PROCEEDINGS

9th International CONGRESS of
Food Technologists, Biotechnologists and Nutritionists

International CONGRESS of Food Technologists, Biotechnologists and Nutritionists

HOTEL INTERNACIONAL, ZAGREB, CROATIA
October 03-05, 2018

Published by:

Croatian Society of Food Technologists, Biotechnologists and Nutritionists,
Pierottijeva 6, 10 000 Zagreb, Croatia

Editor-in Chief:
Karin Kovačević Ganić

Editorial board:
Karin Kovačević Ganić, Verica Dragović-Uzelac, Sandra Balbino

Printed by:
MGM studio Novel d.o.o., Zagreb

ISSN 2584-5292

Organized by:

CROFOST - Croatian Society of Food Technologists, Biotechnologists and Nutritionists, Zagreb, Croatia

PBF - University of Zagreb, Faculty of Food Technology and Biotechnology, Zagreb, Croatia

Co-sponsored by:

Organized under the auspices of:
Kolinda Grabar-Kitarović, President of the Republic of Croatia
Ministry of Science, Education and Sports of the Republic of Croatia
Ministry of Agriculture of the Republic of Croatia
Ministry of Health of the Republic of Croatia
Croatian Chamber of Economy

CONGRESS COMMITTEES

Organizing Committee

President of the Congress and Organizing Committee:
Verica Dragović-Uzelac

Congress Secretary:
Sandra Balbino

Damir Ježek
Jasna Mrvčić
Dubravka Škevin
Helga Medić
Kata Galić
Irena Colić Barić
Božidar Šantek
Zoran Herceg
Antonio Starčević
Marina Krpan
Mladen Brnčić
Ivančica Delaš
Filip Dujmić
Sven Karlović
Ivona Elez Garofulić
Davor Valinger

Sandra Balbino
Marina Tomašević
Zoran Zorić
Sandra Pedisić
Drago Šubarić
Dragan Kovačević
Marin Roje
Igor Jerković
Dijana Gluhak Spajić
Blaženka Kos
Anita Slavica
Tomislav Bosiljkov
Tullia Gallina Toschi, Italy
Roland Ludwig, Austria
Mirjana Gurinović, Serbia

Scientific Programme Committee

President of the Scientific Programme Committee:
Karin Kovačević Ganić

Irena Barukčić
Nada Vahčić
Branka Levaj
Duška Ćurić
Danijela Bursač Kovačević
Predrag Putnik
Jadranka Frece
Zvonimir Šatalić
Suzana Rimac Brnčić
Tonči Rezić
Anet Režek Jambrak
Ksenija Marković
Ivana Radojčić-Redovniković
Jurislav Babić
Stela Jokić
Rozelindra Čož-Rakovac

Jelena Đugum
Karolina Brkić Bubola
Jagoda Šušković
Mirna Habuda-Stanić
Marin Roje
Božidar Šantek
Vladimir Mrša
Krešimir Gjuračić
Jelka Pleadin
Gustavo Barbosa-Cánovas, USA
Francesco Marra, Italy
Francisco J. Barba, Spain
Tullia Gallina Toschi, Italy
Olivier P. Thomas, Ireland
Roland Ludwig, Austria
Mirjana Gurinović, Serbia

The official language of the Congress is English.

Acknowledgement to reviewers
The Editors of the Proceedings express deepest gratitude to the manuscript reviewers who maintained the professional standards of our Proceedings.

TABLE OF CONTENTS

Effect of non-thermal processing techniques on the aroma compounds in white wine Graševina

Marina Tomašević, Katarina Lukić, Karla Kelšin, Damir Ježek, Tomislava Vukušić, Natka Ćurko, Vlatka Poturica, Stela Križanović, Karin Kovačević Ganić

University of Zagreb Faculty of Food Technology and Biotechnology, Zagreb, Croatia (mtomasevic1@pbf.hr)

ABSTRACT

The non-thermal technologies have been extensively studied in past decade, especially in terms of food safety and preservation. In wine industry, safety is often related with sulfur dioxide due to its potential allergic properties and, importance of its reduction or even elimination has been increasingly emphasized. The aim of this study was to evaluate the effect of two non-thermal techniques: high hydrostatic pressure (HHP) and non-thermal plasma (NTP) on the changes of aroma compounds in sulfur dioxide-reduced white wine Graševina (Vitis vinifera L.). During HHP treatment different pressures (200, 400 and 600 MPa) and treatment durations (5, 15 and 25 min) were applied, while NTP treatment included variations of frequency (60, 90 and 120 Hz) as well as treatment duration (3, 5 and 10 min). Immediately after the treatments, wines were subjected to solid-phase microextraction and gas chromatography-mass spectrometry analyses (SPME-GC/MS). Results showed changes in concentrations of the main aroma groups after applied techniques. But, in the case of HHP those changes were pronounced in a much lesser extent than after NTP, where significant decrease in concentrations of acetate esters along with increase of higher alcohols content was observed. Furthermore, longer treatment duration of NTP resulted in more noticeable changes than higher applied frequency. Consequently, obtained results suggest that these techniques, especially HHP, could have an important role in winemaking, primarily for microbial stabilization of wines with reduced sulfur dioxide content.

Keywords: aroma compounds; high hydrostatic pressure; non-thermal plasma; white wine; GC/MS

INTRODUCTION

There is a growing interest for the non-thermal processing of foods, especially fruits and vegetables and their products, primarily due to the preservation of the heat-sensitive bioactive compounds. Moreover, the presumption that non-thermal processing is energy efficient and environmentally friendly additionally makes them very attractive. Other benefits to the food industry include the provision of food safety and new market opportunities (Tokuşoğlu, Swanson, 2014). In recent years, many investigations are related with non-thermal processing technologies where, among others, the application of high hydrostatic pressure (HHP) and non-thermal plasma (NTP) is often emphasized.

HHP represents process that employs pressures in range of 200-1200 MPa with only small increase in processing temperature. It is interesting alternative to traditional food processing and preservation due to its limited effects on covalent bonds that results in minimal modifications in nutritional and sensory quality (Oey et al., 2008). Recent studies showed that HHP was successfully applied on wine for the inactivation of undesired microorganisms (Buzrul, 2012; Briones-Labarca et al., 2017), for increasing the extraction of polyphenolic compounds from grapes and improving the overall quality of wine (Morata et al., 2015), as well as for accelerating the aging process of wine (Santos et al., 2015; Sun et al., 2016). On the other hand, and to the best of our knowledge, there is a lack of information about the effect of NTP technique on wine. Only researches available are our recent investigations (Lukić et al., 2017a; Lukić et al., 2017b) where slight changes in physicochemical properties, chromatic characteristics and phenolic composition of treated wine were estimated. Generally, plasma is described as partially or completely ionized gas with characteristic electrical, chemical and physical properties, which can be generated by many methods such as electrical discharges (corona, spark, glow, arc, microwave discharge, plasma jets and radio frequency plasma) and electrically, magnetically and chemically driven shocks (Petitpas et al., 2007). The most important physical effects are a high electric field, intense UV radiation and overpressure shock waves (Zhang et al., 2009), while major chemical

effect is manifested through the generation of reactive species, such as hydrogen peroxide, hydroxyl, oxygen and hydrogen radicals (Locke et al., 2006).
Despite mentioned studies, there is a lack of information about influence of these technologies on aroma compounds, as one of the main quality parameters of wine. Hence, the aim of this research was to investigate their effect on the main aroma groups in white wine Graševina (*Vitis vinifera* L.).

MATERIALS & METHODS

Wines
The wines used in this study were young white wine Graševina (*Vitis vinifera L.*), vintage 2016, obtained from winery Erdutski vinogradi (Erdut, Croatia). Physicochemical properties of wine were: alcoholic strength, by volume 11.4 vol %, total acidity (as tartaric acid) 5.1 g/L, volatile acidity (as acetic acid) 0.31 g/L, pH 3.37, reducing sugars 2.8 g/L, total extract 20.2 g/L, malic acid 1.2 g/L.

Chemicals
Ethanol was HPLC grade and purchased from J.T. Baker (Deventer, Netherlands), sodium chloride p.a. was purchased from Carlo Erba (Val de Reuil, Spain), while the aroma reference standards were purchased from Sigma Aldrich (St. Louis, USA).

High hydrostatic pressure (HHP) treatment
High hydrostatic pressure system FPG7100 (Stansted Fluid Power, Harlow, UK) was used to perform HHP treatments. The 100 mL of wine was poured into plastic bottle, vacuum sealed in bag and placed in the pressure chamber (maximum capacity of 2 L) with propylene glycol as the compression fluid. Different processing parameters were applied in order to assess the possible effects of the HHP treatment: pressures (200, 400 and 600 MPa) and pressure holding times (5, 15 and 25 min). All the treatments were carried out in triplicate and at room temperature (25 °C).

Non-thermal plasma (NTP) treatment
The plasma treatments were conducted with a point to point electrode configuration in a so-called hybrid reactor with discharges in and above the liquid with 1000 mL glass vessel. Configuration and parameters of NTP were in detail described by Lukić et al. (2017b). 300 mL of wine was treated with plasma at the combination of following processing parameters: frequency at 60, 90 and 120 Hz and treatment duration of 3, 5 and 10 min. Similar to HHP, all treatments were conducted in triplicate and at room temperature (25 °C).

Analysis of aroma compounds
Aroma compounds were analyzed by gas chromatography coupled with mass spectrometry (GC/MS) using an Agilent Gas Chromatography 6890 series equipped with an Agilent 5973 Inert mass selective detector (Agilent Technologies, Santa Clara, USA), with prior extraction by solid-phase microextraction (SPME). Applied method was in detail described by Tomašević et al. (2017).

RESULTS & DISCUSSION

GC/MS analyses resulted in quantification of over 20 different aroma compounds in treated, as well as in non-treated, control Graševina wine. Due to the large quantity of obtained data, quantified aroma compounds were grouped, according to the chemical group they belong to, in acetate esters, ethyl esters, higher alcohols and volatile fatty acids, and the results were presented in Figures 1 and 2.
The influence of process parameters of HHP treatment on the main aroma groups were shown in Figure 1. As can be seen, applied treatment influenced slight changes in concentration of analyzed aroma compounds. Primarily, those changes were negligible decrease of acetate esters and volatile fatty acids (Figure 1.a and d), as well as more pronounced decrease of ethyl esters (Figure 1.b). Furthermore, opposite trend was established regarding the concentration on higher alcohols (Figure 1.c) where application of HHP influenced slight increase in their concentration. Also, it can be seen that higher pressures along with longer treatment duration resulted in more pronounced effect. In other words, the

lowest pressure applied (200 MPa) during 5 min influenced minor changes, while the highest pressure (600 MPa), especially during 25 min, resulted in the most notable ones.
Contradictory results regarding the effect of this technique on changes in wine aroma could be observed in published literature. For example, application of HHP did not affected changes in concentration of several aroma compounds (aldehydes, ethyl acetate, propanol, *i*-butanol, butanol and *i*-amyl alcohol) in research conducted by Briones-Labarca et al. (2017). On the other hand, Santos et al. (2015) determined significant changes of aroma compounds after applied treatment. Main difference between those investigation was the time of analysis, wherein the latter authors investigated long-term effect of HHP (9 months after treatment) and upper investigation was conducted, similar to our case, immediately after the treatment. Hence, this technique could have an important role for wine aging since the compounds found after 9 months of storage were acetals, ketones, furans and aldehydes, compounds that are usually characteristic for wine aging aroma (Santos et al., 2015). Additionally, Briones-Labarca et al. (2017) also investigated the short-term effect of HHP on several aroma compounds (mainly higher alcohols) and they found no changes in concentrations of analyzed compounds after the treatment, which is in line with our conclusions. It is, therefore, necessary to investigate the long-term effect of this technique prior to making general conclusions.

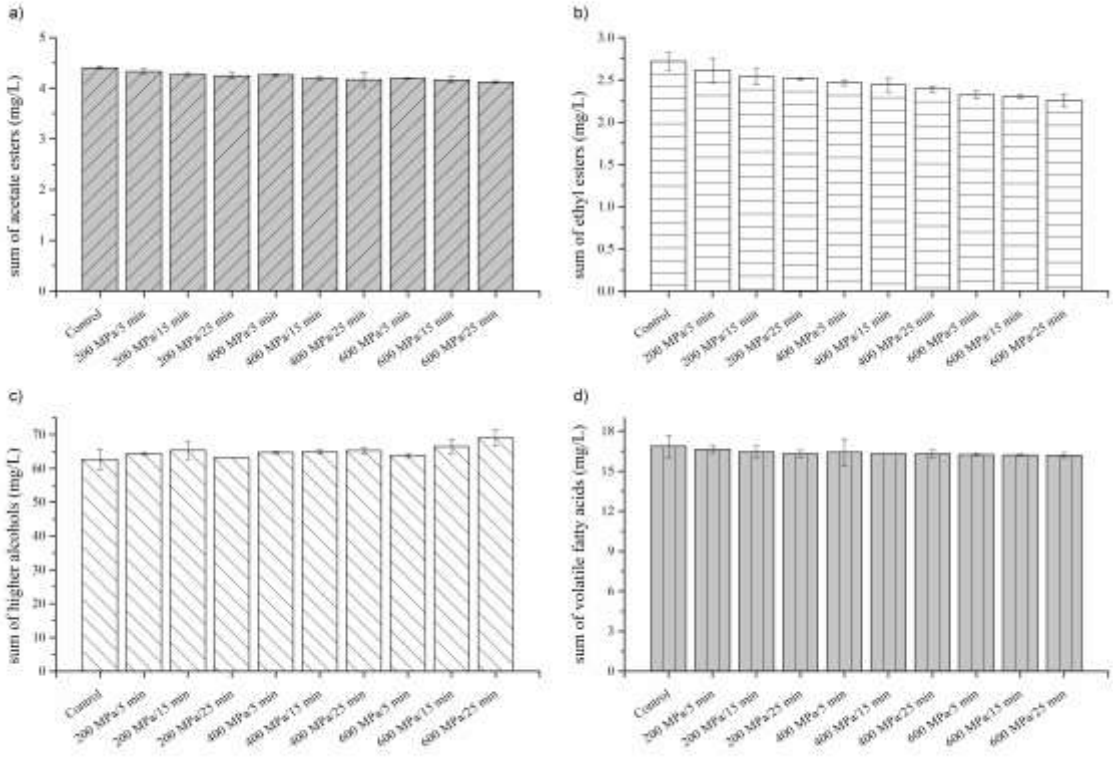


Figure 1. Influence of HHP treatments on the concentrations of main groups of aroma compounds in Graševina wine: a) sum of acetate esters, b) sum of ethyl esters, c) sum of higher alcohols and d) sum of volatile fatty acids.

Figure 2. shows the effect of NTP process parameters on the changes of previously stated aroma compounds. It can be seen that this technique resulted in more pronounced changes when compared to HHP treatments. Primarily, those changes are related to the higher decrease of acetate esters and volatile fatty acids, as well as higher increase of higher alcohols, while decrease in concentrations of ethyl esters was similar as for HHP treatment. Opposite to the HHP, the trend established in the case of NTP was not uniformly correlated with applied process parameters. For example, concentrations of acetate esters (Figure 2.a) decreased in correlation with treatment duration, while higher frequencies did not result in their lower concentrations. Furthermore, different trends could be observed in the case of ethyl esters and volatile fatty acids (Figure 2.b and d), where the longest treatment duration (10 min) resulted in

increment of these compounds. Also, as stated for acetate esters, observed changes are more pronounced at longer treatment duration in comparison to the applied frequency. Finally, like HHP effect, increase in concentration of higher alcohols (Figure 2.c) was also observed after NTP application. Similar to the other groups of aroma compounds, changes were more pronounced in correlation with longer treatment duration, while applied frequency was established as less important factor. These changes could be due to the plasma mechanisms such as produced shock waves and generation of hydroxyl radicals during water photodissociation (Boussetta, Vorobiev, 2014). In the case of application of NTP in wine production, to the best of our knowledge, no studies have investigated the influence of this technology on the volatile aroma compounds of wine. As more, no studies investigated its influence on aroma of other fruit beverages. There is only one research dealing with the effect of NTP on volatile components of tomato juice (Ma, Lan, 2015) where they found that NTP treatment increased concentrations of several compounds (trans-2-hexenal, m-xylene and glycerol). On the other hand, some components, such as ester ethyl laurate were lost after NTP which is in line with our results where the decrease in concentration of esters was established, respectively. As in the case of HHP, it is very important to make further investigation of the NTP influence on overall wine quality as well as to examine its long-term effect.

