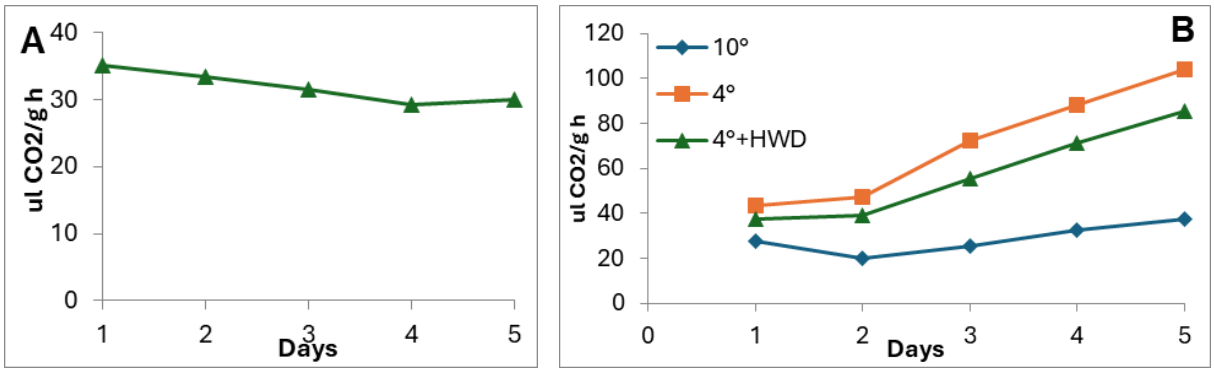


Figure 1. Respiration rate of "Duga bela" pepper fruit during a period of 5 days in: fresh fruits (A), fruits after cold storage (B)

Histological analysis of pericarp

The most important functions of the fruit cuticle are to reduce water loss, regulate gas transport, and protect against pathogens (Lara et al., 2014; Marinov et al., 2023). Konishi et al. (2022) showed a positive correlation between the postharvest quality of pepper fruits and the thickness of the cuticle. The histological analysis of fruit cross-sections showed that "Duga bela" has a thin cuticle (Figure 2A). This result was in accordance with the results of weight loss, which increased with time under all conditions (Table 1). The thicker pericarp and higher skin wax content in "Selika" fruit contributed to its water retention and firmness during storage (Ilić et al., 2014). Cutin and waxes, detected in the cuticle of "Duga bela" by staining with a general Tucakov reagent, ensure the structural integrity of the cuticle (Figure 2B). It can be argued that the treatment with hot water melted the wax layer and sealed the cracks in the epidermis. In this way, the surface of the fruit is strengthened and better tolerates storage at low temperatures (Kantakhoo & Imahori, 2021).

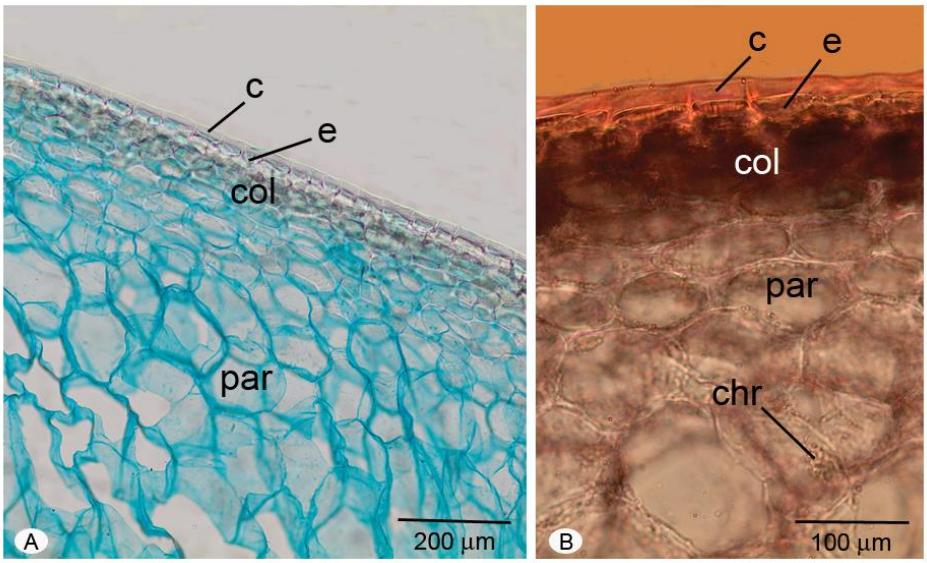


Figure 2. Fruit cross-sections through the pericarp of "Duga bela" pepper fruit Permanent slide for LM, 10x (A); Temporary slide for LM, 20x (B), c – cuticle, e – epidermis, col – collenchyma, par – parenchyma, chr – chromoplasts.

Color

During storage and shelf life, the color of "Duga bela" peppers underwent noticeable changes across all treatments (Table 2). Lightness (L*) consistently increased from initial values of 38.7 - 41.7 to a maximum of 43.6 under 4 °C + HWD, indicating a brighter appearance post-storage. Redness (a*) also raised, particularly at 10 °C, though it was stabilized or slightly decreased after extended storage. Also, yellowness (b*) generally decreased during storage and shelf life, especially in peppers stored at 10 °C, with an

exception of fruits stored at 4 °C. Among the treatments, 4 °C + HWD was the most effective at maintaining overall color quality, with balanced lightness, redness, and yellowness, suggesting its suitability for preserving the visual appeal of peppers during storage. These results can be compared to reports by Majomot et al. (2019), where HWD did not affect the fruit color of "Sweet Cayenne," and Abdullah (2019) where the pepper hybrid "7802 F1" exhibited significant color variations, indicating that the cultivar plays a crucial role in HWD-related color stability.

Table 2. Changes of L, a* and b* during cold storage and shelf life of "Duga bela" pepper fruit*

	L*			**a***			**b***		
days/temp.	10 ºC	4 ºC	4 ºC + HWD	10 ºC	4 ºC	4 ºC + HWD	10 ºC	4 ºC	4 ºC + HWD
0	38.7	41.3	41.7	31.1	24.0	26.1	26.2	22.7	23.5
21	40.9	41.4	42.1	33.2	29.8	27.4	21.4	21.5	22.1
21+3	42.4	42.6	43.6	28.4	28.8	26.3	21.1	23.3	22.6

Chemical composition

Initial color intensity evaluated in ASTA units increased from 18.4 to 21.2 at 10 ºC after 21 days, and then rose to 28.2 after 3 additional days. On the contrary, the storage at 4 ºC caused a significant decrease to 12.5 units, with partial recovery to 19.6 after 3 days of shelf life. The HWT+4 ºC treatment led to a slight initial decrease to 15.7 units, stabilizing at 16.2 units. Overall, 10 ºC storage best preserved and increased ASTA units, while 4 ºC initially reduced color intensity, with HWT+4 ºC maintaining moderate color intensity throughout the experiment. These findings might be interesting since the study by Ko et al. (2022) revealed a strong correlation between ASTA and total carotenoid content (r = 0.965), as well as correlation with antheraxanthin (r = 0.964), capsanthin (r = 0.946), and capsorubin (r = 0.858) in 226 different varieties of pepper.

The level of quinic acid increased across all storage conditions, starting from an initial 13.9 mg/100 g and reaching 16.5 mg/100 g at 10 ºC, 17.8 mg/100 g at 4 ºC, and 16.8 mg/100 g under HWT+4 ºC after 21 days. After 3 days of shelf life, the levels slightly decreased in all treatments except for HWT + 4 ºC, suggesting the letter as the most effective treatments in enhancing and preserving the contents of quinic acid over time.

The initial level of succinic acid was at 43.7 mg/100 g, which decreased significantly at 10 ºC to 18.5 mg/100 g after 21 days, followed by a recovery to 27.8 mg/100 g after 21+3 days. At 4 ºC induced a dropped of this organic acid to 29.3 mg/100 g, and after 3 days of shelf life to 27.6 mg/100 g. The treatment with hot water (HWT+4 ºC) led to a continuous decrease, dropping to 25.0 mg/100 g after 21 days, and eventually falling to 23.2 mg/100 g after an additional 3 days. The treatments at 4 ºC and 10 ºC had the similar and at the same time the highest succinic acid levels after 21+3 period of storage, while the combined treatment showed the opposite effect.

Table 3. Changes of ASTA, quinic and succinic acid during cold storage and shelf life of "Duga bela" pepper fruit

days	**ASTA UNITS**			**QUINIC (mg/100 g)**			**SUCCINIC (mg/100 g)**		
0	18.4			13.9			43.7		
	10 ºC	4 ºC	HWT+4 ºC	10 ºC	4 ºC	HWT+4 ºC	10 ºC	4 ºC	HWT+4 ºC
21	21.2	12.5	15.7	16.5	17.8	16.8	18.5	29.3	25.0
21+3	28.2	19.6	16.2	15.6	16.1	20.0	27.8	27.6	23.2

CONCLUSIONS

The study examined the efficacy of hot water dipping (HWD) as a postharvest treatment for preserving the quality and storability of the traditional Serbian "Duga bela" pepper. The results suggested that HWD, when combined with storage at 4 ºC, effectively minimizes postharvest losses, reduces chilling injury, and maintains the appearance the peppers. HWD treatment led to better retention of color intensity (ASTA units) and quinic acid content. Compared to storage at 10 ºC and 4 ºC without HWD, the combined treatment consistently resulted in peppers with superior overall quality, highlighting its potential as a valuable tool for extending the shelf life and marketability of traditional cultivars like "Duga bela". This approach not only helps preserve the nutritional and sensory qualities of the peppers but also supports the conservation of culturally significant agricultural heritage.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (under Grants: 451-03-66/2024-03/200222 and 451-03-66/2024-03/200007).