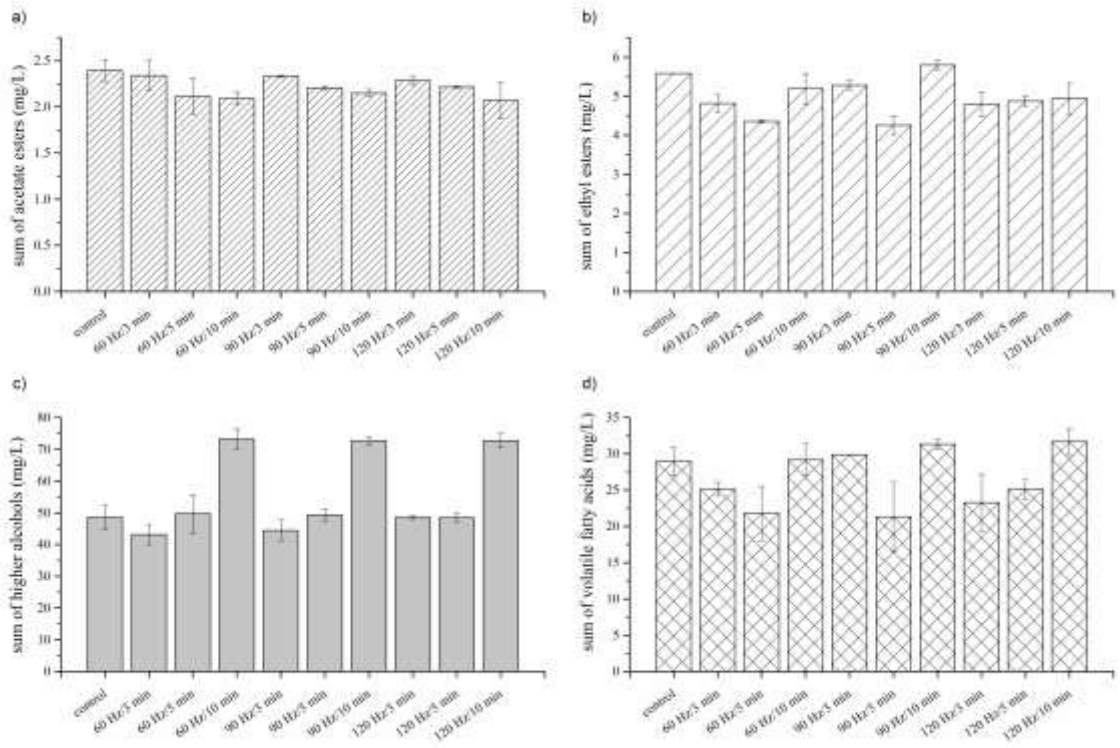


Figure 2. Influence of NTP treatments on the concentrations of main group of aroma compounds in Graševina wine: a) sum of acetate esters, b) sum of ethyl esters, c) sum of higher alcohols and d) sum of volatile fatty acids.

ACKNOWLEDGEMENT

This paper was fully funded by Croatian Science Foundation under the project IP-09-2014-3796.

CONCLUSION

Based on obtained results, it can be concluded that applied non-thermal techniques influenced changes in concentration of the main aroma groups. HHP treatment resulted in changes that were pronounced in

a much lesser extent than after NTP, where significant decrease in concentrations of acetate esters along with increase of higher alcohols content was observed. Regarding the applied process parameters, higher pressures as well as longer treatment duration of HHP resulted in more noticeable changes of analyzed compounds, while duration of NTP treatment was more important factor than applied frequency. Despite the observed changes, obtained results suggest that these techniques, especially HHP, could have an important role in winemaking, primarily for microbial stabilization of wines with reduced sulfur dioxide content, wherein further researches are obligatory in order to investigate their effect on overall wine quality.

REFERENCES

Boussetta, N., Vorobiev, E. 2014. Extraction of valuable biocompounds assisted by high voltage electrical discharges: a review. Comptes Rendus Chimie 17, 197-203.

Briones-Labarca, V., Perez-Wom, M., Habib, G., Giovagnoli-Vicuña, C., Cañas-Sarazua, R., Tabilo-Munizaga, G., Salazar, F. N. 2017. Oenological and Quality Characteristic on Young White Wines (Sauvignon Blanc): Effects of High Hydrostatic Pressure Processing. Journal of Food Quality 2017.

Buzrul, S. 2012. High hydrostatic pressure treatment of beer and wine: A review. Innovative food science & emerging technologies 13, 1-12.

Locke, B., Sato, M., Sunka, P., Hoffmann, M., Chang, J.-S. 2006. Electrohydraulic discharge and nonthermal plasma for water treatment. Industrial & engineering chemistry research 45, 882-905.

Lukić, K., Tomašević, M., Vukušić, T., Kelšin, K., Gracin, L., Ganić, K. K. 2017a. Influence of high voltage electrical discharge plasma treatment on the physicochemical characteristics of wine. Radovi Poljoprivredno-prehrambenog fakulteta Univerziteta u Sarajevu 67, 516-524.

Lukić, K., Vukušić, T., Tomašević, M., Ćurko, N., Gracin, L., Kovačević Ganić, K. 2017b. The impact of high voltage electrical discharge plasma on the chromatic characteristics and phenolic composition of red and white wines. Innovative Food Science & Emerging Technologies.

Ma, T. J., Lan, W. S. 2015. Effects of non-thermal plasma sterilization on volatile components of tomato juice. International Journal of Environmental Science and Technology 12, 3767-3772.

Morata, A., Loira, I., Vejarano, R., Bañuelos, M. A., Sanz, P. D., Otero, L., Suárez-Lepe, J. A. 2015. Grape processing by high hydrostatic pressure: Effect on microbial populations, phenol extraction and wine quality. Food and Bioprocess Technology 8, 277-286.

Oey, I., Lille, M., Van Loey, A., Hendrickx, M. 2008. Effect of high-pressure processing on colour, texture and flavour of fruit-and vegetable-based food products: a review. Trends in Food Science & Technology 19, 320-328.

Petitpas, G., Rollier, J.-D., Darmon, A., Gonzalez-Aguilar, J., Metkemeijer, R., Fulcheri, L. 2007. A comparative study of non-thermal plasma assisted reforming technologies. International Journal of Hydrogen Energy 32, 2848-2867.

Santos, M. C., Nunes, C., Rocha, M. a. M., Rodrigues, A., Rocha, S. M., Saraiva, J. A., Coimbra, M. A. 2015. High pressure treatments accelerate changes in volatile composition of sulphur dioxide-free wine during bottle storage. Food chemistry 188, 406-414.

Sun, X., Li, L., Ma, T., Zhao, F., Yu, D., Huang, W., Zhan, J. 2016. High hydrostatic pressure treatment: An artificial accelerating aging method which did not change the region and variety non-colored phenolic characteristic of red wine. Innovative Food Science & Emerging Technologies 33, 123-134.

Tokuşoğlu, Ö., Swanson, B. G. 2014. *Improving food quality with novel food processing technologies*, CRC Press.

Tomašević, M., Gracin, L., Ćurko, N., Kovačević Ganić, K. 2017. Impact of pre-fermentative maceration and yeast strain along with glutathione and SO2 additions on the aroma of *Vitis vinifera* L. Pošip wine and its evaluation during bottle aging. LWT - Food Science and Technology 81, 67-76.

Zhang, J.-F., Chen, J.-R., Li, X.-Y. 2009. Remove of phenolic compounds in water by low-temperature plasma: A review of current research. Journal of Water Resource and Protection 1, 99-109.

Compare between biscuits produced with Hull-less and Hulled barley

Gjore Nakov[a], Marko Jukić[b], Viktorija Stamatovska[c], Nastia Ivanova[a], Ana Šušak[b], Daliborka Koceva Komlenić[b]

[a] *Department of Biotechnology and Food Technology, University of Ruse "Angel Kanchev", Branch Razgrad, Aprilsko vastanie Blvd. 47, Razgrad 7200, Bulgaria (gore_nakov@hotmail.com ; nivanova@uni-ruse.bg)*
[b] *Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 20, HR-31000 Osijek, Croatia (marko.jukic@ptfos.hr ; ana.susak@ptfos.hr ; dkoceva@ptfos.hr)*
[c] *"St. Kliment Ohridski" University of Bitola, Faculty of Technology and Technical Sciences, Dimitar Vlahov bb, 1400 Veles, Republic of Macedonia (vikistam2@gmail.com)*

ABSTRACT

The use of non-traditional raw matetrials and flour and replacement of wheat flour with them, follows the modern trend of production of healthy bakery products.
The aim of this paper is to show the difference between flour from hull-less and hulled barley and biscuits produced form 100% hull-less and hulled barley flour.
The following analyzes were made on flour and produced biscuits (Method AACC 10-50D), moisture (ISO 6540), ash (ISO 5984:2002), antioxidant activity (DPPH method) and total polyphenols (determined with Folin-Ciocalteu reagent). After baking in biscuits was measuring the color of the biscuits in CIE L*a*b* system by using colorimeter (Konica Minolta Chroma Meter CR-400, Japan).
From the analyses made, we have found that hulled barley flour has higher moisture, ash, antioxidant activity and total polyphenols than hull-less barley flour.
Chemical analysis in biscuits show that heat treatment (baking) reduces the moisture, ash, antioxidant activity and total polyphenols in biscuits.
The produced biscuits with hulled barley flour was more lighter, less redder and yellower then biscuits produced with hull-less barley flour.

Keywords: biscuits; barley; hulled; hull-less; functional food;

INTRODUCTION

Today it is evident that functional foods can become an important portion of people's dietary intake (Lahouar et al., 2016). Functional foods are conventional foods containing substances that give them functional properties (Nakov et al., 2018). In this line, the great number of potentially active nutrients and their multifunctional properties make barley (*Hordeum vulgare*) are functional component for the production of health-promoting food. (Lahouar et al., 2016). From a nutritional point of view, the barley is low in fat, high in dietary fiber and contains essential amino acids in an amount equal to or greater than other cereals. Barley flour is a beneficial food for health. In particular, barley β-glucans are proven to reduce blood cholesterol and glycemic load (Behall et al., 2004; AbuMweis et al., 2010; Kourkoutaet al., 2017).
Barley is usually classified as spring and winter types, two-rowed or six-rowed and hulled or hulless (Gangopadhyay et al., 2015). Hulled barley is covered with palea and lemma, require dehulling to remove the tough inedible outer hull. Hulless barley has an outer hull that's so loosely attached to the kernel that it generally falls off during harvesting (Lahouar et al., 2016; Das, Kaur, 2016). Hull-less barley requires little or no effort to remove the hull during threshing or processing and would be more suitable for soaking and cooking purpose and comparatively more nutritious than hulled barley. Along with that, hull-less barley is superior in nutritional characteristics such as protein, starch, β-glucan, total dietary fiber and limiting amino acids compared with hulled barley (Mangan et al., 2015; Noaman, 2017).
Several researchers have suggested that barley can be incorporated into several food products like yogurt, bread, cookies, noodles, muffins and etc. (Gupta et al., 2011; Frost et al., 2011; Hamed, 2013; Beitane,

2013; Pejcz et al., 2016; Džafić et al., 2017; Badawi et al., 2017; Shehry, 2017; Alka et al., 2017). Biscuits, also known as cookies, are a snack food consumed extensively all over the world. (Gernah, Anyam, 2014). Biscuits are a diverse group bakery product ranging from varieties high and low in fat, high and low in sugar in more or less combination (Ahmad, Ahmed, 2014).

MATERIALS & METHODS

Materials

The raw materials used for preparing the biscuits include: hull-less barley, hulled barley, sugar, salt, sodium bicarbonate, butter, distilled water, glucose solution. All raw materials for production of biscuits were bought from local shops.

Methods

Two types of biscuits were produced. Biscuits were prepared according to the procedure described in AACC Method 10-50D (2000). The flour and biscuit samples were analysed for moisture, ash, total phenolic and corresponding antioxidant activities. Moisture content was determined according to ISO 6540:1980. Ash content was carried out according to ISO 5984:2002. Total phenolic contents and antioxidant capacities were determined according to the methods described by Wang and Ryu (2013). The total phenolics were assessed spectrophotometrically using the Folin-Ciocalteu reagent. The antioxidant activity of the samples was measured on the basis of the scavenging activities of the stable DPPH radical.

After baking in biscuits was measuring the color of the biscuits in CIE L*a*b* system by using colorimeter (Konica Minolta Chroma Meter CR-400, Japan).

Statistical analysis of calculated weighted grades has been made with the help of XLSTAT 2017 and Microsoft Office Excel 2013 programs. During processing the results in XLSTAT 2017 program, analysis of variance (ANOVA) and Fisher`s Least Significant Difference test (LSD) with an importance factor (significance) of 95% ($p<0.05$) have been used.

RESULTS & DISCUSSION

Table 1 presents the results of the chemical analysis of the flour produced by hull-less and hulled barley.

Table 1. Chemical characteristics of the flour produced by hull-less and hulled barley

Parameters	Hull-less barley flour	Hulled barley flour
Moisture (%)	9.92 ± 0.06^{a}	12.33 ± 0.04^{b}
Ash (%)	1.65 ± 0.04^{a}	3.16 ± 0.05^{b}
Antioxidant activity (%DPPH)	2.51 ± 0.03^{a}	12.12 ± 0.53^{b}
Total polyphenols (mgGAE/g)	0.14 ± 0.01^{a}	0.31 ± 0.00^{b}

**Values in the same row with different exponents (a-b) have statistically significant difference ($p<0.05$) ANOVA, Fisher`s LSD.*

The table shows that the flour produced by hull-less barley has lower moisture content (9.92±0.06%) and this is statistically significant ($p<0.05$) compared to the moisture content of the flour produced by hulled barley (12.33±0.04%). The amount of moisture in the flour is significant because of the possibility of storing it for longer. It is recommended for the moisture content of the flour to be less than 14%, or even better if it is below 12% (Gaćeša, 1997).

The ash does not contain organic compounds and its composition includes all the mineral matter contained in food, and nutritionally wise it helps in the metabolism of carbohydrates and other organic compounds (Mousa, Mousa, 2014). From the results obtained, it can be noted that the flour produced by hulled barley has a higher ash content (3.16±0.05%) and is statistically significant ($p<0.05$) compared to the flour produced by the hull-less barley (1.65±0.04%). The higher ash content (mineral matter) in the hulled barley flour is due to the minimal processing of the barley grain itself, and most of the coating and

endosperm remain unchanged. The hull-less barley grain partially loses hull containing the largest amount of mineral matter during the harvest and the amount of mineral matter is determined by the minerals contained in the endosperm of the grain and the parts left over from the hull (Hou and Jimeney, 2012).
Antioxidants and antioxidant systems prevent acute damage to health by "capturing" free radicals that can damage the cellular components (Webb, 2006). Regarding antioxidant activity, the flour produced from hulled barley distinguishes itself with greater antioxidant activity (12.12±0.53%DPPH). The antioxidant activity of this flour is statistically significant compared to the antioxidant activity of flour produced from hull-less barley (2.51±0.03%DPPH).
Polyphenols are metabolites present in plants. These compounds are known to have antioxidant activity and it is assumed that the antioxidant activity of the plants is due precisely to these compounds (Ivanišova et al., 2018). Apart from their role in plants, these nutrient-based compounds act positively on people's health primarily through reduced risk of chronic illness (Gani et al. 2012). From the results shown (table 1), the hulled barley flour has a larger total polyphenols content (0.31±0.00 mgGAE/g) and is statistically significant ($p<0.05$) compared to the total polyphenols content determined in the flour from the hull-less barley (0.14±0.01mgGAE/g).
The results from the analysis of the produced biscuits are provided in table 2. The moisture content in the biscuits is reduced in comparison to the moisture content in the used flour.

Table 2. Chemical characteristics of the produced biscuits

Parameters	Biscuits form 100% hull-less barley flour	Biscuits form 100% hulled barley flour
Moisture (%)	7.99 ± 0.54^{a}	9.10 ± 0.34^{b}
Ash (%)	1.65 ± 0.04^{a}	2.19 ± 0.01^{b}
Antioxidant activity (%DPPH)	2.38 ± 0.03^{a}	11.23 ± 0.04^{b}
Total polyphenols (mgGAE/g)	0.08 ± 0.01^{a}	0.15 ± 0.02^{b}

**Values in the same row with different exponents (a-b) have statistically significant difference ($p<0.05$) ANOVA, Fisher`s LSD.*

During thermal processing (baking), a heat and mass transfer occurs and at the same time the temperature in the biscuits increases. This thermal process affects the qualitative parameters of the produced biscuits, especially the moisture content and color of the biscuits (Nakov et al., 2017a). It can be seen from Table 2 that biscuits produced from 100% hulled barley flour have a higher moisture content (9.10±0.34%) and are statistically significant ($p<0.05$) compared to biscuits produced from 100% hull-less barley flour (7.99±0.54%). The ash content in biscuits produced from 100% hulled barley flour is higher (2.19±0.01%) and statistically significant ($p<0.05$) compared to biscuits produced from 100% hull-less barley flour (1.65±0.04%).
If comparing the ash content in the used flour, it can be noted that the amount of ash in the hulless barley flour remains unchanged after the thermal processing (baking). From the obtained results on the antioxidant activity of the obtained biscuits (table 2), it can be noted that biscuits with 100% hulled barley flour have a higher antioxidant activity (11.23±0.04%DPPH, which is statistically significant ($p<0.05$) compared to biscuits made with 100% hull-less barley flour (2.38±0.03%DPPH). If the antioxidant activity of the used flour and manufactured biscuits is compared, it can be noted that the process of thermal processing affects the antioxidant activity of the biscuits. The biscuits produced from 100% hulles hull-less s barley flour and 100% hulled barley flour have a lower antioxidant activity compared to the flour used (respectively). In terms of total polyphenols content determined in biscuits, the biscuits produced from 100% hulled barley flour are characterized by a greater total polyphenols content (0.15±0.02 mgGAE/g) which is statistically significant ($p<0.05$) compared to biscuits produced from 100% hull-less barley flour (0.08±0.01% mgGAE/g). The process of thermal processing affects the total polyphenols content. In biscuits, the total polyphenols thermal processing is approximately twice as low as the total polyphenols content in the used flour.

The basic ingredients for biscuit production are flour, sugar and fat. Dough is baked at high temperature (205 ^{0}C) for several minutes, to obtain low water content and brown surface of biscuits. During the baking process, heat and mass transfer occurs due to high temperatures. The heat is transferred with the warm air from the surface to the interior of the biscuits and the moisture from the interior to the surface. During the thermal processing (baking), several reactions (Maillard reaction and caramelization) take place which have a direct effect on the formation of the color of biscuits (Nakov et al., 2017b; Stamatovska et. al. 2017).

Table 3. The parameters for biscuits color (L *a *b*)

Time	L*		a*		b*	
	Hulled barley	Hull-less barley	Hulled barley	Hull-less barley	Hulled barley	Hull-less barley
5 min	57.51±2.26	68.27±1.20	2.29±0.34	1.80±0.12	19.98±1.60	23.79±0.47
6 min	57.39±1.80	67.91±1.03	2.33±0.56	2.45±0.12	22.59±1.04	25.01±0.78
7 min	56.46±0.36	67.32±0.78	2.43±0.64	3.37±0.65	23.22±2.37	27.35±1.46
8 min	55.47±2.30	63.73±3.91	3.08±0.57	4.87±0.33	24.55±1.60	27.73±2.59
9 min	54.81±2.77	61.94±0.87	5.48±0.56	7.74±0.78	27.13±0.54	30.95±0.45
10 min	53.43±2.91	58.68±0.94	6.06±0.88	9.53±0.76	28.84±1.49	31.00±0.48

• *The results are an average value of 5 measurements ±SD.*

Table 3 presents the parameters for biscuits color from 100% hulled barley flour and 100% hull-less barley flour, determined by the CIE L *a *b* system, at different baking times (5 to 10 min). Table 3 shows that the values of the L* parameter for biscuits produced from 100% hulled barley flour and 100% hull-less barley flour are reduced by extending the baking time from 5 to 10 min (57.51±2.26 - 53.43±2.91; 68.27±1.20-58.68±0.94, respectively). The closer the values for the L* parameter are to 100, the lighter the color of the biscuits is, and the closer these parameters are to 0, the darker the color is (Konica Minolta (09.03.2018)).

Values for the a* parameter for biscuits from 100% hulled barley flour and 100% hull-less barley flour are increased during baking from 5 to 10 min (2.29±0.34-6.06±0.88; 1.80±0.12- 9.53±0.76, respectively). The more negative the values of the parameter a* are, the greener the color of the biscuits is, the more positive the values of the parameter a* are, the redder the color is (Konica Minolta (09.03.2018)).

The values for parametar b* show the presence of blue or yellow color in the biscuits, i.e. the more negative the values of this parameter are, the bluer the biscuits are, and the more positive the values of this parameter are, the more yellow the biscuits are (Konica Minolta (09.03.2018)). The low moisture content and increased temperature influences the appearance of the yellow color (Bošnjaković, 2014). The values for the b* parameter for biscuits from 100% hulled barley flour and 100% hull-less barley flour shown in Table 3 increase from 5 to 10 minutes of baking (19.98±1.60-28.84±1.49; 23.79±0.47-31.00±0.48, respectively).

CONCLUSION

On the basis of the conducted analysis, it can be concluded that the flour obtained from hulled barley is characterized by higher moisture content, ash and total polyphenols, as well as greater antioxidant activity compared to 100% for biscuits from 100% hulled barley flour and 100% hull-less barley flour barley flour. Higher content in relation to the same parameters is found in biscuits produced from 100% hulled barley flour compared to biscuits produced from 100% for biscuits from 100% hulled barley flour and 100% hull-less barley flour barley flour. Hence, we can conclude that the results obtained for the flour correspond to the results obtained for biscuits but are generally lower due to the impact of thermal processing. From the obtained values for the color parameters of the obtained biscuits, determined using

the CIE L*a*b* system during baking from 5 to 10 minutes, it can be concluded that the values of the parameter L* are reduced, and the values for the parameters a*.

ACKNOWLEDGMENTS

The study was supported by contract of University of Ruse "Angel Kanchev", № BG05M2OP001-2.009-0011-C01, " Support for the development of human resources for research and innovation at the University of Ruse "Angel Kanchev". The project is funded with support from the Operational Program " Science and Education for Smart Growth 2014 - 2020" financed by the European Social Fund of the European Union.

REFERENCES

AACC 10-50D. 2000. Baking Quality of Cookie Flour, Approved Methods of the Association of Cereal Chemists, 10th ed. AACC, St. Paul, MN.

Abu Mweis S.S., Jew S., NP Ames P. N. 2010. β -glucan from barley and its lipid-lowering capacity: a meta-analysis of randomized, controlled trials. European Journal of Clinical Nutrition 64, 1472–1480.

Ahmad S., Ahmed M. 2014. A review on biscuit, a largest consumed processed product in India, its fortification and nutritional improvement. IJSIT 3(2), 169-186.

Alka V., Pinky B., Neelam K. 2017. Grab a healthy bite: Nutritional evaluation of barley based cookies. Asian J. Dairy & Food Res 36(1), 76-79.

Badawi S. I., Shahein R. M., Metwally M.M. 2017. Influence of Additives White Oat Flour and Barley Flour on Some Quality Characteristics of Yoghurt. World Journal of Dairy & Food Sciences 12 (2), 108-114.

Behall M. K., Scholfield J.D., Hallfrisch G.J. 2004. Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. Am J Clin Nutr 80(5), 1185-93.

Beitane I. 2013. The Chemical Composition of Yoghurt Enriched with Flakes from Biologically Activated Hullless Barley Grain and Malt Extract. International Journal of Nutrition and Food Engineering 7(3), 181-184.

Bošnjaković N. 2014. Application of digital image analysis to colours changes on tea biscuits made from wheat flour. Graduate thesis, Universitry Josip Juraj Strossmayer in Osijek, Faculty of Food Technology, Osijek.

Das M., Kaur S. 2016. Status of Barley as a Dietary Component for Human. Research & Reviews: Journal of Food and Dairy Technology, An Update on different dietary practices and recent trends in Dairy Industry - S1, 25-30.

Džafić A., Oručević-Žuljević S., Spaho N., Akagić A. 2017. Effects of barley flour addition and baking temperature on β-glucans content and biscuits properties. Technologica Acta 10(1), 35-44.

Frost J. D., Adhikari K., Lewis S. D. 2011. Effect of barley flour on the physical and sensory characteristics of chocolate chip cookies. J Food Sci Technol 48(5), 569–576.

Gaćeša S. 1997. MEBAK. Metodi analize u pivarstvu, knjiga 1, 3 izdanje, 74.

Gani A., Wani S.M., Masoodi F.A., Hameed G. 2012. Whole-Grain Cereal Bioactive Compounds and Their Health Benefits: A Reviw. Journal of Food Processing & Technology 3 (3):146.

Gernah I. D., Anyam K. 2014. Production and quality assessment of protein–rich biscuits from blends of wheat and defatted sesame flours. International Journal of Food Processing Technology 1(1), 27-31.

Gupta M., Bawa S. A., Abu-Ghannam N. 2011. Effect of barley flour and freeze–thaw cycles on textural nutritional and functional properties of cookies. Food and bioproducts processing 89, 520–527.

Hamed A. 2013. Effect of barley flour rich in β-Glucan on rheological properties of frozen dough and quality of bread and cookies, Master Thesis. University of Guelph, Ontario, Canada.

Hou G., Jimeney V..2012. Developing Barley – Fortified Wheat Based Foods. (http://www.barleyfoods.org/documents/barleyinbagelsbw.pdf).

International Standard Organisation (ISO): Maize – Determination of moisture content (on milled grains and on whole grains). ISO 6540:1980.

International Standard Organisation. 2002. ISO 5984:2002: Animal feeding stuffs - Determination of crude ash.

Konica Minolta https://sensing.konicaminolta.us/blog/identifying-color-differences-using-l-a-b-or-l-c-h-coordinates/ (09.03.2018)

Kourkouta L., Koukourikos K., Iliadis C., Ouzounakis P., Monios A., Tsaloglidou A. 2017. Bread and Health. Journal of Pharmacy and Pharmacology 5, 821-826.

Lahouar L., Arem E.L. A., Achour L. 2016. Bioactive compounds in whole grain barley: Nutraceutical properties and health benefitsJournal of Bioresources Valorization 1(1), 5-15.

Mangan B., Hui L., Lashari S. M., Shah N. A., Licao C., Weining S. 2015. Nutritional characteristics and starch properties of Tibetan barley. Int. J. Agri. Policy & Res. 3(7), 293-299.

Mousa R., Mousa A. 2014. Nutritional Assessment of Biscuits Formulated by Simultaneous Substitution with Sweet White Lipinoil and Extracted Flour after Germination. American Journal of Food and Nutrition 2(6), 108-116.
Nakov Gj., Ivanova N., Damzanova S., Stamatovska V., Saveski A., Koceva Komlenić D., Kosović I., Šušak A. 2017a. The influence of time of baking on the moisture and color of biscuits. International scientific conference of young scientist and student "Youth scientific achievements to the 21st centru nutrition problem solution", 14.
Nakov Gj., Koceva Komlenić D., Stamatovska V., Šušak A., Jukić M. 2017b. Influence on time of baking and different role of barley flour on the colour of the biscuits. Journal of Hygienic Engineering and Desing 21, 90-95.
Nakov Gj., Stamatovska V., Ivanova N., Damyanova S., Godjevargova Tz., Koceva Komlenić D. 2018. Physicochemical characteristics of functional biscuits and *In vivo* determination of glucose in blood after consumption of functional biscuits. Journal of Hygienic Engineering and Desing, 22, 25-32.
Noaman M.M. 2017. New Utilization of Barley as Human Healthy Food. J Plant Breed Agric. 1(1:2).
Pejcz E., Wojciechowicz-Budzisz A., Gil Z., Czaja A., Spychaj R., 2016. Effect of naked barley enrichment on the quality and nutritional characteristics of bread – Part I. The effect on wheat bread. Engineering Sciences & Technologies 2(21), 38-45.
Shehry A. A. G. 2017. Preparation and Quality Evaluation of Pasta Substituted with Hull-less Barley Aust. J. Basic & Appl. Sci. 11(1), 98-106.
Stamatovska V., Nakov Gj., Dimov I. Taneva I., Kalevska T., Uzunoska Z. 2017. The changes in colour of biscuits during baking depending on time and different proportion of barley flour. Conference Book of Abstract, 6th Annual International Conference, International Conference for Food Science and Technology IC-FST 2017, October 27-29, Durrës, Albania, 282.
Wang, Y.Y., Ryu, G.H. 2013. Physicochemical and antioxidant properties of extruded corn grits with corn fiber by CO_2 injection extrusion process. Journal of Cereal Science 58 (1), 110-116.
Webb P.G. 2006. Dietary Supplements and Functional Foods, In: Chapter 5. Free radicals and antioxidant, 107.

MATHEMATICAL MODELING OF AIR DRYING STRAWBERY WITH OSMOTIC PRETREATMENT

Ivan Pavkov[a], Zoran Stamenković[a], Milivoj Radojcin[a], Kešelj Krstan[a], Siniša Bikić[b], Monika Lutovska[c],

[a]Faculty of Agriculture, University of Novi Sad, 21000 Novi Sad, Serbia (ivan.pavkov@polj.uns.ac.rs)
[b]Faculty of Technical Science, University of Novi Sad, Bulevar cara Lazara 6, 21000 Novi Sad, Serbia (bika@uns.ac.rs)
[c] Faculty of Technical Sciences, University 'Mother Teresa', 1000 Skopje, Macedonia, (monika.lutovska@unt.edu.mk)

ABSTRACT

The aim of this study was to develop mathematical model for predicting the convective thin layer drying kinetics of strawberry slices, fresh and osmotic pretreated, to calculate the effective diffusivity and activation energy.
Fresh, untreated strawberries are cut on slices and dry in sucrose solution on temperature 50 ^{o}C and concentration 55 and 65 ^{o}Bx. After osmotic dehydration slices are dried in thin layer on air temperature 60, 70 and 80 ^{o}C and air velocity 1 m/s. The experimental data was fitted to five thin layer models, that are widely used in most food and biological materials. The determination of coefficient (R^2), reduced chi – square (χ^2) and root mean square error (RMSE) were used as the primary criterion to select the best equation to account for variation in the drying curves of the dried samples. The Page model was found to be a model for describing the characteristic of strawberry for all of experimental conditions.