REFERENCES

Abdullah, M. A. (2019). Enhancement of Sweet Pepper Fruits Quality and Storability by Some Postharvest Treatments. *Annals of Agricultural Science, Moshtohor*, 57(2), 447-454.

Brizzolara, S., Manganaris, G. A., Fotopoulos, V., Watkins, C. B., & Tonutti, P. (2020). Primary metabolism in fresh fruits during storage. *Frontiers in plant science*, 11, 80.

Fallik, E., & Ilić, Z. (2017). Hot water treatments. In: S. Pareek (ed.) *Novel postharvest treatments of fresh produce*. (pp. 241-258). CRC Press, Boca Raton. ISBN: 9781498729918 - CAT# K26101

Fallik, E., Grinberg, S., Alkalai, S., Yekutieli, O., Wiseblum, A., Regev, R., Beres, H. and Bar-Lev, E (1999). A unique rapid hot water treatment to improve storage quality of sweet pepper. *Postharvest Biology and Technology*, 15(1), 25-32.

Hameed, R., Malik, A. U., Khan, A. S., Imran, M., Umar, M., &Riaz, R. (2015).Evaluating the effect of different storage conditions on quality of green chillies (*Capsicum annuum* L.).*Tropical Agricultural Research*, 24(4).

Ilić, Z. S., Kevrešan, Ž., Mastilović, J., Zorić, L., Tomšik, A., Belović, M., Pestorić, M., Karanović, D., & Luković, J. (2017). Evaluation of mineral profile, texture, sensory and structural characteristics of old pepper landraces. *Journal of Food Processing and Preservation* 41, 5: e13141.

Ilić, Z. S., Ljubomir, Š., Mirecki, N., & Fallik, E. (2014). Cultivars differences in keeping quality and bioactive constituents of bell pepper fruit during prolonged storage. Journal: *Journal Of Advances In Biotechnology*, 4(1).

Ilić, Z. S., Milenković, L., Vasić, M., Girek, Z., Zdravković, M., & Zdravković, J. (2013). Old cultivars and populations from traditional pepper-growing regions of Serbia as breeding potential. *Journal of Agricultural Science*, 5(6), 132-140.

Kader, A. A. (2002). Postharvest technology of horticultural crops (Vol. 3311). University of California Agriculture and Natural Resources.

Kantakhoo, J., & Imahori, Y. (2021). Antioxidative responses to pre-storage hot water treatment of red sweet pepper (*Capsicum annuum* L.) fruit during cold storage. *Foods*, 10, 3031.

Ko, HC., Haile, M., Lee, S., Hwang A., Lee, GA., Choi YM., Hahn, BS., & Ro, N. (2022). Correlation of Carotenoids Content and ASTA Values of Pepper (*Capsicum chinense*) Genetic Resources. *Horticulturae* 8, no. 6: 486.

Konishi, A., Terabayashi, S., & Itai, A. (2022). Relationship of cuticle development with water loss and texture of pepper fruit. *Canadian Science Publishing*, 102, 103 – 111.

Kundaković, TD., Drobac, MM., Petrović, SD., Maksimović, ZA., & Marčetić, MD. (2017) Makroskopska i mikroskopska analiza biljnih droga. *Priručnik za praktičnu nastavu iz predmeta Farmakognozija*. Univerzitet u Beogradu, Farmaceutski fakultet, Beograd.

Lakušić, B., Stevanović, B., Jančić, R., & Lakušić, D. (2010). Habitat-related adaptations in morphology and anatomy of *Teucrium* (*Lamiaceae*) species from the Balkan peninsula (Serbia and Montenegro). *Flora-Morphology, Distribution, Functional Ecology of Plants*, 205(10), 633-646.

Lara, I., Belge, B., & Goulao L. F. (2014). The fruit cuticle as a modulator of postharvest quality. *Postharvest Biology* and Technology, 87, 103-112.

Lim, C. S., Kang, S. M., Cho, J. L., Gross, K. C., & Woolf, A. B. (2007). Bell pepper (*Capsicum annuum* L.) fruits are susceptible to chilling injury at the breaker stage of ripeness. *HortScience*, 42(7), 1659-1664.

Majomot, A. M. C. C., Secretaria L. B., & Bayogan, E. R. V. (2019). Effect of Hot Water Treatment and Evaporative Cooling on Some Postharvest Characteristics of Sweet Pepper (*Capsicum annuum* cv.'Sweet Cayenne'). *Mindanao Journal of Science and Technology*, 17.

Marinov, O., Nomberg, G., Sarkar, S., Arya, GC., Karavani, E., Zelingrer, E., Manasherova, E., & Cohen, H. (2023). Microscopic and metabolic investigations disclose the factors that lead to skin cracking in chili-type pepper fruit varieties. *Horticulture Research*, 2023, 10: uhad036.

Milenković, L., Mastilović, J., Kevrešan, Ž., Bajić, A., Gledić, A., Stanojević, L., Cvetković, D., Šunić, L., & Ilić, Z. S. (2020). Effect of shading and grafting on yield and quality of tomato. *Journal of the Science of Food and Agriculture*, 100(2), 623-633.

SENSORY ANALYSIS OF MILK CHOCOLATE WITH INULIN AS A SUGAR SUBSTITUTE

Mishela Temkov*, Katerina Stoilova, Bojan Ginoski, Irma Djurdjevic, Sara Kechovska, Marija Gligorova, Milica Arizanova, Elena Velichkova

Ss. Cyril and Methodius University in Skopje, North Macedonia, Faculty of Technology and Metallurgy
Department of Food Technology and Biotechnology

*Corresponding author:
E-mail address: mishela@tmf.ukim.edu.mk

ABSTRACT

Overconsumption of sugars in diets is linked to various health issues, including dental diseases, diabetes, and obesity. Despite this, eliminating sugar from products like chocolate remains a challenge for manufacturers. The objective of this research was to examine the impact of inulin on the sensory characteristics of milk chocolate. Two types of milk chocolate with partial (50 % sugar and 50 % inulin – 50S-50I) and total (100 % inulin – 100I) substitution of sugar with inulin were compared to its conventional counterpart (100 % sugar – 100S), all produced on the laboratory. Chocolate products were subjected to a consumer test using a 10 cm VAS (Visual Analogue Scale) structured scale for overall acceptability, while the intensity of different sensory attributes were examined on 5-point JAR "just about right" scale. The survey involved 72 healthy random participants, who recognized the terminology “alternative sweeteners”, but only half of them were aware about their health benefits. When buying the product 80.56% of judges will choose the product according to the taste, texture and appearance and much less will select a product beneficial to health. Newly developed chocolates containing reduced amount or no sugar at all were well accepted in the consumers test with no significant difference compared to the chocolate produced by the ordinary formulation. The intensities of butter and cocoa flavors were less pronounced in inulin containing chocolates and the sweetness was described as not enough. On the other hand, acidity, gloss on the surface, snap and melting behavior in the mouth were perceived in “just about right” range by highest percentage of consumers than the control sample.
Keywords*: chocolate, functional product, inulin, sensory acceptance, JAR test*

INTRODUCTION

Chocolate is a complex confectionary product consisting of a fat-based suspension that incorporates sugar particles and various non-fat cocoa solids. Sucrose, which typically comprises 40-50% of chocolate's composition, has multiple crucial functions: it provides sweetness, enhances the cocoa flavor, acts as a natural preservative, and serves as a bulking agent (Aidoo et al. 2013). However, the global rise in sucrose consumption has led to increased concern about associated health issues, particularly obesity and diabetes. This growing health awareness drove extensive research into developing 'sucrose-free' chocolate alternatives (Kiumarsi et al. 2021). A food product can be labeled as "light" if it provides fewer than 40 calories per serving, or as "sugar-free" if it contains less than 0.5 grams of sugars per serving (Food Label Claims and Guidelines: Provided by MyFoodDiary.com; 2024). Nevertheless, removing sugar from products such as chocolate is still a challenge for manufacturers, which have the opportunity to use nutritive and non-nutritive sweeteners. Nutritive sweeteners are ingredients that replace both the physical bulk and sweetness of sugar. These products, often referred to as “sugar replacers” or “bulk sweeteners,” whereas non-nutritive sweeteners are substances with an intense sweet taste used in small quantities to replace the sweetness of a much larger amount of sugar or sucrose (Aidoo et al. 2013).
Inulin represents a linear carbohydrate composed of fructose units linked by β-2,1-glycosidic bonds, with or without a terminal glucose residue with a high degree of polymerization and consequently a higher molecular weight. Due to the absence of hydrolyzing enzymes in the human upper gastrointestinal tract, these compounds are non-digestible (Mao et al. 2019). However, they are hydrolyzed and extensively fermented by the microbiome in the large

intestine into short-chain fatty acids such as butyrate, propionate, and acetate. Consequently, their caloric value is estimated to be 25–35% of that of fully digested fructose (Temkov et al. 2020). Inulin is commonly used as an additive in functional foods, often as a substitute for lipids and a supplement for sugar. It is also utilized in products with increased dietary fiber content, such as bread, providing a bifidogenic effect (Farzanmehr and Abbasi 2009). The main challenge for the food industry is to meet consumers' high expectations, as they demand foods that taste great, are reduced in fat and/or calories, and offer added health benefits. Additionally, these foods must be convenient and affordable. In chocolate formulation, inulin is commonly used for its nutritional benefits, such as lowering calorie content, reducing the glycemic index, and providing a source of prebiotic fiber, as well as for its functional advantages, like enhancing texture and mouthfeel.