Keywords: strawberry; osmotic dehydration; air drying; mathematical modeling.

INTRODUCTION

Strawberry is a very popular seasonal fruit available in Serbia during the spring and early summer period. Compared to other fruits, strawberry is highly appreciated for excellent organoleptic properties, such as red colour, fine texture, and unique flavour and taste. Also, strawberry is a good source of ascorbic acid and other antioxidants. They have a very short post harvest shelf life, which effects market potential and consumer access. Because strawberry is seasonal, most of the harvested fruits in Serbia are preserved by freezing or by processing into concentrated juices, jams, jellies, puree and marmalades. Strawberry are mainly used as dairy products or as fruit ingredients in biscuits, cookies etc.
Dried strawberry fruits are perfect as components of fruit and herbal teas, in muesli and desserts but also as healthy dried snacks. It can be preserved by drying processes such as air drying, osmotic dehydration with air drying, microwave and freeze drying (Adak et al., 2017; Agnieszka and Lenart, 2010; Piotrowski et al., 2004; Ferrando and Spiess, 2003).
Research carried out in last decade proves that osmotic dehydration in processing of fruits has very good effect on decreasing water content with simultaneous increasing of dry matter content and achieving attractive tasty and engaging product (Radojčin et al., 2010*)*. Osmotic drying as pre-treatment in fruit drying technology has influence on decreasing draying rate values during air and microwave drying *(*Pavkov et al., 2010; Piotrowski et al., 2004). Combination of osmotic dehydration with convective drying appears as good drying technology for dried fruits with reasonable energy consumption, a good shelf life and quality. The aim of this study was examining kinetics of air drying of strawberries in fresh state or after initial osmotic dehydration. Detailed analysis concerned influence of osmotic dehydration on drying curves, drying rate, rehydration and colour changes.

Mathematical models have provide to be very useful in design and analysis of heat and mass transfer processes during drying. All parameters used by simulation models are directly related to the drying conditions. Furthermore the drying conditions, as directly related to the drying time are effecting the energy demands (Menges and Ertekin; 2006). Thin layer drying equations are used to estimate drying times of several products and also to generalise drying curves. The drying kinetics of

all fruit and vegetables cannot be described by the same equation due to the difference in moisture content and typical transport phenomena during drying. Recently, there are lot of studies on drying kinetics of fruit and vegetables (Kingsly et al., 2007, Si et al., 2015, Menges and Ertekin, et al., 2006, Pavkov et al., 2017). However, work on the effect of pretreatmnents on drying kinetics of strawberries slices has not yet been reported. The aim of this study was to develop mathematical model for predicting the convective thin layer drying kinetics of strawberry slices, fresh and osmotic pretreated, to calculate the effective diffusivity and activation energy.

MATERIALS & METHODS

Fresh strawberry (Jolly) were obtained from local producer Intersad, Rumenka, Serbia and stored in a refrigerator at 4^{o}C until used. The stage of fruit ripeness was the beginning of technological ripeness with the value pH = 3.24. Strawberries of uniform size were selected for the experiment: length (31.55 ± 2.75 mm), width (30.1 ± 3.28 mm), thickness (25.9 ± 2.78 mm), mass 11,75± 2,68 g and moisture content 87.14% ±0.49% (wet basis). The average values of the main physical properties of fresh strawberry measured on a random sample of 20 fruits.

After washing in tap water and removing the external impurities, strawberries were cut into 4,0 ±0,5 mm thick slices along longitudinal axis. Slices were dipped in 5% ascorbic acid solution at room temperature (≈25 ^{o}C) during 3 min.

Strawberries for drying were prepared in two ways:

- untreated (in fresh state were introduced in to convective dryer) and
- pre-treated – OD (strawberries after osmotic dehydration were introduced in to convective dryer).

Osmotic dehydration was carried out in 55 ^{o}Bx and 65 ^{o}Bx sucrose solution at temperature 50 ^{o}C with steering for 2h. The mass ratio of the raw material to osmotic solution was 1:4.

The air drying experiments of untreated and pre-treated strawberries were carried out at air temperatures of 60, 70 and 80 ^{o}C and 1,0 m/s air velocity. The samples were placed on trays in thin layer in experimental convective dryer "IVA – 2" (Pavkov et al., 2009). Experimental dryer continuously recording mass changes of strawberry samples and air temperature. Specific weight of strawberry slices on trays was 1.56 kg/m^2. Each sample utilized in the experiment air drying weighed about 800 g. The amount of water removed during the air drying process was recorded at 5 minutes intervals by mass sensor integrated in drying chamber, with an accuracy of ± 1 g. Drying is continued until equilibrium moisture content reached. The drying data from air drying test were then expressed as moisture content on dry basis (X_t) and moisture ratio (MR) versus drying time.

The moisture ratio (MR) of air drying samples at any time (t) is calculated according to the following equation:

$$MR = \frac{X_t - X_{eq}}{X_o - X_{eq}} \quad (3)$$

where X_t, X_0 and X_{eq} are moisture content at any time (kg/kg d.b.); initial moisture content (kg/kg d.b.) and equilibrium moisture content (kg/kg d.b.).

The drying curves (MR – t) were fitted by means of five different moisture ratio models that are widely used in most food and biological materials (Table 1). Those models are generally derived by simplifying the general series solution of Ficks second law. Non-linear regression analysis was used to estimate the parameters of models.

Table 1 Mathematical models applied to the drying curves

No.	Model	Name of model	References
1.	MR = exp(−kt)	Newton	Bon et al., 2007
2.	MR = exp(−kt^n)	Page	Hassan-Beygi, et al., 2009
3.	MR = aexp(-kt)	Handerson and Pabis	Handesron and Pabis, 1961
4.	MR = aexp(−kt) + c	Logarithmic	Bon et al., 2007
5.	MR = 1 +at +bt^2	Wang and Singh	Wang and Singh, 1978

* k – drying constant, n – model exponent, a, b, c – model coeficinets aan t – drying time (h)

The determination of coefficient (R^2), reduced chi – square (χ^2) and root mean square error (RMSE) were used as the primary criterion to select the best equation to account for variation in the drying curves of the dried samples. Reduced χ^2 is used to determine the goodness of the fit. The lower the values of the reduced χ^2, the better goodness of the fit. The RMSE gives the deviation between the predicted and experimental values and it is required to reach zero (Serdar and Bese, 2016, Zhengfu et al., 2007, Ertekin and Megnes, 2006).

RESULTS & DISCUSSION

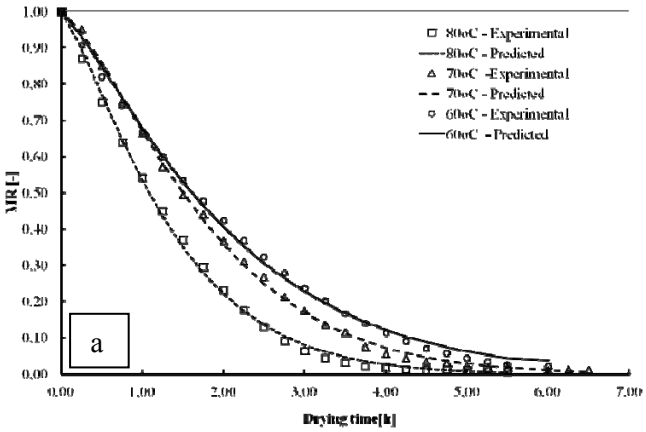
The results of air drying behavior fresh and osmotic treated strawberries slices are shown on the figure 1 (Pavkov et al., 2018). The five thin-layer drying models (Table 1) were evaluated in terms of the statistical parameters R^2, χ^2 and RMSE. In all cases, the R^2 values for the models were greater than 0.975, indicating a good fit (Table 2.). The R^2 values varied between 0,975 and 0,999, χ^2 values between 0,0248 and 9,84E-05, and RMSE values between 0.038 and 0,0043. Generally, Page model gave higher R^2 and lover χ^2 and RMSE values. Thus, it was selected to represent the thin-layer drying characteristics of strawberries satisfactorily. When the Page model analyzed according to the different drying air temperature and velocity conditions, individual constants could be obtained (Table 3.). Figures 1 (a, b and c) compare experimental data with the predicted ones using Page model for strawberries at air temperature 60, 70, 80 ^oC, air velocity 1.5 m/s, untreated and with osmotic treatment (OD).

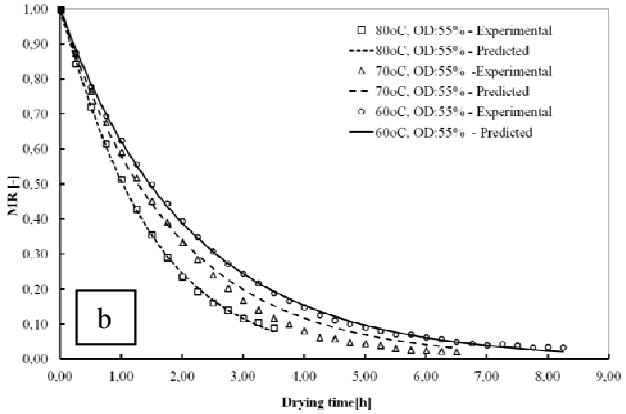
Table 2. Statistical results of five models at different drying conditions

Name of model	Treatment	Drying air temperature								
		60 ^oC			70 ^oC			80 ^oC		
		R^2	χ^2	RMSE	R^2	χ^2	RMSE	R^2	χ^2	RMSE
Newton	untreated	0.985	0.00135	0.0359	0.976	0.00248	0.0488	0.985	0.00142	0.03686
	OD: 55 %	0.999	4.3E-05	0.00648	0.9985	0.000117	0.0106	0.9985	0.000117	0.010421
	OD: 65 %	0,998	0.000128	0.01116	0.99714	0.000221	0.01464	0.994	0.00049	0.0218
Page	untreated	0.997	0.00025	0.0151	0.999	9.12E-05	0.009177	0.998	0.00017	0.012423
	OD: 55 %	0,999	4.20E-05	0.0063	0.9985	0.000117	0.0104	0.999	5.95E-05	0.007184
	OD: 65 %	0.997	6.08E-05	0.0076	0.999	6.51E-05	0.007825	0.995	0.00044	0.020005
Henderson and Pabis	untreated	0.989	0.00109	0.0317	0.985	0.00158	0.03815	0.989	0.00114	0.0322
	OD: 55 %	0.999	3.43E-05	0.0057	0.998	0.000120	0.0096	0.9987	0.00012	0.0100
	OD: 65 %	0.998	0.00012	0.0106	0.998	0.0131	0.00018	0.995	0.0197	0.000430
Logari-thmic	untreated	0.999	0.00010	0.00946	0.993	0.00076	0.0260	0.993	0.0007	0.0260
	OD: 55 %	0.999	2.45E-05	0.00688	0.999	7.98E-05	0.00842	0.999	9.84E-05	0.00887
	OD: 65 %	0.9988	6.82E-05	0.0119	0.998	0.00016	0.0119	0.996	0.00037	0.0178
Wang and Singh	untreated	0.999	3.68E-05	0.00567	0.999	0.000357	0.0178	0.994	0.00068	0.0243
	OD: 55 %	0.984	0.00106	0.0313	0.988	0.000965	0.0293	0.9987	0.000130	0.0102
	OD: 65 %	0.983	0.00136	0.0354	0.975	0.00206	0.0433	0.993	0.00064	0.0234

Table 3. Statistical results of Page model and its constants and coefficients at different drying conditions

Treatment	Drying air temperature					
	60 ^oC		70 ^oC		80 ^oC	
	k	n	k	n	k	n
untreated	-0.3868	1.2234	-0.3988	1.3599	-0.6269	1.2695
with OD:55 %	-0.4758	0.9896	-0.5462	0.9871	-0.6775	1.0546
with OD:65 %	- 0.4080	1.0581	-0.5265	1.0941	-0.6070	1.0557





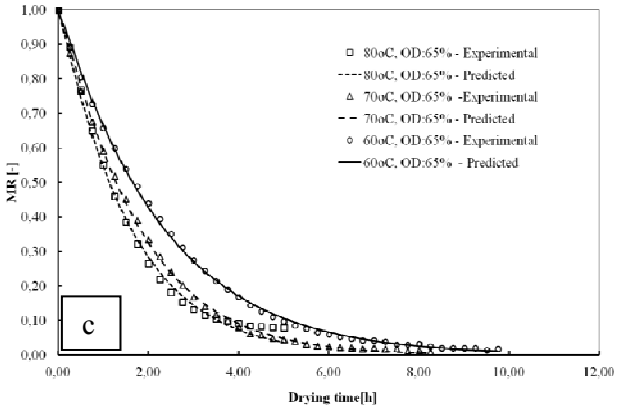


Figure 1. Experimental and predicted moisture ratio strawberries with drying time at different drying conditions: a - untreated, b – with osmotic treatment 55 %, c – with osmotic treatment 65 %

CONCLUSION

In order to explain the drying behaviour of thin layer drying strawberries slices, untreated and with osmotic pretretament five different thin layer drying model were compared according to their RMSE, chi-square and R^2. According to the results of thin layer drying of strawberries, Page model could be used to predict moisture content of the product at any time of drying process with high ability between

drying air temperatures 60 and 80oC, velocity of 1,5 m/s, untreated and with osmotic pretreatment between 55 and 65% sucrose.

ACKNOWLEDGEMENTS

This research was supported by the Serbian Ministry of Science and Technological Development – projects “Combined Technology of Integrated and Organic Fruit and Vegetable Drying” (Project no. TR 31058).

REFERENCES

Bon J., Rossello C., Femenia A., Eim V., Simal S. 2007. Mathematical modeling of drying kinetics for apricots: Influence of the external resistance to mass transfer. Drying Technology, 25, 1829–1835.
Crank J. 1995. The mathematics of diffusion, 2nd Ed., Clarendon Press, Oxford, U.K.
Doymaz I., Ismail O. 2011. Drying characteristics of sweet cherry. Food and Bioproducts Processing. 89, 31-38.
Ertekin C., Menges O., H. 2006. Mathematical modeling of thin layer drying of Golden apples. Journal of Food Engineering, 77, 119-125.
Ertekin C., Yaldiz O. 2004. Drying of eggplant and selection of a suitable thin layer drying model, Journal of Food Engineering, 63, 349-359.
Gamboa-Santos J., Montilla, A, Andres C.J., Villamiel M., Garcia-Perez, J.V. 2014. Air-borne ultrasound application in the convective drying of strawberry, Journal of Food Engineering, 128, 132-139.
Hassan-Beygi S.R., Aghbashlo M., Kianmehr M.H., Massah J., 2009. Drying characteristics of walnut (Juglans regiaL.) during convection drying. International Agrophysics, 23, 129–135.
Henderson S.M., Pabis S., 1961. Grain drying theory. I.Temperature effect on drying coefficient. Journal ofAgricultural Engineering Research, 6(3), 169–174.
Panchariya P.C., Popović D., Sharma A.L., 2002. Thin layer modeling of black tea drying pocess, Journal of Food Engineering, 52, 349-357.
Pavkov I., Babić Lj., Babić M., Radojčin M. 2009. Kinetics of the combined drying technology of pear slices (Pyrus). Journal on Processing and Energy in Agriculture, 13 (2), 111-116.
Pavkov I., Stamenković Z., Radojčin M., Kešelj K., Bursić V., Bikić S., Mitrevski V. 2018. Osmotic and convective drying of strawberries: Effects of experimental parameters on the drying kinetics color and rehidration, Journal on processing and energy in agriculture 22(2), 58-64.
Serdar, A. and Bese, A., V. (2016). Convetive drying of hawthorn fruit (Crataegus spp.): Effect of experimental parameters on drying kinetics, color, shrinkage and rehydration capacity. food Chemistry, 210, pp. 577-584.
Soponronnariti S., Swasdisevi, T., Wetchacama S., Wutiwiwatchai W. 2001. Fluid bed drying of soybeans, Journal of Stored Products Research, 37(2), 133-151.
Verma L. R., Bucklin R.A., Endan J.B., Wratten F.T. 1985. Effects of drying air parameters on rice drying models, Transactions of the ASAE, 28, 296-301.
Wang C.Y., Singh, R.P. 1978. A single layer drying equation for rough rice. ASAE Paper No: 3052 ASAE, St Joshep, MI
Zhengfu W., Junhong S., Xiaojun L., Fang C., Guanghua Z., Jihong W., Xiaosong H. 2007. Mathematical modeling on hot air drying of thin layer apple pomace. Food research International, 40, 39-46.

Change of anthocyanins content during production of jam from different species of fruit

Viktorija Stamatovska[1], Gjore Nakov[2], Ljubica Karakasova[3], Frosina Babanovska-Milenkovska[3], Tatjana Kalevska[1], Vezirka Jankuloska[1]

[1]"St. Kliment Ohridski" University of Bitola, Faculty of Technology and Technical Sciences, Dimitar Vlahov bb, 1400 Veles, Republic of Macedonia (vikistam2@gmail.com; tkalevska@gmail.com; vezirka.jankuloska@gmail.com)

[2]University of Ruse "Angel Kanchev" – branch Razgrad, Department of Biotechnology and Food Technology, blv. Aprilsko Vastanie, 47 Razgrad 7200, Bulgaria (gore_nakov@hotmail.com)

[3]University of Ss. Cyril and Methodius, Faculty of Agricultural Sciences and Food, Department of Food Processing, Edvard Kardelj bb, 1000 Skopje, Republic of Macedonia (karakasoval@yahoo.com; frosibm@gmail.com)

ABSTRACT

Anthocyanins represent soluble pigments. From them comes the red, blue and purple color of fruit. Anthocyanins are very easily degraded by heat treatment, which can have a major impact on the color quality and nutritional characteristics of the finished product. In order to determine how the heat treatment affects the content of the anthocyanins, jams were produced from peach and two varieties of raspberry. The jams were produced by boiling the prepared fruits, in open stainless steel vats on direct fire, under atmospheric pressure, temperature of ≈ 100 °C, for a period of 15 minutes. As sweeteners were used: sucrose, fructose, sorbitol and agave syrup. The analysis of anthocyanins was carried out on fresh fruits and obtained jams (spectrophotometrically at pH differential method with UV-Vis spectrophotometer). The research was repeated three times, for a period of three years.
The values obtained for the average content of anthocyanins in jams (8.49 - 27.96 mg CGE/100 g FW) are lower compared to the established values in fresh fruits (18.45 - 108.43 mg CGE/100 g FW).
Based on the results obtained, it can be concluded that with this method of heat treatment of the fruit (high temperature, presence of oxygen and light) part of the anthocyanins present in the fruits are lost during the processing. The obtained jams still possess the required quality in accordance with the standards.

Keywords: anthocyanins; peach; raspberry; jams;

INTRODUCTION

During the last few years the interest in anthocyanins was growing. Anthocyanins are colored water-soluble pigments with known antioxidant activity which may be responsible for some biological activities including the prevention or lowering the risk of cardiovascular disease, diabetes, arthritis and cancer (Miguel, 2011). Fruit is good source of anthocyanins. The color and stability of these pigments are influenced by pH, light, temperature, oxygen, sugar, structure etc. (Rababah et al., 2011; Khoo et al., 2017).
There is a considerable demand for fresh fruits as well as their products. Since many types of fruits are seasonal and their shelf lives are limited, they must be processed to keep the quality (Rababah et al., 2011). Fruits can be preserved by preparing jam. Jam is one of the most popular food products because of their low cost, all year long availability and organoleptic properties (Arya et al., 2017). Jam structure is determined by the equilibrium between the pectin, sugar and acid contents present. It is known that quality parameters of fruit jams, such as color, acidity, soluble solids, texture and nutraceutical content, can be affected during processing and storage (Rababah et al., 2011). Jam making involves thermal processing, which degrades chemical components and reduces biological activities, to produce a desirable shelf life (Lee et al., 2013).
The objective of this study was to evaluate changes in anthocyanins of fresh fruit and its jam during jam processing.

MATERIALS & METHODS

Cultivared peach Cresthaven from Rosoman, cultivated raspberry Willamette from Krusevo region and wild raspberry from the mountain region of Skopska Crna Gora were processed into jams at the factory Vitalia Nikola Ltd., Skopje, by a traditional method. Fruits were harvested at full technological maturity. Jams were prepared with different sweeteners: sucrose (reduced amount), fructose, sorbitol, and agave syrup. Commercial low-esterified pectin (GENU pectin type LM 115AS from the company CPKelco) and Ca^{2+} in the form of calcium citrate ware added at the final stage of the jam cooking. Citric acid was added towards the end of cooking to providing the necessary acidity. Jam processing was conducted following the procedure described in our previous study (Stamatovska et al., 2017). The thermal processing time was 15 min, at ≈ 100 °C. The quantity of dry matter was controlled (refractometrically, using a handheld refractometer ATAGO, HSR-500) in order to get the content of max 42 - 44 °Brix in the final product.

The content of anthocyanins in the fresh fruits and produced jams were determined according to the pH differential method (Guisti, Wrolstad, 2001). All measurements were performed with UV-Vis spectrophotometer Helios omega. Results were expressed as mg of cyanidin 3-glucoside equivalents per 100 g of fresh weight of fruit or jam (mg CGE/100 g FW). The research was repeated three times, in a period of three years (2011, 2012, 2013). The tests were carried out in the laboratories of the Faculty of Technology in Plovdiv, Bulgaria (University of Food Technologies).

RESULTS & DISCUSSION

The results obtained for the content of anthocyanins in fresh fruits of peach cultivar Cresthaven and on both cultivars of raspberry (Willamette and wild raspberry) in the three years of testing are shown in Figure 1. From the results shown, it can be seen that there is a variation in the values obtained for the content of anthocyanins, both between the different types of fruits, and between the same types of fruits, but different cultivars. This is according to the fact that the chemical composition of fruits is specific.The anthocyanin contents can vary by types and cultivars of fruits, degree of maturity, geographical origin, climatic condition and handling procedures (Ivanović et al., 2016; Stamatovska et al., 2017). Cresthaven peaches in the three years of testing were characterized by lower content of anthocyanins (19.98 mg CGE/100 g FW, 18.81 mg CGE /100 g FW, 16.57 mg CGE /100 g FW) compared to the fruits of the Willamette raspberry (111.23 mg CGE /100 g FW, 109.93 mg CGE /100 g FW, 104.14 mg CGE /100 g FW) and the wild raspberry 46.36 mg CGE /100 g FW, 52.86 mg CGE /100 g FW, 48.76 mg CGE /100 g FW).

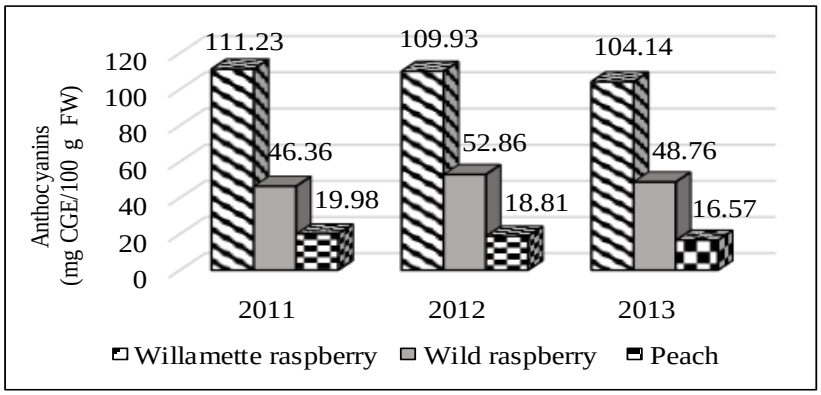


Figure 1. The content of anthocyanins in the fresh fruits

For the content of anthocyanins in the fruits of raspberries and peaches, many investigations have been conducted by several authors (Guerrero et al., 2010; Marjanovic-Balaban et al., 2012; Stajčić et al.,

2012; Kivi et al., 2013; Kostecka-Gugała et al., 2015; Nakov et al., 2015; Probst, 2015; Ivanović et al., 2016; Oancea, Călin, 2016; Ye et al., 2017; Alibabić et al., 2018). Based on the obtained data from these investigations, it can be concluded that the fruits of raspberry contain a larger amount of anthocyanins compared to the fruits of peach, which is in accordance with the obtained results from our analyzes.

Figure 2 shows the results obtained for the content of anthocyanins in the produced jams of peach cultivar Cresthaven and of the two cultivar of raspberry with reduced amounts of sucrose (Surc), fructose (Fru), sorbitol (Sorb) and agave syrup (AgS) each year. Results obtained indicate relationships between levels of anthocyanins in fruits used for jam processing and the content of anthocyanins in the produced jams. Peach jams in the three years of testing were characterized by lower content of anthocyanins compared to the jams of the two cultivars of raspberry, which is in accordance with the determined values for the content of anthocyanins in fresh fruits.

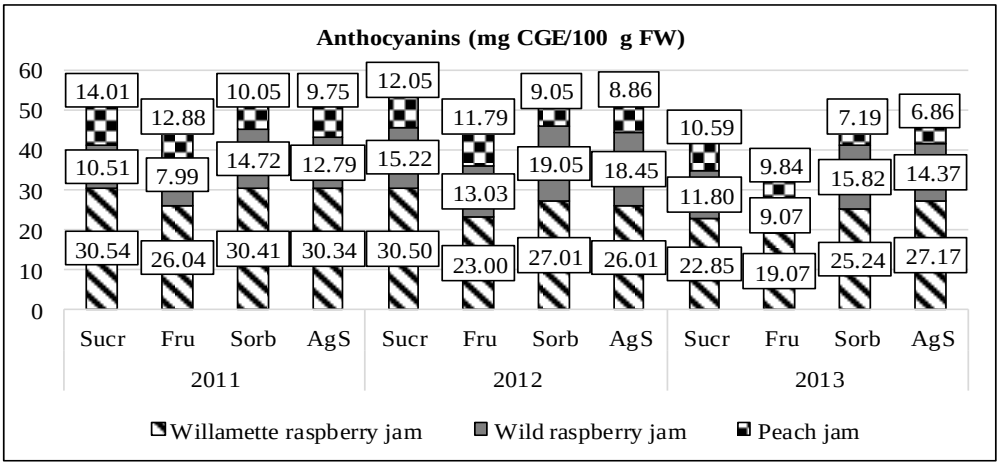


Figure 2. The content of anthocyanins in produced jams

Also, the influence of thermal processing of fruits, which can cause certain chemical reactions, degradation and creation of new compounds, should not be neglected, and in doing so can cause changes in the contents of the anthocyanins in the produced jams.The jams produced are characterized by lower concentrations of anthocyanins than fresh fruits (Figure 2).

Anthocyanin pigments readily degrade during thermal processing which can have a dramatic impact on colour quality and may also affect nutritional properties (Patras et al., 2010). The data indicate great instability of anthocyanins. Oxygen, pH, temperature, light, presence of sugars and their degradation products, enzymes, ascorbic acid and metals are considered to be important factors influencing anthocyanins stability (Patras et al., 2010; Rababah et al., 2011; Poiana et al., 2012; Tepić, 2012; Oancea, Călin, 2016). When the fruits are thermally treated at higher temperatures in the presence of oxygen, some of the present anthocyanins may be degraded. Sugars in higher concentrations increase the stability of anthocyanins and this stability is thought to be a consequence of a decrease in water activity. Fructose has a greater degradation effect on glucose, sucrose and maltose anthocyanins (Ścibisz, Mitek, 2009; Tepić, 2012).

The calculated average values for the content of anthocyanins (Figure 3) in the produced jams (Willamette raspberry jam, Wild raspberry jam, Peach jam) for the three years of production are lower (22.70 – 27.96 mg CGE/100 g FW, 10.03 – 16.53 mg CGE/100 g FW, 8.49 – 12.22 mg CGE/100 g FW, respectively) than the average values for the content of anthocyanins in fresh fruits (108.43 mg CGE/100 g FW, 49.33 mg CGE/100 g FW, 18.45 mg CGE/100 g FW). Part of the anthocyanins present in fresh fruits have been lost during the processing. Our previous study showed that heat processing during jam making from fresh fruit of plum Stanley decreased the amounts of anthocyanins (Stamatovska et al., 2017).

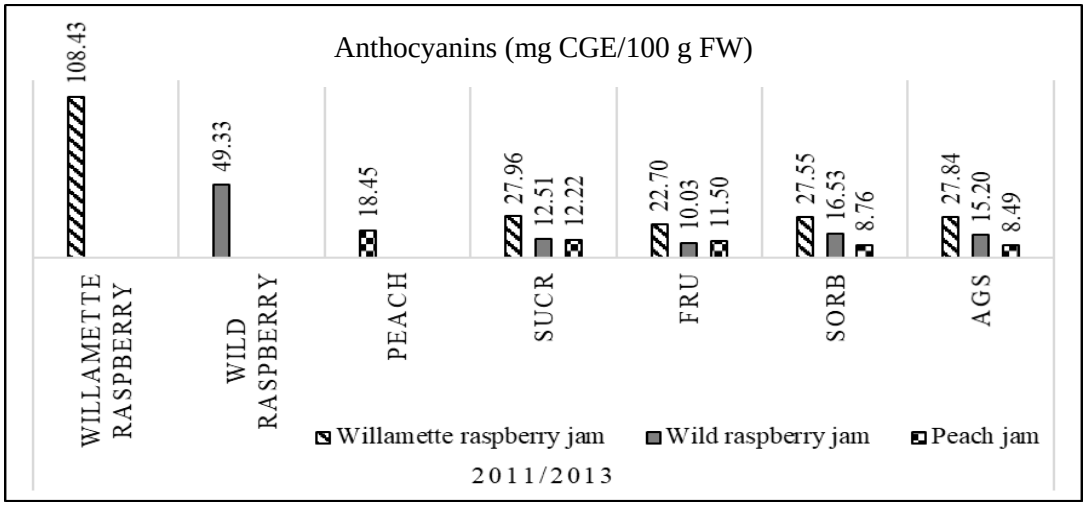


Figure 3. Average values for the content of anthocyanins in fresh fruits and in the produced jams

Previous results obtained by many other authors proved that anthocyanins are lost during the process of jam production and the jams produced are characterized by lower content than the starting raw materials, indicating that anthocyanins are sensitive to the jam production process (Patras et al., 2010; Poiana et al., 2011; Levaj et al.,2012; Lee et al., 2013; Oancea, Călin, 2016; Rodrigues et al., 2017). The reasons for this phenomenon are probably the previously mentioned factors which affect the stability of anthocyanins (oxygen, pH, T, light, presence of sugars and their degradation products etc.).

CONCLUSIONS

From the results obtained, it can be concluded that fresh raspberries (Willamette and wild raspberries) are characterized by higher content of anthocyanins compared to peaches (Cresthaven). The differences in the content of the anthocyanins between the jams are due to the different content of the anthocyanins in the fruits from which they were obtained. Regarding the applied technological procedure, the results obtained confirmed that part of the anthocyanins present in the fruits were lost during the processing. In all jams, a lower content of anthocyanins is noted in comparison with the content of fresh fruit anthocyanins due to the heat treatment of the fruits (high temperature, presence of oxygen, light, present sugars). However, further studies are needed to fully understand the stability of anthocyanins during the jam production process. Based on the statements and conclusions made we can summarize that the jam can also be a good source of anthocyanins, although in comparison with fresh fruit, losses occur.