In this study, sugar in the control sample was either completely (100%) or partially (50%) replaced with inulin on a 1:1 basis, despite the lower sweetening power of inulin compared to sugar. The primary objective was to evaluate both the sensory intensity and consumer acceptance of various attributes using hedonic and Just-About-Right (JAR) scales. These sensory methods provide indicator perceptions that are valuable for product reformulation, particularly when balancing taste with nutritional improvements. The ultimate aim of this research is to offer practical guidance for manufacturers who seek to improve the nutritional profile of their chocolate products or develop alternatives tailored to consumers with specific dietary restrictions.

MATERIAL AND METHODS

Materials

For the production of milk chocolate, ingredients such as cocoa mass, cocoa butter, lecithin, and milk powder (all obtained from Moringa Healthy Food in Skopje), along with inulin Frutafit OFP (Sensus, Roosendaal, The Netherlands), were used. The sugar was sourced from a local store in North Macedonia.

Milk Chocolate Production

To prepare 100 g of milk chocolate, 28 g of cocoa mass was first melted in a water bath at 60°C. Then, 30 g of powdered sugar (or a mix of 15 g sugar and 15 g inulin for partial sugar substitution, or 30 g of inulin for total sugar substitution, based on the formulation) and 12 g of milk powder were added to the melted cocoa mass. The mixture was then conched using a rotor glass agitator (designed in our lab, with a blade diameter of 30 mm) at 30 rpm for 1.5 hours. At the start of the conching process, one-third of the total 18 g of cocoa butter was added, followed by the second third after 15 minutes. The remaining 6 g of cocoa butter, along with 0.6 g of lecithin, were added after 1 hour, and conching continued for an additional 30 minutes.The milk chocolate was then tempered in a water bath by cooling it to 29°C over 20–25 minutes, holding it for 10 minutes, and then heating it to 32°C for 5 minutes. The chocolate was poured into polycarbonate molds (150 x 40 x 20 mm), and the molded chocolate was cooled overnight in the refrigerator at 7°C. The following day, the chocolate bars (7 x 20 x 12 mm) were unmolded, wrapped in aluminum foil, and stored in the refrigerator before analysis. The formulation containing 100% sugar was used as the reference sample.

Ethics and Evaluation Conditions

The sensory tests were conducted in the food laboratory at Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, North Macedonia in accordance with the Declaration of Helsinki (Adopted by the 18 WMA General Assembly, Helsinki, Finland, June 1964 and amended by the 64 WMA General Assembly, Fortaleza, Brazil, October 2013). Assessors provided written consent after reviewing detailed information about the study. All relevant institutional and governmental regulations regarding the ethical use of human volunteers were followed and approved by the Faculty Committee of the

Faculty of Technology and Metallurgy (Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia, 09-1273/1).

Study design

Product survey

The research data were gathered using a structured questionnaire comprising a total of seven multiple-choice questions organized into several sections: sociodemographics (three questions), chocolate consumption habits (one question), knowledge of alternative sweeteners (two questions), and purchase intention (one question). The sociodemographic section included inquiries about age, gender, education, and employment status, while the chocolate consumption habits section focused solely on frequency of consumption. Data related to nutrition and health examined participants' understanding of alternative sweeteners, including terminology and associated health benefits, while the consumer purchase behavior section aimed to identify their purchase intentions.

Sensory Analysis

Hedonic Test

Seventy-two participants were asked to evaluate their liking of three chocolates: chocolate with 100% sugar (100S), chocolate with a 50/50 mix of sugar and inulin (50S-50I), and chocolate with 100% inulin (100I). The hedonic appreciation was assessed using visual analog scales (VAS) consisting of a 10 cm structured scale with 'dislike extremely' on the left anchor and 'like extremely' on the right anchor. It combines the simplicity of the traditional VAS that allows great sensitivity with structured anchors to guide the respondents and to give consistency in responsess. Participants, after being instructed on how to use the scale, indicated their preference by marking a point on the scale. The distance from the far-left extreme to the mark was measured and converted into numerical scores.The demographic composition of the participants was 69% female and 31% male. The majority of participants were aged 18 to 25 years (63.89%), with 6.94% aged 26 to 35 years, 12.50% aged 36 to 45 years, 11.11% aged 46 to 55 years, and 5.56% over the age of 55.

Just About Right (JAR) Test

For each cholocolate seven attributes were evaluated: butter and cocoa flavor, gloss, sweetness, acidity, snap and melting behavoir in the mouth. A 5-point JAR scale was used to indicate whether a particular attribute was perceived at an acceptable or non-acceptable level. Participants determined the intensity of each attribute, indicating whether it fell on the extreme ends ("much too low," “not enough” or “too much” and "much too much") or in the middle of the scale ("just about right").

Statistical Analysis

Mean liking scores, standard deviation and significances ($\alpha = 0.05$) were calculated for each formulation. A two-way analysis of variance (ANOVA) was performed for the hedonic evaluation (formulation, panelists). When significant overall difference at a confidence level of 0.05 was found, a post-hoc (Least Significant Difference) test was employed to conduct pairwise comparisons between groups. Penalty analysis was applied to the JAR results using XLStat 2022.3.1 (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

SENSORY ANALYSIS

Product survey and Hedonic test

Twenty-five percent of the panelists consumed chocolate daily, 27.78% ate chocolate 2 to 3 times per week and 33.33 % only once per week. Only small proportion (4.17%) consumed chocolate 2 to 3 times per month, while 9.72% preferred the pleasure of eating chocolate only once a month. A significant proportion (73.61%) were acquainted with the terminology alternative sweeteners, but only 47.22 % had any knowledge about their health benefits. Price of the product will be detrimental 36.11%, 45.83 % will choose the product according to the producer, 38.89% decide to buy the chocolate according to the packaging, the taste, texture and appearance are important for 80.56% of judges, but only 25.00% are concerned about health benefits of different compounds included in the formulation. The consumer acceptability of chocolates with sucrose substituted partialy (50S-50I) and totally (100I) with inulin did not differ significantly ($p=0.57$, $p=0.339$, respectively) when compared to the control sample where sucrose was used as sole sweetener (100S). The mean score was the highest for the 100S sample (6.65±2.052), followed by the chocolate made with inulin as sole sweetener 100I (6.29±2.328), whereas 50S-50I scored a mark of 5.93±2.427, eventhough all the samples belong to the same population due to the same level of acceptance (Figure 1). Moreover, the results obtained in this research have shown that the new product – a functional chocolate was accepted quite well among consumers. The increased fiber content and 50% reduction in sugar are significant factors that make this product recommendable for both the general healthy population and diabetics. Similar results were obtained by (Golob, Mičović et al. 2004). These findings have several implications for product development. First, they suggest that future formulations should prioritize high-sugar reduction while incorporating alternative sweeteners like inulin to enhance consumer acceptance. Additionally, emphasizing the functional benefits of increased fiber could appeal to health-conscious consumers and those managing diabetes. Moreover, these insights could guide marketing strategies by highlighting the health benefits and reduced sugar content, potentially attracting a broader audience.

Just about right test

The JAR test results are given in Figure 2 (A-G). The percentage of consumers responds for the intensity (“much too low”, “JAR”, or “much too high”) for each attribute are given separately to emphasize the comparison between chocolates with inulin (50% and 100% addition) and the control sample. The results show distinct differences in butter and cocoa flavor perception between chocolates with inulin and the control sample (100S). Butter flavor was considered insufficient by 33.3% of respondents for 50S-50I ($p= 0.037$) and 31.9% for 100I, compared to only 13.9% for 100S. Similarly, 13.9% of respondents found the cocoa flavor lacking in both inulin-containing chocolates, while only 4.2% felt the same for the control ($p=0.025$). These findings highlight a reduced intensity of butter and cocoa flavors in the inulin-sweetened chocolates compared to the control sample.