ACKNOWLEDGEMENTS

The study was supported by contract of University of Ruse “Angel Kanchev”, № BG05M2OP001-2.009-0011-C01, " Support for the development of human resources for research and innovation at the University of Ruse “Angel Kanchev”. The project is funded with support from the Operational Program " Science and Education for Smart Growth 2014 - 2020" financed by the European Social Fund of the European Union.

REFERENCES

Alibabić V., Skender A., Bajramović M., Šertović E., Bajrić E. 2018. Evaluation of morphological, chemical, and sensory characteristics of raspberry cultivars grown in Bosnia and Herzegovina. Turk J Agric For 42 (1), 67-74.

Arya A., Doke A., Jaishree G., Bhalerao G. J., Shinde S. R. 2017. Development of value added fruit jams. Food Sci. Res. J. 8(1), 1-6.
Barbalho M.S., Otoboni M.A., Marinelli P., Bezerra N.O.C., Meneghini A., Oliva É., Lopes A.R.R, Souza R. T., Oshiiwa M., Nicolau C.T.C., Moura B.M. 2016. Brazilian Peach (*Prunus Persica*) and Passion Fruit (*Passiflora Edulis*) Nectars: Good Source of Vitamin C and Anthocyanins?.Journal of Food Research 5(2), 50-57.
Giusti M.M., Wrolstad, E.R. 2001. Characterization and measurement with UV-Visible spectroscopy. In: Current Protocols in Food Analytical Chemistry; Wrolstad, E. R., Ed.; John Wiley & Sons: New York, F1.2.1-F1.2.13.
Guerrero C.J., Ciampi P.L., Castilla C.A. Medel S.F., Schalchli S.H., Hormazabal U.E., Bensch T.E., Alberdi L.M. 2010. Antioxidant capacity, anthocyanins, and total phenols of wild and cultivated berries in Chile. Chile J Agric Res 70, 537-544.
Ivanović M., Pavlović A., Mitić M., Pecev-Marinković E., Krstić J., Mrmošanin J. 2016. Determination of total and individual anthocyanins in Raspberries grown in south Serbia. Proceedings of the "21th International symposium on biotechnology", Čačak, Serbia, March 2016. 263-267.
Khoo H. E., Azlan A., Tang T. S., Lim M. S., 2017. Anthocyanidins and anthocyanins- colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food & Nutrition Research 61, 1361779.
Kivi R. A., Khavari-Nejad R.A., Dehgan G., Najafi F., Hajilo J. 2013. Antioxidant capacity and phytochemical properties of raspberry species in Iran, International Journal of Biosciences 3(6), 145-152.
Kostecka-Gugała A., Ledwożyw-Smoleń I., Augustynowicz J., Wyżgolik G., Kruczek M., Paweł K. 2015. Antioxidant properties of fruits of raspberry and blackberry grown in central Europe. Open Chemistry 13(1), 1313–1325.
Lee D.J., Lee H., Lee S.H., Kim D. 2013. Effects of Jam Processing on Anthocyanins and Antioxidant Capacities of Rubus coreanus Miquel Berry. Food Sci Biotechnol 22(6), 1607-1612.
Levaj B., Kovačević D.B., Bituh M., Dragović-Uzelac V. 2012. Influence of Jam Processing Upon the Contents of Phenolics and Antioxidant Capacity in Strawberry fruit (*Fragaria ananassa* × Duch.). Croatian Journal of Food Technology, Biotechnology and Nutrition 7, 18-22.
Marjanovic-Balaban Z., Grijic S., Jasic M., Vujacinovic D. 2012. Testing of chemical composition of wild berries. Proceedings of Agrosym 2012 Third International Scientific Symposium „Agrosym 2012", Jahorina, November 2012. 154-161.
Miguel G. M., 2011. Anthocyanins: Antioxidant and/or anti-inflammatory. JAPS 01(06), 07-15.
Nakov Gj., Stamatovska V., Necinova Lj., (2015). Incidence of anthocyanins in different types of fruit (review), Scientific works of University of Food Technologies-Plovdiv LXII, Plovdiv, 304-309.
Oancea S., Călin F. 2016. Changes in total phenolics and anthocyanins during blackberry, raspberry and cherry jam processing and storage. Romanian Biotechnological Letters 21(1), 11232-11237.
Patras A., Brunton N.P., O'Donnell C., Tiwari B.K. 2010. Effect of thermal processing on anthocyanin stability in foods; mechanisms and kinetics of degradation. Trends Food Sci. Tech. 2, 3-11.
Poiana M. A., Alexa E., Mateescu C. 2012. Tracking antioxidant properties and color changes in low-sugar bilberry jam as effect of processing, storage and pectin concentration. Chemistry Central Journal 6(4), 1-11.
Poiana M.A., Moigradean D., Dogaru D., Mateescu C., Raba D., Gergen I. 2011. Processing and storage impact on the antioxidant properties and color quality of some low sugar fruit jams. Romanian Biotechnological Letters 16(5), 6504-65012.
Probst, Y. 2015. A review of the nutrient composition of selected Rubus berries. Nutrition and Food Science 45(2), 242-254.
Rababah T. M., Al-u'datt M. H., Al-Mahasneh M. A., Feng H., Alothman M. A. , Almajwal A., Yang W., Kilani I., Alhamad M. N., Ereifej K., Abu-Darwish M. 2011. Effect of storage on the physicochemical properties, total phenolic, anthocyanin, and antioxidant capacity of strawberry jam. JFAE 9(2), 101-105.
Rodrigues M. L., de Souza F.D., da Silva A. E., de Oliveira O.T., de Lima P. J. 2017. Physical and chemical characterization and quantification of bioactive compounds in berries and berry jams. Semina: Ciências Agrárias 38(4), 1853-1864.
Ścibisz I., Mitek M. 2009. Effect of processing and storage conditions on phenolic compounds and antioxidant capacity of highbush blueberry jams, Pol. J. Food Nutr. Sci. 59(1), 45-52.
Stajčić M.S, Tepić N.A., Đilas M.S., Šumić M.Z., Čanadanović-Brunet M.J., Ćetković S.G.,Vulić J.J., Tumbas T.V. 2012, Chemical composition and antioxidant activity of berry fruits, Acta Periodica Technologica 2012(43), 93-105.
Stamatovska V., Karakasova Lj., Babunovska-Milenkovska F., Nakov Gj., Blazevska T., Durmishi N. 2017. Production and characterization of plum jams with different sweeteners. JHED 19, 67-77.
Tepić A. 2012. Colored matter of fruit and vegetables. University of Novi Sad, Faculty of Technology, Novi Sad.
Ye J., Xu F., Wang G., Chen Q., Tao T., Song Q. 2017. Molecular Cloning and Characterization of an Anthocyanidin Synthase Gene in *Prunus persica* (L.) Batsch. Not Bot Horti Agrobo 45(1), 28-35.

Influence of aging of wine in Slavonian oak barrels with different toasting levels on organoleptic and physico-chemical characteristics

Zrinščak, S.[a], Zrinščak, I.[b], Wendelin, S.[c], Eder, R.[c]

[a] *Polytechnic in Požega, Požega, Croatia (szrinscak@vup.hr)*
[b] *Vinogradastvo i vinarstvo Zrinščak, Požega, Croatia (szrinscak@vup.hr)*
[c] *Higher secondary school in Klosterneuburg, Austria (Reinhard.Eder@weinobst.at)*

ABSTRACT
Modern wine production is unimaginable without aging of wine in wooden barrels and the most used are the "barrique" barrels of 225 L and made from oak wood with different toasting levels. The Slavonian part of Croatia was long known for the production of high quality oak barrels however in the last decades this knowledge was almost lost. Therefore, we investigated the influence of different barrels made from Slavonian oak on red wine characteristics. We used two different sizes of barrels: barrique (225 liters) and Halbfüder (500 liters) and three different toasting levels: light, medium and heavy. Two single variety red wines from varieties Cabernet Sauvignon and Merlot grown in Kutjevo area were stored in the different barrels and in a stainless steel vessel. Samples were taken after 6, 12 and 18 months of aging and physico-chemical and sensorial analyses were performed. The sensorial evaluation of the wines was performed by descriptive analysis with 11 certified wine tasters from Austria according to eight descriptors

Keywords: Slavonian oak; wine; Cabernet Sauvignon; Merlot; aroma

INTRODUCTION

The production of wood barrels is almost 2000 years old, and through history, only the shape of the barrel, the tree species, the thickness of the barrel (the wall of the barrel) has changed. The method of making barrels, bending by fire and water has remained the same today (Lantschauer et al., 2001). In modern enology, the 225 L barrique barrels are most widely used, which in different degrees of toasting show an optimal contribution on the taste and aroma of wine (Fehlow, 1994).

The preparation and the way of drying the wood, as well as the high temperature during wood toasting play a very important role for barrel quality and properties. Three forms regarding the toasting intensities are most widely spread: light, medium and heavy toasting. Many authors have been investigated these factors and concluded that the choice of toasting level will have a decisive impact on wine sensory properties and overall quality (Díaz-Plaza et al, 2002; Fernández de Simón et al., 2003). Furthermore, the period of aging in barrels along with the cellar conditions such as temperature, relative humidity and air flow, also affect the final properties of barrel-aged wines. Also, in production of high-quality wines there is often a recommendation for using only new barrique barrels. In order to achieve good quality of wines, some winemakers are working on a combination of new barrels for a certain period of time with previously used ones for the next period (MacNeill, 2015).

Each region or, more precisely, every tree growing area in the world has a specific character, so that the tree differs according to its characteristics, its chemical composition and the amount of substance that will be extracted into the wine during aging process. The possibilities of using these characteristics could be in producing of specific wines with pronounced attributes that are not found in other regions. Therefore, the region could be presented through unique impression of wine that is remembered and consequently a new, higher value of wine could be achieved.

In addition to the world's most famous species of American and French oak woods, research has shown that other world regions have also a very high quality wood and that there is no monopoly on quality. Two varieties of oak are mostly spread in Europe: *Quercus robur* L. known as common, penduculate oak and *Quercus petreae* L. known as sessil oak, and often they are emphasized as exclusive ones for wine production (Schisser, 2001). Regarding the literature data, sessile oak is richer on aromatic components such as vanillin and methyl octalactone, while penduculate oak is characterized as richer in the phenolic components such as ellagitanins and catechol (Domine, 2003).

The aim of this research was to investigate the influence of Slavonian oak, as most important in Croatia, on physico-chemical, aromatic and sensory characteristics of Merlot and Cabernet Sauvignon wines during 6, 12 and 18 months of aging in oak barrels.

MATERIALS & METHODS

The paper presents the results of the research of physico-chemical and organoleptic influences on wines of different varieties, aged in different barrel sizes during different aging period of 6, 12 and 18 months. Barique barrels (225 L) and Halbfüder barelles (500 L), with different toasting intensities: light (LT), medium (MT) and heavy (HT), were used for aging of Merlot wines, while latter barrel size was used for aging of Cabernet Sauvignon wines. Wood, used for production of previously mentioned barrels, was Slavonian oak. Control wines represent those aged in stainless steel vessels. Standard physico-chemical parameters of wine were analyzed by FTIR spectroscopy (WineScan, Foss Analyticals, Hilleroed, Denmark). Wood-associated aroma compounds were detected by GC-MS analysis (Agilent GC 7890A with mass spectrometer 5975C, Agilent Technologies, Santa Clara, USA). The sensorial evaluation of the wines was performed by descriptive analysis with 11 certified wine tasters from Austria according to eight descriptors.

RESULTS & DISCUSSION

Table 1 shows the basic physico-chemical analysis of wines where it can be seen that the amount of alcohol in both analyzed wines is relatively high, which is desirable for wine aging in wooden barrels. Also, it can be seen that malolactic fermentation has been carried out since the higher concentration of lactic acid was determined. Furthermore, the determined volatile acidity is within sensory acceptable limits.

Table 1. Basic chemical composition of red wines – Merlot and Cabernet Sauvignon (CS)

Variety	Sample	Alcohol (vol %)	Reduced sugars (g/L)	Total acidity (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Volatile acidity (g/L)
Merlot	LT225	15.3	1.5	5	1.4	0.4	1.3	0.9
Merlot	HT225	15.3	1.5	5	1.3	0.4	1.3	0.9
Merlot	LT500	15.3	1.8	4.9	1.4	0.3	1.3	0.8
Merlot	HT500	15.1	2.4	5.2	1.2	0.4	1.2	0.8
Merlot	INOX	14.5	1	4.7	1.3	0.4	1.3	0.7
CS	HT500	14.1	2	4.4	1.1	0.3	2.4	0.8
CS	MT500	14.3	1.8	4	1.1	0.3	2.4	0.8
CS	LT500	14.2	1.2	4.1	1.1	0.3	2.4	0.8
CS	INOX	14.2	1.2	4	1.3	0.3	2.4	0.8

LT- light toasting; MT-medium toasting; HT- heavy toasting.

The concentrations of aroma compounds determined in analyzed wines are presented in Table 2. As can be seen in table, after 6 months of aging of Merlot wines the concentrations of furfural in 225 L-barrels, both LT and HT, are much lower than of those determined in heavy toasted (HT) 500 L-barrel where more than two-fold concentrations were determined. But, LT 500 L-barrel showed lower effect than both variations of 225 L-barrel. During longer period of aging (12 months) concentration of this compound significantly increased in all analyzed Merlot wines (around 5500 μg/L) where HT barrels, both 225 and 500 L, showed significantly higher concentration in comparison to LT ones (661.8 and 1321 μg/L). Subsequently, during further storage (18 months) concentrations of this compound were additionally increased, amounting 7788.3-9021.4 μg/L in HT barrels and 916.9-945.3 μg/L in LT barrels. As in previous cases, larger barrel volume (500 L) resulted in higher concentrations of furfural. Regarding the Cabernet Sauvignon wines, lower concentrations of this compound were detected in all cases. But, the increasing trend was similar to the one established for Merlot wines: the highest concentrations were determined after 18 months of aging, with higher concentrations (3739.6 μg/L) determined in wine aged in MT barrels. Control wines aged in stainless steel vessel, as expected, were

characterized with significantly lower concentrations of this compound. Generally, furfural is the most abundant compound determined in all analyzed wines.
Higher concentrations of 5-methyl furfural were also detected in Merlot wines and, similarly to furfural, its concentrations increased during aging period of 18 months (from 63.8 to 2271.4 μg/L). Also, HT barrels of 500 L influenced its higher concentrations in both analyzed wines (2271.4 μg/L in Merlot wine and 1017.4 μg/L in CS wine after 18 months of aging). Regarding the guaiacol, *m*-cresol, vanillin and syringaledhyde concentrations, similar trends as previously noted, were established: higher concentrations found after longer period of aging, as well as in HT barrels. But, opposite to previous compounds, HT barrel of 225 L influenced higher concentrations of these compounds in Merlot wines. The concentration of vanillin determined in both analyzed wines increased during 12 months of aging, but after that period its concentration remain constant. In Merlot wine in LT barrels the values were 479 μg/L after 12 and 503 μg/L after 18 months, and 1500 μg/L after 12 and 1586 μg/L after 18 months of aging in HT barrels. As stated previously, 500 L-barrels influenced lower concentrations than 225 L-barrel, and amounted only 253 μg/L after 12 and 267 μg/L after 18 months of aging in LT barrels and 625 μg/L after 12 and 666 μg/L after 18 months in HT barrels. Regarding Cabernet Sauvignon wines, similar trend was observed with quite low concentrations of 181 μg/L after 12 and 203 μg/L after 18 months of aging in LT barrels. The amount of syringaldehydes, were much higher in HT barrels and practically remain constant after 12 months of aging. In Merlot wine, aged in HT 225 L-barrels, the concentration of 3367 μg/L after 12 and 3643 μg/L after 18 months of aging was determined. Furthermore, its levels in same wine aged in HT 500 L-barrels amounted 1547 μg/L after 12 and 1690 μg/L after 18 months of aging. Cabernet sauvignon also had the highest value in HT barrels, which amounted 895 μg/L after 12 and 1045 μg/L after 18 months of aging. Approximately the same results were obtained by other investigations (Jeromel, 2017; Herjavec 2007; Schelberhofer 2004).
Furthermore, the concentrations of *cis*- and *trans*-whiskey lactone were also determined in analyzed wines, and the concentrations are in accordance with previously published papers (Herjavec et al., 2007; Chatonnet et al., 1994). The concentration of cis-whiskey lactone was higher in HT barrels of 225 L barrels and increased from 6 to 18 months twice, from 15.7 to 30 μg /L in LT and from 40.1 to 82 μg/l in HT barrel. On the other hand, the determined values in 500 L- barrels were higher after 6 than after 18 months of aging, where reduction in concentration from 71.1 to 32.4 μg /L in LT, and from 66.8 to 18.5 μg/L in HT barrels was observed. In Cabernet Sauvignon the highest *cis*-whiskey lactone values were determined after 12 months of aging in HT barrel. Furthermore, the concentration of *trans*-whiskey lactone determined in Merlot wine was increased by longer period of aging in both 225 L-barrels and 500 L-barrels. The highest concentration of *trans*-whiskey lactone was determined in wine aged in LT 225 L-barrel amounting 605 μg/L, while in LT 500 L-barrel was 309 μg/L. In Cabernet Sauvignon, the concentrations of *trans*-whiskey lactone after 18 months of aging, was quite similar in LT and MT barrels and amounted 348 μg/L and 341 μg/L, respectively.
The concentrations of *m*-cresol were similar after 6, 12 and 18 months of aging in wood. Furthermore, its concentrations were also similar regarding the barrel volume used (225 and 500 L). Only slightly higher concentrations were determined in HT barrels, when compared to other toasting levels.
Determined concentrations of eugenol were higher in LT barrels and do not increase after 12 months of aging. The range from 64.5 μg/L in Merlot in a LT 225 L-barrel to 54.2 μg/L in a LT 500 L-barrel in Cabernet Sauvignon wine was determined.
Finally, large amount of 4-ethyl phenol was determined in Merlot wine after 18 months aging in stainless steel tanks, where the amount of 466.6 μg/L was detected, implying the possibility of microbiological contamination with *Brettanomyces bruxellensis* yeasts, which is in accordance with investigation of Lemperle (2000).
Obtained analytical data are in accordance with conducted descriptive sensory analysis (Figure 1) that involved Cabernet Sauvignon wines. It can be seen that the wines aged in stainless steel tank was ranked with lowest scores for most of the attributes. Furthermore, wine aged in LT barrel was characterized with moderate scores of majority of sensory attributes, while wine aged in MT barrel was scored with the highest intensities of tobacco/toast and coconut. Finally, wine aged in HT barrel was characterized with highest scores of majority of sensory attributes (mouthfeel, coffee/cacao, caramel/vanilla and dry fruity/flowers), implying that heavy toasting result in most pronounced oak aromas.

Table 2. Oak volatile concertation (µg/L) in Merlot and Cabernet Sauvignon (CS) after 6, 12 and 18 months in barrels with different toasting intensities and volumes

Variety	Sample	Furfural	5-Methyl furfural	Guaiacol	*cis*-Whiskey lactone	*trans*-Whiskey lactone	*m*-Cresol	Eugenol	4-ethyl phenol	Vanillin	Syringaldehyde
Merlot	6 LT225	669.7	63.8	7.7	15.7	342.7	1.4	29.0	1.2	307	243
Merlot	6 HT225	625.5	186.9	31.9	40.1	136.3	3.6	12.5	3.0	700	1468
Merlot	6 LT500	506.8	97.7	7.9	71.1	168.3	1.3	25.7	1.2	152	183
Merlot	6 HT500	1256.9	438.5	17.9	66.8	133.7	1.6	11.1	1.4	313	786
Merlot	6 INOX	143.5	23.0	3.4	0.0	5.0	1.0	1.3	3.2	10	43
Merlot	12 LT225	661.8	120.4	9.6	24.1	609.1	1.3	66.0	1.4	479	466
Merlot	12 HT225	5500.9	1324.3	61.8	9.4	261.2	2.7	24.4	1.5	1500	3367
Merlot	12 LT500	1321.7	312.2	10.6	20.1	291.3	1.1	50.0	1.0	253	317
Merlot	12 HT500	5551.6	1710.6	26.2	25.7	246.5	1.5	23.7	1.6	625	1547
Merlot	12 INOX	291.0	65.8	4.1	2.0	16.0	0.9	2.2	78.1	10	43
Merlot	18 LT225	916.9	126.6	9.4	30.0	605.5	1.2	64.5	20.4	503	523
Merlot	18 HT225	788.3	1588.9	63.0	82.0	276.5	2.6	25.6	19.4	1586	3643
Merlot	18 LT500	945.3	196.4	9.1	32.4	309.6	1.0	53.6	12.7	267	361
Merlot	18 HT500	9021.4	2271.4	30.0	18.5	289.9	1.6	29.0	10.2	666	1690
Merlot	18 INOX	352.9	71.7	4.8	5.7	16.1	0.8	2.2	466.6	10	58
CS	6 HT500	596.0	208.0	12.6	6.3	184.5	1.7	19.1	6.3	221	406
CS	6 MT500	260.1	51.5	11.0	22.9	193.7	1.6	20.5	1.6	134	223
CS	6 LT500	241.4	35.7	11.9	10.3	187.7	1.6	27.8	1.5	83	157
CS	6 INOX	127.3	6.7	4.5	0.1	6.0	1.4	1.7	1.4	10	52
CS	12 HT500	2018.0	827.2	15.7	97.5	302.7	1.7	34.9	1.3	448	895
CS	12 MT500	2090.0	551.0	13.3	40.3	319.9	1.5	35.5	1.1	266	458
CS	12 LT500	1310.0	288.4	14.1	10.9	322.4	1.5	50.6	1.2	181	335
CS	12 INOX	141.6	6.1	4.2	0.2	6.9	1.4	1.8	1.3	10	51
CS	18 HT500	3574.7	1017.4	16.1	20.6	335.7	1.6	37.9	1.3	511	1045
CS	18 MT500	3739.6	740.9	13.8	21.7	341.7	1.4	40.9	1.1	296	525
CS	18 LT500	1979.4	367.6	14.2	91.0	348.1	1.4	54.2	1.2	203	459
CS	18 INOX	147.5	7.3	4.3	0.4	0.4	1.3	2.5	1.3	10	75

LT- light toasting; MT-medium toasting; HT- heavy toasting.

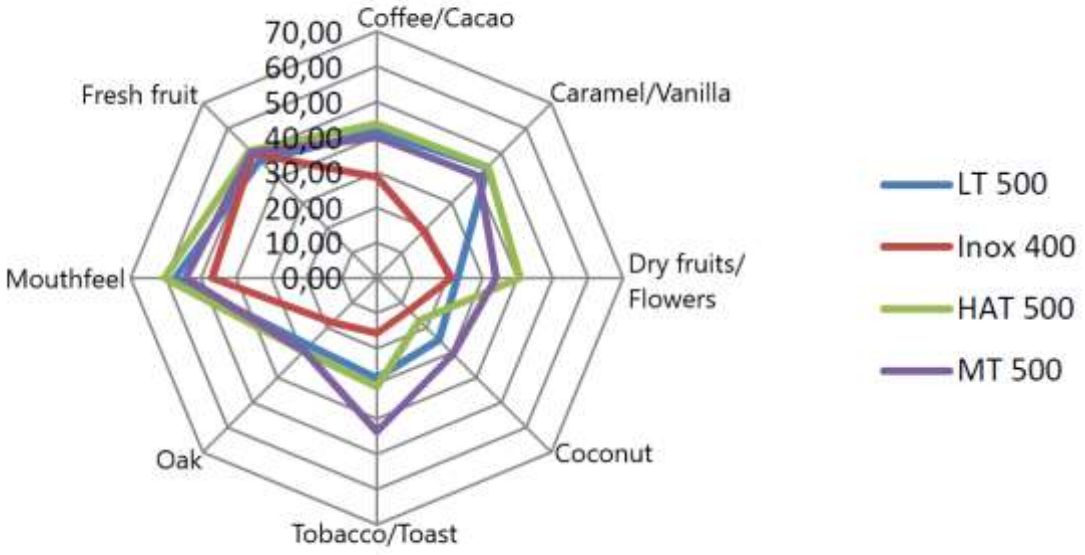


Figure 1. Sensory properties of Cabernet Sauvignon wine using sensory descriptive analysis

CONCLUSION

Based on the obtained results it can be concluded that concentrations of volatile compounds extracted from Slavonian oak barrels have a significant influence on their chemical composition and that heavy toasting (HT) results in the most significant extraction of aromatic compounds such as furfural, 5-methyl furfural, guaiacol, vanillin and syringaldehyde. These results are in accordance with descriptive sensory analysis, where wine aged in HT barrels was characterized with higher scores for coffee/cacao, caramel/vanilla, dry fruits/flower and overall mouthfeel sensations.

REFERENCES

Díaz-Plaza, E. M., Reyero, J. R., Pardo, F., Alonso, G. L., & Salinas, M. R. 2002. Influence of oak wood on the aromatic composition and quality of wines with different tannin contents. Journal of Agricultural and Food Chemistry 50(9), 2622-2626.

Domine, A. 2003. Wine, Konmann, Berlin. p. 130.

Chatonet, P., Boidron, J., Duborduieu, D. Pons, M. 1994 Evolutions de certains composes volatils du bois dechene au cours de son sechage premiers resultats. OENO One 28(4), 359-38.

Fehlow, C. 1994. Eichenholz als Werkstoff fur die Produktion von Klenfassern (Barriques). Diplomarbeit am Fachbereich Weinbau und Getranketechnologie der Fachhochschule Wiesbaden.

Fernández de Simón, B., Cadahía, E., Jalocha, J. 2003. Volatile compounds in a Spanish red wine aged in barrels made of Spanish, French, and American oak wood. Journal of Agricultural and Food Chemistry 51(26), 7671-7678.

Herjavec, S., Jeromel, A., Orlic, S., Kozina, B. 2007. Changes in volatile and sensory properties of vugava wines aged in croatia oak barrels. Journal of Central European Agriculture 8(2), 195-202.

Jeromel, A., Orbanić, F., Tomaz, I. 2017. Kakvoća crnih vina dozrijevanih u bačvama od slavonske hrastovine. Glasnik zaštite bilja 6, 19-27.

Lantschauer R., Redl, G. Schonberger, G. 2001. Die Weinwelt der Barriques. Vinothek verlag Graz.

Lemperle, E. 2007. Weinfehler erkennen, Ulmer, Stuttgart. p. 51.

Macneil, K. 2015. The Wine Bible (revised second edition), New York Workman publishing, New York, p. 51.

Schusser, M. 2001. Barriquefasserzeugung aus burgenlandischer Eiche, Diplomarbeit an der Universitat fur Bodenkultur, Wien.

Microwave-assisted extraction of tannins from grape skin pomaces

Natka Ćurko, Karla Kelšin, Verica Dragović-Uzelac, Marina Tomašević, Karin Kovačević Ganić

University of Zagreb Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10000 Zagreb (ncurko@pbf.hr)

ABSTRACT

Microwave-assisted extraction (MAE) is valuable alternative to conventional extraction due to the possibility to reach comparable extraction yield with shorter extraction time. The wine by-products (grape skin and seed pomaces) are rich source of phenolic compounds, among others tannins, with diverse bioactive properties. The aim of this study was to evaluate a single and joint effects of different variables under microwave irradiation i.e. solvent concentration, time and temperature on the extraction of tannins from grape skin pomaces. Concentrations of tannins were determined by spectrophotometry using acid hydrolysis method. All tested variables showed significant impact on the extraction yields of total tannins from grape skin pomaces, but solvent concentration and temperature showed to be the most responsive ones. Highest concentrations were extracted with 60% (v/v) of methanol at 60 °C during 9 min. Application of consecutive MAE cycles on three cultivars (Cabernet Sauvignon, Merlot and Teran) revealed that 98.8-99.7% of total tannins were extracted within six cycles. Grape skin pomaces of Teran, compared to Cabernet Sauvignon and Merlot, showed significantly higher concentrations of total tannins.

Keywords: grape by-products; grape skin pomaces; microwave-assisted extraction (MAE); tannins

INTRODUCTION

Grape pomace, consisted of skins, seeds and stems, is the main waste from wine industry representing around 20% of processed grape weight (Ky et al., 2014). In recent years, this wine by-product has attracted considerable attention as inexpensive and easily accessible source of bioactive phenolic compounds, that are used for various proposes in cosmetic, pharmaceutical and food industry (Shrikhande, 2000). Beneficial health effects of polyphenols are primary related to their antioxidant properties (Peixoto et al., 2018). Also, clinical and nutritional epidemiological studies showed inverse correlation between the consumption of polyphenol-enriched diets and reduced risk of neuro-degenerative and cardiovascular diseases (Billingsley & Carbone, 2018; Mennen et al., 2004).

Due to the large structural variability of phenolic compounds present in the different parts of grapes and grape pomaces it is necessary to develop and optimize the extraction method for each matrix. Grape skin represents 45 to 65 % of grape pomace, and is an exceptional source of phenolic acids (hydroxycinnamic and hydroxybenzoic acids), stilbens, anthocyanins (free and acylated anthocyanins), polymeric pigments, flavonols, flavanol monomers and oligomers and particularly flavanol polymers (tannins or proanthocyanidins) (Kammerer et al., 2004). Skin tannins contain lower portion of galloylated sub-units and higher degree of polymerization than seed tannins, and due to their complex structure, conventional extraction methods most commonly include time consuming two-step processes that combine different organic solvents (Chira et al., 2009; Ky et al., 2014).

Microwave-assisted extraction (MAE) is considered as novel extraction method that combines energy of microwave radiation and conventional solvent extraction. This technique is used to extract active compounds from range of raw materials using energy of microwaves to heighten the temperature of the solvent successfully and rapidly (Chan et al., 2011). Also, compared to conventional techniques, MAE can decrease solvent consumption and the extraction time (Casazza et al., 2010; Li et al., 2011; Medouni-Adrar et al., 2015). Nevertheless, very few papers deal with the MAE of phenols from complex matrix of grape skin or skin pomace, while the full potential of this technique in the extraction of tannins to the best of our knowledge has not been systematically studied.

The aim of this work was to study effect of different MAE parameters as solvent concentration, extraction temperature and time as well as effects of sequential repetitive extraction cycles in order to develop the method for the MAE of tannins from grape skin pomaces.

MATERIALS & METHODS

Sample preparation

Grape pomace of Cabernet Sauvignon, Merlot and Teran, were provided from Agrolaguna winery (Poreč, Croatia) in vintage 2014. Samples were first frozen (-80 °C) and afterwards freeze-dried (using Christ Alpha 1-4 LSC Plus freeze-dryer (Osterode am Hatz, Germany). Freeze-dried skins were manually separated from seeds and pulp and ground with electric grinder. Samples were stored at -20 °C before subsequent analyses.