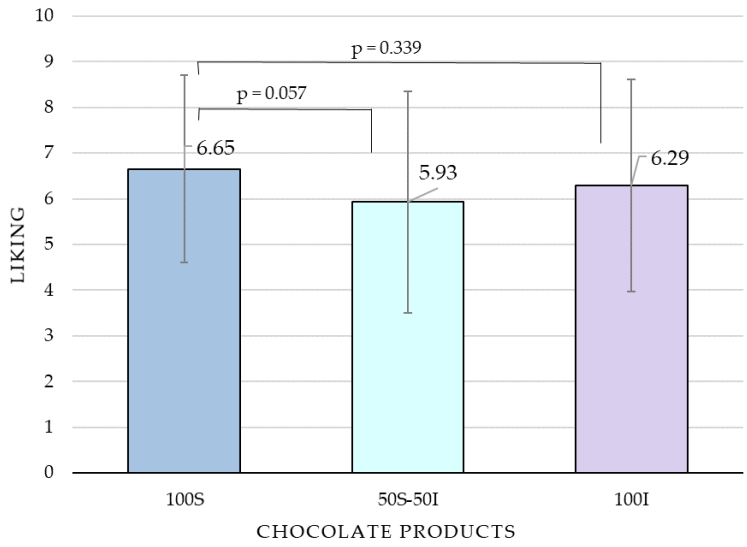


Figure 1. Hedonic liking scores for three types of chocolate: with 100% sucrose (100S), with 50% sucrose and 50% inulin (50S-50I), and with 100% inulin (100I), compared to a control sample with 100% sucrose (100S). Data is presented for n=72 panelists

Sugar enriches the flavor profile of chocolate by amplifying the aroma of other flavors and counteracting the bitterness commonly associated with cocoa. However, sucrose substitutes often fail to replicate the physical properties that sugar contributes to chocolate processing (Torrico, Sharma et al. 2021). The sweetness of the chocolate is an attribute that acquire great deal of attention. When developing a new functional product that has reduced or no content of sugar, the intention is to create a product that will not be dull in sweetness. Approximately 60% of respondents rated the sweetness of the 100S sample as "just about right", while 33% found it too sweet. A larger portion of the panel (65.3%) considered the partially substituted sample (50S-50I) to be in the JAR range for sweetness. In contrast, only about half of the respondents (48.6%) rated the sugar-free chocolate as JAR in sweetness ($p=0.011$), with 40.2% finding it not sweet enough. However, surface gloss and appearance were rated as JAR by a higher percentage of respondents for the inulin-containing chocolates (70.1% for 50S-50I and 62.5% for 100I) compared to 55.6% for the standard 100S chocolate.

In addition, the perception of optimal acidity improved as the percentage of panelists increased from 25% for 100S to 30.1% for 50S-50I and 40.2% for 100I. High-quality chocolate breaks with a clean edge and produces an audible cracking sound called a snap. The chocolate with 100 % inulin had the JAR snap according to the 55.6 % of the respondents, compared to 48.6% for the standard 100S chocolate.

Mouthfeel in chocolate refers to the sensory experience of texture sensations perceived in the mouth as the chocolate melts and mixes with saliva. It characterizes the manner in which the chocolate breaks, melts, and coats the palate, all of which contribute to the overall pleasure and satisfaction of eating chocolate. Chocolates that exhibit high viscosity often have a thick, pasty mouthfeel that persists in the mouth (Afoakwa, Paterson et al. 2007). The viscosity of chocolate is affected by various factors, including its composition, processing methods, and the distribution of particle sizes. The melting behavior for the produced chocolates was JAR for the majority of the panelists, ranging from 61.1% for 100I to 72.2% for 50S-50I indicating that the high DP inulin that was used contributed to the creaminess and smoothness of the chocolate.

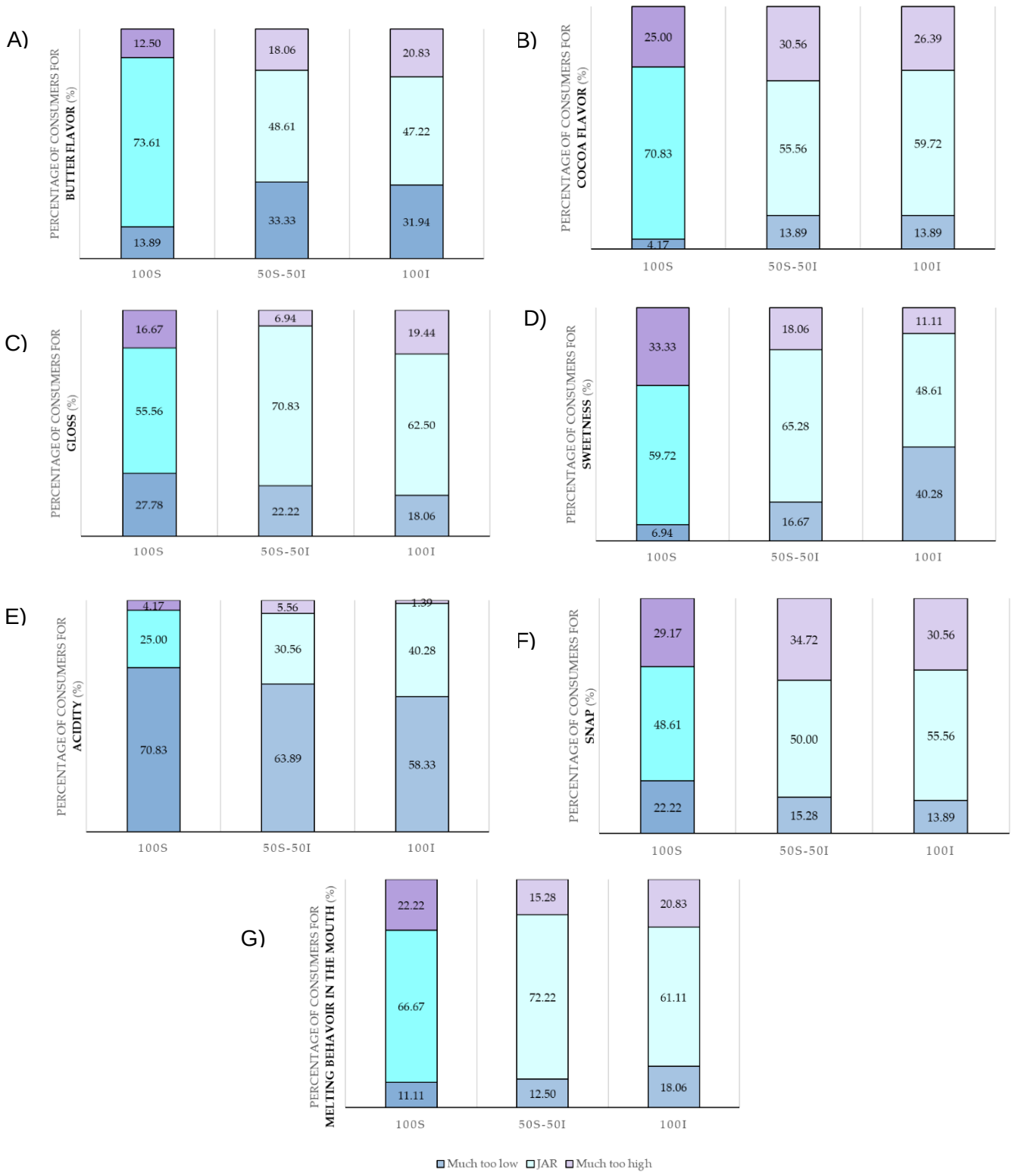


Figure 2. Just-about-right (JAR) scale percentages of responses in three levels ("Much too low", "Just about right", "Much too much") of A) butter odour; B) cocoa odour; C) gloss; D) sweetness; E) acidity snap; F) mouthfeel (n=72)

CONCLUSIONS

The study explored the production and sensory evaluation of milk chocolate with varying sugar and inulin content. For the purposes of the research three types of chocolate were produced: ordinary chocolate no sugar substitution (100S) and chocolates with partial (50S-50I) and full (100I) sugar substitution. Sensory analysis, including consumer preference and

attribute intensity (JAR test), revealed that overall acceptability of chocolates with partial (50S-50I) and total (100I) sugar substitution did not significantly differ from the control (100S). While the control sample received the highest overall liking scores, followed by the 100% inulin chocolate, the 50/50 sugar-inulin mix had the lowest score. Despite these variations, the functional chocolate with increased fiber and reduced sugar was generally well accepted, making it a promising option for health-conscious consumers and diabetics. The JAR test revealed sensory differences: the control chocolate (100S) was preferred for its butter and cocoa flavors, whereas the inulin-sweetened chocolates had distinct sweetness and acidity profiles. Notably, the 100% inulin chocolate (100I) excelled in texture, with a majority of panelists praising its optimal snap and creamy melting behavior in the mouth. Overall, the study highlights that while traditional milk chocolate remains popular, chocolate formulations incorporating inulin can be well-received particularly due to their nutritional benefits, despite some sensory differences compared to standard products.

ACKNOWLEDGEMENTS

The authors would like to acknowledge University's Student Parliament for funding this project.

REFERENCES

Afoakwa, E. O., et al. (2007). "Factors influencing rheological and textural qualities in chocolate – a review." Trends in Food Science & Technology 18(6): 290-298.

Aidoo, R. P., et al. (2013). "Industrial manufacture of sugar-free chocolates – Applicability of alternative sweeteners and carbohydrate polymers as raw materials in product development." *Trends in Food Science & Technology* 32(2): 84-96.

Farzanmehr, H. and S. Abbasi (2009). "Effects of inulin and bulking agents on some physicochemical, textural and sensory properties of milk chocolate." *Journal of Texture Studies* 40: 536–553.

Golob, T., et al. (2004). "Sensory acceptability of chocolate with inulin." *Acta agriculturae Slovenica* 83(2).

Kiumarsi, M., et al. (2021). "Comparative study of instrumental properties and sensory profiling of low-calorie chocolate containing hydrophobically modified inulin. Part II: Proton mobility, topological, tribological and dynamic sensory properties." *Food Hydrocolloids* 110.

"MyFoodDiary." Retrieved 08.08., 2024, from https://www.myfooddiary.com/Resources/label_claims.asp

Temkov, M., et al. (2020). "Inulinase immobilisation in PAA/PEG composite for efficient fructooligosaccharides production." *Biocatalysis and Biotransformation* 40(1): 50-63.

Torrico, D. D., Sharma, C., Dong, W., Fuentes, S., Viejo, C. G., & Dunshea, F. R. (2021). Virtual reality environments on the sensory acceptability and emotional responses of no-and full-sugar chocolate. *Lwt*, 137, 110383.

SENSORY QUALITY OF FRESH AND COOKED EGGS OBTAINED FROM HENS FED A SPECIALLY DESIGNED FEED MIXTURE

Nedeljka Spasevski*, Tatjana Peulić, Slađana Rakita, Jasmina Lazarević, Ivana Čabarkapa, Olivera Đuragić, Bojana Kokić

Institute of Food Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000 Novi Sad, Serbia

*Corresponding author:
E-mail address: nedeljka.spasevski@fins.uns.ac.rs

This study aimed to assess the influence of dietary inclusion of flaxseed, camelina seed, and hempseed in hens' diets on the sensory attributes of both fresh and cooked eggs. Two hundred and forty Lohmann Brown laying hens were divided into eight groups (two control groups, K1 and K2, and six experimental groups, L1, L2, C1, C2, H1, and H2) and fed for four weeks. The control groups were fed a corn-soybean meal based diet, while the experimental groups received co-extrudates: flax-corn meal (13.5% - L1, 22.5% - L2), camelina-corn meal (16.6% - C1, 27.6% - C2), and hemp-corn meal (18.4% - H1, 30.7% - H2). K1 contained up to 3% fat without pigments, while K2 contained up to 5% fat and synthetic pigments. All experimental groups included natural pigments (1% carrot and 0.5% paprika). In the sensory evaluation [Roche Yolk Color Fan (RYCF), acceptability, homogeneity and color (hue)] of fresh egg yolks, experimental groups (L1, L2, C1, C2, H1, H2) with 1% carrot and 0.5% paprika had similar ($p>0.05$) RYCF values, ranging from 12.20 to 12.80. Control group K1 (without synthetic pigments) had the lowest RYCF value (8.00), while K2 (with synthetic pigments) had the highest RYCF value (14.20), both significantly different from the experimental groups ($p<0.05$). Color acceptability and yolk color hue were similar across the experimental groups but significantly lower ($p<0.05$) in the control groups (K1 and K2). In the sensory evaluation (smell, taste, acceptability, homogeneity, and hue) of cooked eggs, control groups K1 and K2 also had lower color acceptability and yolk color hue compared to the experimental groups. The addition of co-extrudates did not affect yolk color, smell, or taste, except for flaxseed co-extrudates (L1 and L2), which negatively impacted yolk taste ($p<0.05$).
Keywords*: sensory quality, egg yolk, natural pigments, flaxseed, camelina seed, hemp seed*

INTRODUCTION

The sensory evaluation of egg yolks is based on their color, smell, and taste. The color of the yolk is very important when it comes to consumer acceptance. It is even considered that this characteristic of the egg is the most significant in terms of consumer choice and evaluation (Delgado-Vargas et al., 1998). Although it has not been proven that the color of the yolk affects the taste of the egg or its nutritional content, the acceptability of the product and its desirability strongly depend on its appearance, especially the color, which is perceived as one of the priority criteria for egg quality. The preference for a certain shade of yolk color varies depending on geographical areas and consumer habits (Nys and Guyot, 2011). This characteristic is an extremely important quality factor in some countries, such as France, the United Kingdom, and Germany, while in other countries, like Spain and Italy, it is not considered crucial (Hernandez, 2005). The taste and smell of eggs can be influenced by the diet of laying hens through the addition of oilseeds rich in polyunsaturated fatty acids. Adding a higher amount of oil rich in omega-3 fatty acids to the feed can negative effect on the eggs, as they become more susceptible to oxidation, leading to changes in their taste and smell (Surai et al., 2001). A negative impact on the sensory qualities of eggs (taste, smell, and acceptability) has been observed even with the addition of 3% fish oil to the feed mixtures for laying hens (Škrtić et al., 2006).

Eggs enriched with omega-3 fatty acids that have poor sensory ratings (such as a "fishy" smell and taste) are not acceptable to processors or consumers (Kralik et al., 2014). Like fish oil, flaxseed amounts greater than 10% negatively affect the sensory quality of eggs (Jiang et al., 1992; Hayat et al., 2010; Coorey et al., 2015).

However, as noted by Goldberg et al. (2012), the addition of hemp seed (10% and 20%) and hemp oil (4%, 8%, and 12%) does not affect the sensory properties of eggs. Additionally, the addition of larger amounts of camelina does not impact the sensory properties of eggs

(Rokka et al., 2002; Valkonen et al., 2007). These findings open up new possibilities for balancing and combining different sources of oil that can be added to the feed of laying hens, allowing for a significant increase in desirable omega-3 fatty acids in the eggs, while also ensuring no negative impact on the organoleptic acceptability of the product. Preventing the oxidation process in eggs rich in omega-3 fatty acids, and thus the development of undesirable smell and taste, can be achieved by simultaneously introducing vitamin E and carotenoids as antioxidants, which enhance the stability of polyunsaturated fatty acids in the eggs (Surai and Sparks, 2001).
The main goal of the present study was to investigate the impact of incorporating co-extrudates made from flaxseed, camelina seed, and hempseed, as well as natural pigments, on the sensory quality of fresh and cooked eggs.

MATERIAL AND METHODS

This experiment included eight groups (two control groups and six experimental groups) of 240 Lohmann Brown laying hens. The control groups (K1 and K2) were provided with a diet based on corn and soybean meal. The experimental groups were given co-extrudates of different compositions: flaxseed-corn meal (13.5% for L1 and 22.5% for L2), camelina-corn meal (16.6% for C1 and 27.6% for C2), and hemp-corn meal (18.4% for H1 and 30.7% for H2). The K1 diet included up to 3% fat without pigments, while the K2 diet contained up to 5% fat with synthetic pigments. All experimental diets were supplemented with natural pigments (1% carrot and 0.5% paprika), based on the previous experiment described in the study by Spasevski et al. (2018), which achieved an optimal yolk color of 12.2 to 13.4 per RYCF. Feed and water were available ad libitum. The composition of the diet used to feed the laying hens is presented in Table 1.

Table 1. The composition of the control and experimental diets

Ingredients (%)	K1	K2	L1	L2	C1	C2	H1	H2
Corn	57.60	55.40						
Co-extruded			13.50	22.50				
Co-extruded camelina seed					16.60	27.60		
Co-extruded							18.40	30.70
Soybean oil	1.30	3.00						
Corn grits			46.70	40.30	45.80	38.80	42.50	33.50
Soybean cake	20.00	20.00	17.20	14.60	15.00	11.00	16.50	13.20
Sunflower cake- 33%	8.50	9.00	8.50	8.50	8.50	8.50	8.50	8.50
Feed yeast	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Monocalcium	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Natrium bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Commercial premix	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Calcium carbonate	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Nacl	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Paprika			0.5	0.5	0.5	0.5	0.5	0.5
Carrot			1.0	1.0	1.0	1.0	1.0	1.0
Synthetic pigment [g/kg]		0.055*						

*Control diet K2 contains: 0.04 g/kg carophyll red and 0.015 g/kg carophyll yellow

Sensory analysis

Sensory evaluation of fresh and boiled egg yolk was performed by a panel of 7 assessors experienced in sensory analysis of egg (6 females and 1 male, 31−59 years old) recruited from the Institute of Food Technology, according to SRPS EN ISO 8586 (2015). The color of fresh egg yolk was visually assessed using the Roche Yolk Color Fan (Hoffman-La Roche

Ltd, Basel, Switzerland). The color was rated based on a scale of 15 sample shades, ranging from 1 (light pale) to 15 (dark orange). Homogeneity, acceptability, color (hue), smell, and taste were evaluated using a 5-point scale ranging from 1 (unacceptable) to 5 (acceptable) for acceptability; from 1 (highly non-homogenous) to 5 (homogenous) for homogeneity; from 1 (highly noticeable differences) to 5 (optimal (orange)) for color (hue); from 1 (uncharacteristic, foreign smell) to 5 (optimally expressed, characteristic smell) for smell and from 1 (unpleasant, foreign taste) to 5 (exceptionally pleasant, characteristic taste) for taste. All characteristics were assessed visually under laboratory conditions that met the SRPS EN ISO 8589 (2012) standard. Eggs were served on plastic plates marked with three-digit numbers for the assessors.

Statistical analysis

The data obtained from the sensory analysis of fresh and cooked eggs was conducted using IBM SPSS Statistics 25 (IBM, Chicago, IL, USA). To assess the differences between the results, Tukey's multiple comparison test was used. Identical letters in rows or columns indicate that there are no significant differences between the groups at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

The results of sensory evaluation of fresh egg yolk are shown in the Figure 1.

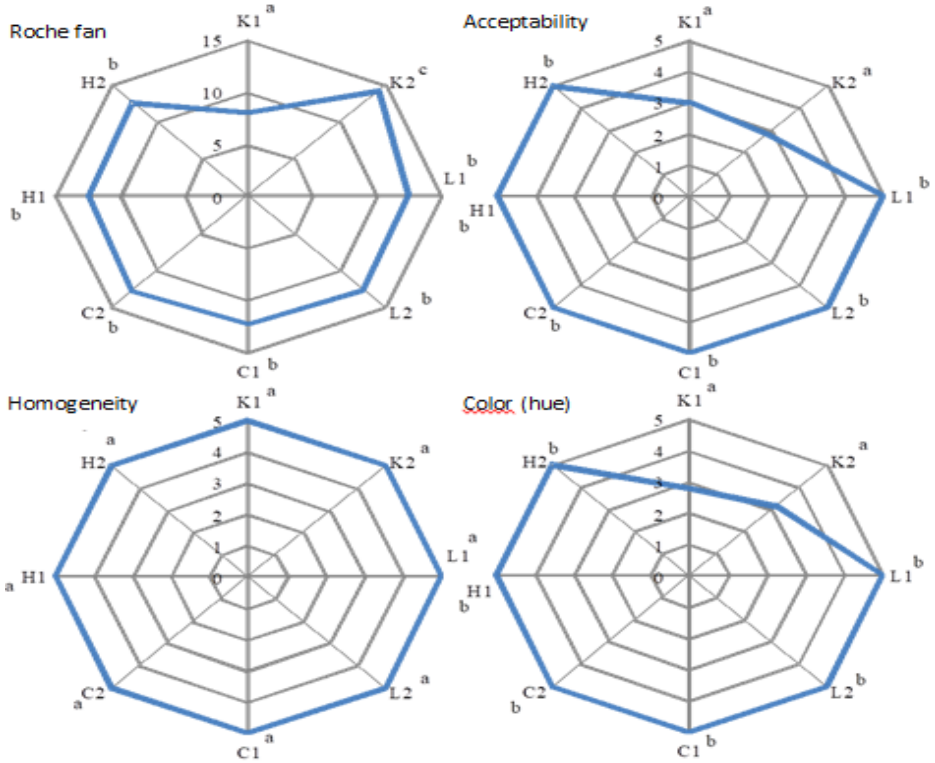


Figure 1. Sensory evaluation of fresh egg yolks from control and experimental groups after 30 days of feeding. K1 - control group with 3% and synthetic pigment; K2 - control group with 5% and without synthetic pigment; L1 - flax-corn meal (13.5%); L2 - flax-corn meal (22.5%); C1 - camelina-corn meal (16.6%); C2 - camelina-corn meal (27.6%); H1 - hemp-corn meal (18.4%); H2 - hemp-corn meal (30.7%). All experimental groups contain 1% carrot and 0.5% paprika.

Based on the results shown in Figure 1, the values of the sensory attributes for the acceptability and hue of fresh yolks were statistically significantly different ($p<0.05$) between the experimental and control groups, indicating that do not prefer light-colored yolks (K1, below 10 on the RYCF) or very dark yolks (K2, above 14 on the RYCF). The most

acceptable yolks were from the experimental groups, with values on the RYCF ranging from 12.50 to 13.39. The values for color uniformity did not differ statistically between the experimental and control groups, indicating that natural pigments can be used as a substitute for synthetic pigments in laying hens' diets.

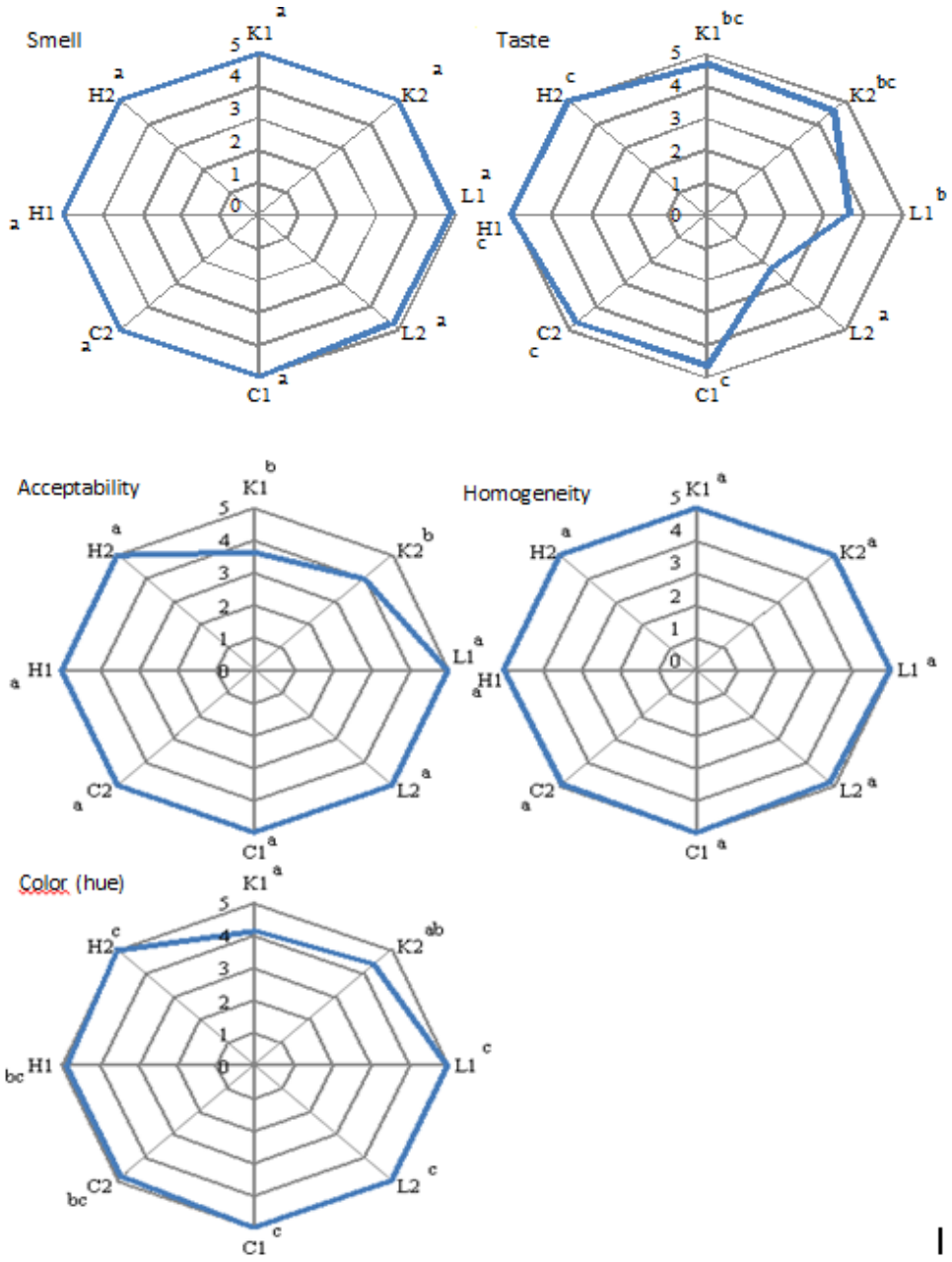


Figure 2. Sensory evaluation of boiled egg from control and experimental groups after 30 days of feeding. K1 - control group with 3% and synthetic pigment; K2 - control group with 5% and without synthetic pigment; L1 - flax-corn meal (13.5%); L2 - flax-corn meal (22.5%); C1 - camelina-corn meal (16.6%); C2 - camelina-corn meal (27.6%); H1 - hemp-corn meal (18.4%); H2 - hemp-corn meal (30.7%). All experimental groups contain 1% carrot and 0.5% paprika.

Based on the results shown in Figure 2, the sensory attributes for acceptability and color uniformity of boiled eggs did not differ statistically significantly ($p>0.05$) between experimental and control groups, while a statistically significant difference was observed in color hue among them ($p<0.05$), except for K2 compared to H1 and C2. This indicated that

noticeable differences in the color of fresh yolks were not easily observed after boiling the eggs. The only sensory issue that may arise when manipulating the diet of laying hens is the development of a "fishy" taste and smell when a higher amount of polyunsaturated fatty acids, particularly fish oil in larger percentages (Gonzalez-Esquerra and Leeson, 2000), or plant oils such as flaxseed oil, is added to the feed.

According to the results shown in Figure 2, eggs from all groups had an egg-like smell, meaning there were no statistically significant differences between groups. However, eggs from L1 and L2 groups differed significantly ($p<0.05$) in taste compared to all other groups. In these groups, the laying hens were fed with co-extrudates of flaxseed at amounts of 13.5% and 22.5%, which aligns with literature suggesting that adding flaxseed in amounts greater than 10% negatively affects the sensory properties of eggs (Jiang et al., 1992; Hayat et al., 2010; Coorey et al., 2015).

In contrast to flaxseed, adding co-extrudates of camelina seed at amounts of 16.6% and 27.6% did not negatively impact the sensory properties of eggs, which is consistent with available literature (Rokka et al., 2002; Valkonen et al., 2007). Additionally, adding co-extrudates of hempseed at amounts of 18.4% and 30.7% did not negatively affect the sensory evaluation of eggs; rather, eggs from these groups were rated as the most desirable with maximum sensory scores for taste (5.00). The obtained results are consistent with available literature (Goldberg et al., 2012).

CONCLUSIONS

The inclusion of co-extrudates from flaxseed, camelina seed, and hempseed, as well as natural pigments (carrot and paprika), in hens' diets did not negatively affect the acceptability, homogeneity, or color (hue) of fresh egg yolks, nor the smell, taste, acceptability, homogeneity, or color (hue) of boiled egg yolks, except for co-extrudates from flaxseed, which negatively influenced the taste of boiled eggs ($p < 0.05$). In contrast to co-extrudates from flaxseed, the incorporation of co-extrudates from camelina seeds at concentrations of 16.6% and 27.6%, as well as co-extrudates from hemp seeds at concentrations of 18.4% and 30.7%, did not adversely affect the sensory evaluation of eggs. By adding 1% carrot and 0.5% paprika to all experimental diets, a yolk color ranging from 12.50 to 13.39 on the RYCF scale was achieved, which was rated as optimal (orange), with a maximum score of 5 for acceptability, homogeneity, and color (hue). This suggests that a specifically designed feed formulation for laying hens can yield functional eggs with desirable sensory attributes.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia No. 451-03-66/2024-03/200222.

REFERENCES

Coorey, R., Novinda, A., Williams, H., Jayasena, V., 2015. Omega-3 Fatty Acid Profile of Eggs from Laying Hens Fed Diets Supplemented with Chia, Fish Oil, and Flaxseed. Journal of Food Science 80, 180-187.

Delgado-Vargas, F., Paredes-López, O., Avila-González, E., 1998. Effects of sunlight illumination of marigold flower meals on egg yolk pigmentation. Journal of agricultural and food chemistry 46, 698-706

Goldberg, E.M., Gakhar, N., Ryland, D., Aliani, M., Gibson, R.A., House, J.D., 2012. Fatty acid profile and sensory characteristics of table eggs from laying hens fed hempseed and hempseed oil. Journal of Food Science 77, S153-S160.

Gonzalez-Esquerra, R., Leeson, S., 2000. Effect of feeding hens regular or deodorized menhaden oil on production parameters, yolk fatty acid profile, and sensory quality of eggs. Poultry Science 79, 1597-1602.

Hayat, Z., Cherian, G., Pasha, T., Khattak, F., Jabbar, M., 2010. Sensory evaluation and consumer acceptance of eggs from hens fed flax seed and 2 different antioxidants. Poultry Science 89, 2293-2298.

Hernandez, J., 2005. European consumer surveys about egg quality: How to improve the nutritional value, XIth European Symposium on the Quality of Eggs and Egg Products, pp. 23-26.

Jiang, Z., Ahn, D.U., Ladner, L., Sim, J.S., 1992. Influence of Feeding Full-Fat Flax and Sunflower Seeds on Internal and Sensory Qualities of Eggs. Poultry Science 71, 378-382.
Kralik, I., Kralik, Z., Zelić, S., 2014. Preferencije potrošača konzumnih jaja, 49th Croatian and 9th International Symposium on Agriculture, Poljoprivredni fakultet, Sveučilišta Josipa Jurja Strossmayera u Osijeku, pp. 156-160.
Nys Y, Guyot N. 2011. Egg formation and chemistry. In: Nys Y, Bain M, Vanimmerseel F, editors. Improving the safety and quality of eggs and egg products, vol. 1. Cambridge: Woodhead publishing, p. 83–132.
Rokka, T., Alén, K., Valaja, J., Ryhänen, E.L., 2002. The effect of a Camelina sativa enriched diet on the composition and sensory quality of hen eggs. Food Research International 35, 253-256.
Spasevski, N., Puvača, N., Pezo, L., Tasić, T., Vukmirović, Đ., Banjac, V., Čolović, R., Rakita, S., Kokić, B., Džinić, N., 2018. Optimisation of egg yolk colour using natural colourants. Europ. Poult. Sci. (Archiv für Geflügelkunde) 82: 1-17.
Surai, P.F., Speake, B.K., Sparks, N.H.C., 2001. Carotenoids in Avian Nutrition and Embryonic Development. 1. Absorption, Availability and Levels in Plasma and Egg Yolk. The Journal of Poultry Science 38, 1-27.
Surai, P.F., Sparks, N.H.C., 2001. Designer eggs: from improvement of egg composition to functional food. Trends in Food Science & Technology 12, 7-16.
Škrtić, Z., Kralik, G., Gajčević, Z., 2006. Obogaćivanje jaja s PUFA n-3. Krmiva: Časopis o hranidbi životinja, proizvodnji i tehnologiji krme 48, 95-103.
Valkonen, E., Venäläinen, E., Tupasela, T., Hiidenhovi, J., Valaja, J., 2007. Camelina sativa cake in layer diets improves yolk fatty acid composition, In: WPSA, C.B.o. (Ed.), XVIII European symposium on the quality of poultry meat and XII European symposium on the quality of eggs and egg products, Prague, Czech Republic, pp. 82-84.

HROMATOGRAFIJA

MASENA SPEKTROMETRIJA

GASNA HROMATOGRAFIJA

TRACE 1600 GC

Novu TRACE 1600 seriju gasnih hromatografa odlikuje jedinstveni modularni koncept i mogućnost zamene injektorskih i detektorskih modula od strane korisnika, te omogućava fleksibilnost konfiguracije. Stoga je pogodan kako za rutinske, tako i za najzahtevnije laboratorije.
TRACE 1600 može da se nadoveže sa čitavom paletom MS detektora, gde se kontrola i akvizicija podataka vrši kroz Chromeleon softver.

GC-MS/MS TRIPLE QUADRUPOLE SISTEMI

Najnovija generacija GC-MS/MS sistema TSQ 9610 u pogledu osetljivosti i robusnosti odgovora na najstrožije zahteve EU regulative za analizu dioksina i furana. Zahvaljujući novom jonskom izvoru (AEI) detekcioni limit instrumenta doseže ato opseg. NeverVent Technology omogućava da se bez narušavanja vakuuma promeni hromatografska kolona za svega 35 min, odnosno zameni josnki izvor za svega 5 min.

TSQ 9610 Triple Quadrupole GC-MS

TEČNA HROMATOGRAFIJA

Thermo Scientific portfolio za tečnu hromatografiju čini Vanquish serija, koja obuhvata HPLC, UHPLC, nano LC sisteme:

- Vanquish Horizon UHPLC sistem sa ultravisokim pritiscima do 1500 bara
- Vanquish Flex UHPLC sistem sa pritiscima do 1000 bara i Vanquish Core HPLC do 700 bara
- Vanquish Duo UHPLC sistem – sistem dizajniran za istovremeno izvođenje dve aplikacije

Vanquish Core HPLC system

LC-MS/MS TRIPLE QUADRUPOLE SISTEMI

TSQ Quantis+

Nova generacija triple stage quadrupole masenih spektrometara (TSQ Fortis+, TSQ Quantis+, TSQ Altis+) je bez kompromisa u pogledu osetljivosti i robusnosti. Zahvaljujući jedinstvenoj active ion management (AIM+) tehnologiji, unapređenom jonskom izvoru i elektron multiplajeru, pružaju pouzdanu kvantifikaciju. Model TSQ Altis+ je najprestižniji u seriji i pruža nenadmašnu osetljivost za sve tipove molekula u različitim matriksima.
TSQ maseni spektrometri se prodaju sa trogodišnjim fabričkom garancijom.

JONSKA HROMATOGRAFIJA

Dionex ICS-6000 HPIC sistem

Bilo da imate samo nekoliko uzoraka ili veliki broj, jednostavan analitički zadatak ili pravi izazov, nudimo odgovarajuće rešenje za analizu katjona i anjona:

- Modularni jonski hromatografi za zahtevne IC aplikacije: Dionex ICS-6000 HPIC sistemi
- Integrisani jonski hromatografi za rutinske analize, RFIC sistemi i kapilarni: Dionex Integrion HPIC, Dionex Aquion, Dionex Easion sistemi i Dionex ICS-4000 kapilarni HPIC sistem

ORBITRAP EXPLORIS LC-MS/MS SISTEMI

Orbitrap Exploris HRAM serija masenih spektrometara nudi izuzetnu osetljivosti i selektivnost, te omogućava analize do najnižih nivoa sa visokom kvantitativnom tačnošću i preciznošću. U zavisnost od kompleksnosti analita, možemo da ponudimo tri modela:

- Orbitrap Exploris 120: rezolucija 120.000 m/z, za male molekule
- Orbitrap Exploris 240: rezolucija 240.000 m/z, za male molekule i peptide
- Orbitrap Exploris 480: rezolucija

Orbitrap Exploris 240 MS

ORBITRAP ASTRAL MASENI SPEKTROMETAR

Orbitrap Astral maseni spektrometar kombinuje visoku produktivnost, sveobuhvatnu pokrivenost i kvalitet podataka i veću osetljivost pružajući tačnu i preciznu kvantifikaciju u širokom dinamičkom opsegu. Kombinacija tri masena analizatora (maseni filter visokih rezolucija, Orbitrap™ maseni analizator i novi Astral maseni analizator) omogućava brzu akviziciju izvanrednih HRAM podataka sa visokom osetljivošću i dinamičkim opsegom. Performanse ovog instrumenta čine ga idealnim za tačnu i preciznu kvantifikaciju bez presedana, u analizi kako pojedinačnih ćelija tako i masovnih uzoraka.

HRAM ORBITRAP TRIBRID LC-MS/MS SISTEMI

Orbitrap Eclipse Tribrid MS

Orbitrap Tribrid sistemi su najnapredniji HRAM maseni spektrometri na svetu, koji kombinuju najbolje osobine kvadrupola, Orbitrapa i jonskih zamki i omogućavaju analizu najzahtevnijih uzoraka. Rezolucija i povećana brzina ciklusa čine ove uređaje pogodnim za rad na uzorcima visoke kompleksnosti koji se sreću u proteomici, metabolomici i drugim high-throughput tehnikama. Kroz veliki broj tehnoloških inovacija omogućena je identifikacija više jedinjenja, tačnija kvantifikacija i otkrivanje više strukturnih detalja.

SOFTVER ZA HROMATOGRAFIJU CHROMELEON 7.3 I CHROMELEON CDS ENTERPRISE

Chromeleon 7.3 CDS softver

Chromeleon 7.3 CDS hromatografski softver obezbeđuje najbrži put od uzorka do rezultata. Podržava kontrolu masenog spektrometra i obradu podataka svih separacionih tehnika (LC, GC, IC). Softver je potpuno integrisan sa GC-MS/MS I LC-MS/MS instrumentima kao i hromatografskim sistemima drugih proizvođača opreme.

STELLAR MASENI SPEKTROMETAR

Thermo Scientific™ Stellar™ maseni spektrometar donosi inovacije u osetljivosti, specifičnosti i produktivnosti. Stellar maseni spektrometar kombinuje revolucionarne hardverske i softverske napretke sa robusnom kvantifikacijom. Povećajte pouzdanost rezultata zahvaljujući specifičnosti hiper-brze MSn akvizicije u punom skeniranju iz kvadrupolne-linearne ionske zamke i detektorskog sistema, koji omogućava osetljivost za pojedinačne jone.

Orbitrap Exploris 240 MS

ANALYSIS LABORATORY EQUIPMENT

ANALYSIS d.o.o. • Japanska 4, 11070 Novi Beograd • Tel/fax: +381 11 318 64 46; +381 11 318 64 48
e-mail: info@analysis.rs • **www.analysis.rs**

MOLEKULSKA SPEKTROSKOPIJA

INFRACRVENA SPEKTROSKOPIJA I MIKROSKOPIJA (FT-IR)

Nicolet iS50 FT-IR spektrometar sa Nicolet RaptIR FT-IR mikroskopom

Thermo Nicolet iS5 FT-IR spektrometar obezbeđuje napredne FT-IR performanse u kompaktnoj veličini po pristupačnoj ceni i predstavlja idealno rešenje za industrijske, forenzičke i akademske laboratorije. Model Nicolet iS50 pokriva VIS, NIR, MIR i FAR oblast. Preporučeni model FT-IR mikroskopa: Nicolet iN10 i novi Nicolet RaptIR pogodan za široki spektar industrija.

EXTREVA ASE AUTOMATIZOVANI EKSTRAKTOR RASTVARAČEM (ACCELERATED SOLVENT EXTRACTOR)

Automatizovano bez ikakve intervencije, izvršite ekstrakciju uzoraka, cleanup u ekstrakcionoj ćeliji i evaporaciju—sve u jednoj besprekornoj operaciji—skratite vreme manuelnog rada sa sati na minute i omogućite sebi da se fokusirate na druge laboratorijske prioritete dok koristite do 50% manje rastvarača od drugih tehnika pripreme uzoraka . EKSTREVA ASE ubrzani ekstraktor rastvarača nudi sveobuhvatnu pripremu uzoraka: automatski ekstrahuje i koncentriše uzorke, eliminišući potrebu za ručnim premeštanjem ekstrakta uzoraka na drugi uređaj za isparavanje rastvarača.

UV-VIS SPEKTROFOTOMETRIJA

Evolution One UV-Vis

Thermo Scientific nudi široku paletu UV-Vis spektrofotometara, od jednozračnih do dual i double beam UV-Vis spektrofotometara, koji zahvaljujući svojoj raznovrsnosti nalaze primenu u edukaciji, kontroli kvaliteta i istraživačkom radu.

RAMANSKA SPEKTROMETRIJA

DXR RamanSmart spektrometar dizajniran je za rutinske i procesne analize u analitičkim i razvojnim laboratorijama. Zahvaljujući izuzetnim performansama, nalazi primenu i u istraživačkim, akademskim i forenzičkim laboratorijama. Omogućava jednostavno uzorkovanje i minimalnu priprema uzorka, kao i snimanje kroz pakovni materijal.

DXR3 Raman Mikroskop

KOLONE I POTROŠNI MATERIJAL ZA HROMATOGRAFIJU

Thermo Scientific nudi veliki izbor kolona za tečnu, gasnu i jonsku hromatografiju po povoljnim cenama, kao i veliki izbor potrošnog hromatografskog materijala za pripremu uzorka: špric filtere, Quecherse, SPE kertridže, viale, itd.

ANALITIČKA OPREMA

WE ARE BULDING A TEAM THAT LOVES TO WORK TOGETHER

ELEMENTALNA ANALIZA

thermo scientific

MASENA SPEKTROMETRIJA SA INDUKOVANO-SPREGNUTOM PLAZMOM (ICP-MS)

Thermo Scientific portfolio ICP-MS instrumenata sadrži single quadrupole (ICP-MS SQ), triple quadrupole (ICP-MS TQ) kao i masene spektrometre visoke rezolucije (ICP-MS HR). Fleksibilnost i jednostavnost korišćenja u kombinaciji sa visokom osetljivošću i stabilnošću instrumenta čine ih idealnim za kvalitetne rutinske kao i za zahtevne analize.

iCAP TQ ICP-MS

SPEKTROMETRIJA SA INDUKOVANO-SPREGNUTOM PLAZMOM (ICP-OES)

iCAP PRO XPS ICP-OES

Nova Thermo Scientific iCAP PRO serija ICP-OES obezbeđuje veoma brzu multielementalnu analizu tragova elemenata. Instrument karakterišu napredne performanse, visoka produktivnost, jednostavno korišćenje, pouzdani rezultati u skladu sa propisanim regulativama i standardima. Modeli: iCAP PRO i PRO X - osnovna konfiguracija; iCAP PRO XP - rutinske analize pogodne za QA/QC laboratorije i iCAP PRO XPS - na najzahtevnije analize, namenjene R&D laboratorijama.

FLASH SMART CHNS/O, N-protein, TOC ANALIZATOR

FlashSmart analizator rešava više laboratorijskih zadataka, poboljšava i ubrzava laboratorijski rad i skraćuje vreme trajanja analize, zahvaljujući Dumas metodi koja omogućava visoku fleksibilnost i modularnost u radu, nudeći više od dvadeset konfiguracija u jednom instrumentu. Moćni softver podržava automatizaciju i precizno izveštavanje.

FlashSmart™ elementalni analizator

ANALYSIS LABORATORY EQUIPMENT

ANALYSIS d.o.o. • Japanska 4, 11070 Novi Beograd • Tel/fax: +381 11 318 64 46; +381 11 318 64 48
e-mail: info@analysis.rs • **www.analysis.rs**

MCLABOR

LABORATORIJSKA I PROCESNA OPREMA

Anton Paar

KPM
WE CRAFT ASSURANCE

Brabender®
A brand of Anton Paar

AMS Alliance · BRUINS INSTRUMENTS · CHOPIN TECHNOLOGIES · EyePro System · PROCESS SENSORS CORPORATION · Unity · SIGHTLINE
(a KPM brand)

Hitachi High-Tech

OFFICE@MCLABOR.CO.RS

DOBANOVAČKA 101
11080 ZEMUN

WWW.MCLABOR.CO.RS

EKSLUZIVNI DISTRIBUTER I ZASTUPNIK
PRODAJNA, SERVISNA I APLIKATIVNA PODRŠKA

- MERAČI GUSTINE
- MERAČI GASOVA
- MERAČI PROTOKA
- VISKOZIMETRI
- REOMETRI
- NIR ANALIZATORI (BRZO ODREĐIVANJE HEMIJSKOG SASTAVA)
- MIKROTALASNA PRIPREMA UZORAKA
- MERAČI VELIČINE ČESTICA
- MULTIPARAMETRASKI HEMIJSKI ANALIZATORI
- XRF ANALIZATORI
- PETROHEMIJSKA OPREMA
- EKSTRUDERI
- VIZUELNI INSPEKCIONI SISTEMI ZA KONTROLU ILI SORTIRANJE MATERIJALA
- POLARIMETRI
- REFRAKTOMETRI
- PROCESNA OPREMA ZA INDUSTRIJU PIĆA
- XRD SISTEMI

CIP - Каталогизација у публикацији
Библиотеке Матице српске, Нови Сад

663/664:658.562(082)
614.31(082)

INTERNATIONAL Congress "Food Technology, Quality and Safety" (5 ; 2024 ; Novi Sad)
e-Proceedings [Elektronski izvor] / 5th International Congress "Food Technology, Quality and Safety", 16-18 October 2024, Novi Sad ; [main editor Nemanja Teslić]. - Novi Sad : Institute of Food Technology, 2024

Način pristupa (URL): https://fins.uns.ac.rs/publications/?_sft_publicationss=zbornik. - Opis zasnovan na stanju na dan 22.10.2024. - Bibliografija uz svaki rad.

ISBN 978-86-7994-064-3

а) Животне намирнице -- Контрола квалитета -- Зборници б) Животне намирнице -- Хигијена -- Зборници

COBISS.SR-ID 155103753