Microwave-Assisted Extraction (MAE)

Extraction was performed in a single-mode microwave reactor (Milestone, Start S Microwave Labstation for Synthesis, Sorisole, Italy). Three independent MAE variables were studied: (i) solvent concentration (% of methanol in water, v/v) [20% (X_{11}), 60% (X_{12}) and 100% (X_{13})]; (ii) extraction duration [2 min (X_{21}) and 9 min (X_{22})]; and (iii) temperature [30 °C (X_{31}) and 60 °C (X_{32})] comprising 12 experiments ($X_{11}X_{21}X_{31}$; $X_{12}X_{21}X_{31}$; $X_{13}X_{21}X_{31}$; $X_{11}X_{22}X_{31}$; $X_{12}X_{22}X_{31}$; $X_{13}X_{22}X_{31}$; $X_{11}X_{21}X_{32}$; $X_{12}X_{21}X_{32}$; $X_{13}X_{21}X_{32}$; $X_{11}X_{22}X_{32}$; $X_{12}X_{22}X_{32}$; $X_{13}X_{22}X_{32}$) that were run in duplicate. A portion of 0.5 g freeze-dried Cabernet Sauvignon skin pomace powder and solvent volume of 25 mL were added to 50 mL round bottom flask with double neck and cooling system with adjustable microwave power output, operating at 2.45 GHz. After each MAE experiment, mixture was centrifuged, supernatant was evaporated at 30 °C, the residue was dissolved in water and freeze-dried in order to obtain grape skin pomace extracts.

Spectrophotometric analysis of total tannins

Concentrations of total tannins (proanthocyanidins) were determined by the method of tannin interflavan bond cleavage and formation of colored anthocyanidins by acid hydrolyses at the temperature of 100 °C (Ribéreau-Gayon & Stonestreet, 1966). Grape skin pomace extracts were solubilized in wine model solution (12% ethanol, 4.5 g/L tartaric acid, pH=3.5) at concentration of 0.25 g/L. Two reaction tubes for each sample were prepared, each containing 4 mL of prepared diluted sample, 2 mL of distilled water and 6 mL of hydrochloric acid (12 N), after which both tubes were hermetically closed. One tube was left at the room temperature, while the other was heated in a water bath at 100 °C. After 30 minutes, the heated tube was cooled and 1 mL of absolute ethanol was added to both tubes. The differences between tubes in absorbance at 550 nm were measured in a path length of 1cm with respect to distilled water as blank. The results were expressed in mg per g of dry weight (dw) grape skin pomaces.

MAE in sequential irradiation cycle

Optimal parameters of single MAE cycle (65% methanol, 9 min and 60 °C) obtained by response surface methodology ($p < 0.001$, $R^2 = 0.9653$) were further performed in eight consecutive cycles. A portion of 0.5 g freeze-dried grape skin pomace powder and solvent volume of 25 mL were added to 50 mL round bottom flask with double neck as earlier described. After first MAE run, mixture was centrifuged, solid part was separated and reused for MAE by addition of fresh solvent. Supernatants were separately evaporated at 30 °C and freeze-dried in order to study the effect of each of eight cycles and finally to determine exact number (n) of irradiation cycles for completion of the extraction.

RESULTS & DISCUSSION

Effect of different MAE variables (methanol concentration, extraction time and temperature) were tested are presented in Fig. 1. Significant differences among majority of treatments were established, where all tested variables influenced extraction yields of total tannins ($p < 0.05$). However, methanol concentration and temperature showed to be the most responsive ones. Also, significant joint effects of methanol concentration and temperature ($p < 0.001$), as well as temperature and time ($p < 0.05$) were found.

The lowest concentrations of tannins were extracted using 20% methanol (v/v) during 2 min at temperature of 30 °C, amounting up to 1.45 mg/L (Fig. 1). Increase of methanol concentration to 60 and 100% (v/v) at the similar conditions (2 min and 30 °C) induced 5.7 fold higher concentrations, but no significant differences were found between the two latter experiments. Identical pattern was noticed among the experiments with 20%, 60% and 100% (v/v) methanol, during 9 min and 30 °C. Compared to 20% methanol, increase from 60 and 100% (v/v) at aforementioned conditions extracted 4.0 and 3.8 fold higher concentrations respectively, but again without significant differences among the latter two.

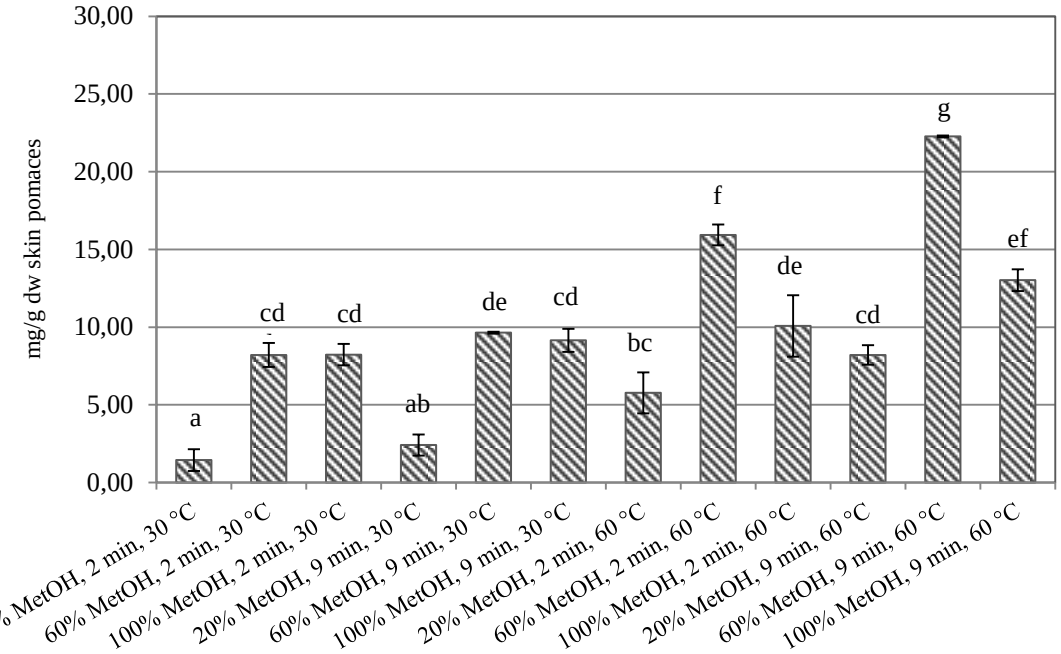


Figure 1. Effect of methanol concentration (20, 60 and 100%, v/v), time (2 and 9 min) and temperature (30 and 60 °C) on the MAE of tannins from grape skin pomaces

On the other hand, opposite trends were noticed among the experiments conducted at 60 °C. Significant differences were found among the extractions using 20%, 60% and 100% (v/v) methanol, for both 2 and 9 min of duration. The highest concentrations were extracted with 60% (v/v) methanol, followed by 100% (v/v) methanol, while the lowest one were extracted with 20 % methanol. Indeed, 60% (v/v) methanol is the most commonly used solvent in the two step conventional extraction of grape tannins (Chira et al., 2009; Ky et al., 2014). Compared to 20% methanol, increase from 60 and 100% (v/v) at 60 °C during 2 min induced 2.8 and 1.7 fold higher concentrations respectively, while at the same conditions and 9 min showed 2.7 and 1.6 fold higher amounts. Obtained results were in accordance to earlier studies that also demonstrated importance of solvent choice and polarity for the extraction of phenolic compounds (Hong et al., 2001; Li et al., 2011). In addition, 1.2 to 4 fold higher concentrations of tannins were extracted at 60 °C, compared to the similar experiments at lower temperature, probably due to the enhancing solubility and the diffusion coefficient (Pinelo et al., 2005). Nevertheless, since flavonoids showed to be sensitive to the temperature degradation (Casazza et al., 2010), this parameter was kept relatively low. Also, prolongation of the extraction time from 2 to 9 min at 30°C extracted 1.1 to 1.7 fold higher concentrations of tannins, while the significant effect of time variable was not noticed at this temperature. The same prolongation at 60 °C induced 1.3-1.4 fold higher concentrations of tannins, while significant effect of time variable, contrary to earlier observe at 30 °C, was noticed in the case 60% and 100% (v/v) methanol.

The highest concentrations of total tannins were extracted using 60% methanol (v/v) during 9 min at temperature of 60 °C, amounting up to 22.27 mg/L (Fig. 1). Comparison of the single MAE cycle results of with other MAE studies conducted on grape skin pomace was difficult, since various operating systems were used, whit different research aims (mostly focused to the total polyphenols), as well as different solvents, liquid:solid ratio (l:s), temperatures, power, time, etc. (Casazza et al., 2010; Medouni-Adrar et al., 2015; Pedroza et al., 2015). For example, extraction with 100% (v/v) methanol, with 5mL/g (l:s), at 110 °C and 60 W during 60 min were optimal parameters performed by Casazza et al. (2010) while 60% (v/v) ethanol, with 4 mL/g (l:s) and 900 W during 1,033 s, were the optimal ones performed by Pedroza et al. (2015).

Table 1. Extraction of total tannins (concentration and relative recovery) by sequential irradiation cycles in Cabernet Sauvignon, Merlot and Teran grape skin pomaces

Cycle number	Grape pomace cultivar					
	Cabernet Sauvignon		Merlot		Teran	
	TT*	TT (%)**	TT*	TT (%)**	TT*	TT (%)**
1st	18.78[a]	59.6	17.70[a]	60.4	23.82[b]	61.9
2nd	27.05[c]	85.8	24.87[b]	84.9	32.99[h]	85.8
3rd	29.16[ef]	92.6	27.50[cd]	93.9	36.10[i]	93.9
4th	30.32[fg]	96.2	28.63[de]	97.8	37.27[ij]	96.9
5th	30.89[g]	98.0	28.86[e]	98.6	38.11[j]	99.1
6th	31.13[g]	98.8	29.06[ef]	99.2	38.34[j]	99.7
7th	31.35[g]	99.5	29.21[ef]	99.7	38.41[j]	99.9
8th	31.51[g]	100	29.29[ef]	100	38.46[j]	100

Data are expressed as average value of three replications ± standard deviation (n=3). ANOVA to compare data among three cultivars; different letters indicate statistical differences between extracts (Tukey's test, $p < 0.05$). Abbreviations: TT: total tannins. *Concentrations of total tannins expressed in mg/g dw grape skin pomaces. **Relative recovery of total tannins expressed in % and calculated relative to the amount obtained after eight cycles.

In order to develop the method for the extraction of tannins that could be applied prior to analytical determination, effects of eight MAE cycles were studied (Fig.1). Sequential irradiation cycles were tested by addition of fresh solvent and by repeating the extraction step following the optimal extraction conditions obtained by response surface methodology as earlier described. The highest concentrations of tannins in all three cultivars (Cabernet Sauvignon, Merlot and Teran) were extracted in the first cycle. Each successive cycle compared to the first one, extracted lower amounts, but at the same time contributed to the overall concentration. Significant increase in concentration of tannins was found in fist six cycles, implying their importance in all three cultivars. On the other side, impact of the last two cycles was negligible. Earlier study of Pedroza et al. (2015) also demonstrated the significance of sequential cycles in the MAE of total polyphenols. However, due to the differences in the parameters used (higher power and shorter time) these authors proposed lower number of cycles.
Finally, our results showed that for completing the extraction of tannins from grape skin pomace optimal MAE conditions (65% methanol v/v, at 60 °C during 9 min) should be applied in six sequential irradiation cycles. Overall extraction time of our method (6×9 min) was comparable with the study of Casazza et al. (2010), where lower power (60 W) and longer time (60 min) were also applied. In addition, compared to time consuming conventional extraction procedures that can take up to 6-24 h, extraction time was importantly shortened. Significant differences among three cultivars were established in the concentrations of total tannins, while contrary, similar trends considering the relative recovery were observed. Grape skin pomace of Teran showed to be rich source of tannins, with significantly higher concentrations than Cabernet Sauvignon and Merlot. Concentrations extracted by final MAE method were in line with the values obtain in grape skin pomaces (different cultivars, vintages, terroir, etc.) by conventional extraction available in the literature data (Deng et al., 2011; Ky et al., 2014), additionally confirming the efficiency of developed method.

CONCLUSION

Microwave assisted extraction (MAE) proved to be efficient and fast method for the extraction of tannins from grape skin pomaces. All tested MAE parameters, methanol concentration, temperature, time, as well as number of irradiation cycles showed significant impact. Highest concentrations of tannins were extracted using 60% (v/v) methanol at 60 °C during 9 min. Application of optimal MAE

parameters in six sequential irradiation cycles was necessary for completing the extraction of tannins from grape skin pomace prior to the analytical determination.

ACKNOWLEDGMENTS

This work was supported by means of the project "The application of innovative technologies in bioactive compounds isolation from organic waste in the wine production", co-financed by the European Union under the call RC.2.2.08: "Strengthening Capacities for Research, Development and Innovation" funded by the European Regional Development Fund, the Regional Competitiveness Operational Program 2007 - 2013.

REFERENCES

Billingsley, H. E., Carbone, S. 2018. The antioxidant potential of the Mediterranean diet in patients at high cardiovascular risk: An in-depth review of the PREDIMED. Nutrition and Diabetes 8(1), 1-13.

Casazza, A. A., Aliakbarian, B., Mantegna, S., Cravotto, G., Perego, P. 2010. Extraction of phenolics from *Vitis vinifera* wastes using non-conventional techniques. Journal of Food Engineering 100(1), 50-55.

Chan, C. H., Yusoff, R., Ngoh, G. C., Kung, F. W. L. 2011. Microwave-assisted extractions of active ingredients from plants. Journal of Chromatography A 1218(37), 6213-6225.

Chira, K., Schmauch, G., Saucier, C. d., Fabre, S., Teissedre, P.-L. 2009. Grape Variety Effect on Proanthocyanidin Composition and Sensory Perception of Skin and Seed Tannin Extracts from Bordeaux Wine Grapes (Cabernet Sauvignon and Merlot) for Two Consecutive Vintages (2006 and 2007). Journal of agricultural and food chemistry 57(2), 545-553.

Deng, Q., Penner, M. H., Zhao, Y. 2011. Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins. Food Research International 44(9), 2712-2720.

Hong, N., Yaylayan, V. A., Vijaya Raghavan, G. S., Paré, J. R. J., Bélanger, J. M. R. 2001. Microwave-assisted extraction of phenolic compounds from grape seed. Natural Product Letters 15(3), 197-204.

Kammerer, D., Claus, A., Carle, R., Schieber, A. 2004. Polyphenol Screening of Pomace from Red and White Grape Varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. Journal of agricultural and food chemistry 52(14), 4360-4367.

Ky, I., Lorrain, B., Kolbas, N., Crozier, A., Teissedre, P. L. 2014. Wine by-Products: Phenolic characterization and antioxidant activity evaluation of grapes and grape pomaces from six different French grape varieties. Molecules 19(1), 482-506.

Li, Y., Skouroumounis, G. K., Elsey, G. M., Taylor, D. K. 2011. Microwave-assistance provides very rapid and efficient extraction of grape seed polyphenols. Food Chemistry 129(2), 570-576.

Medouni-Adrar, S., Boulekbache-Makhlouf, L., Cadot, Y., Medouni-Haroune, L., Dahmoune, F., Makhoukhe, A., Madani, K. 2015. Optimization of the recovery of phenolic compounds from Algerian grape by-products. Industrial Crops and Products 77, 123-132.

Mennen, L. I., Sapinho, D., De Bree, A., Arnault, N., Bertrais, S., Galan, P., Hercberg, S. 2004. Consumption of Foods Rich in Flavonoids Is Related to A Decreased Cardiovascular Risk in Apparently Healthy French Women. Journal of Nutrition 134(4), 923-926.

Pedroza, M. A., Amendola, D., Maggi, L., Zalacain, A., De Faveri, D. M., Spigno, G. 2015. Microwave-assisted extraction of phenolic compounds from dried waste grape skins. International Journal of Food Engineering 11(3), 359-370.

Peixoto, C. M., Dias, M. I., Alves, M. J., Calhelha, R. C., Barros, L., Pinho, S. P., Ferreira, I. C. F. R. 2018. Grape pomace as a source of phenolic compounds and diverse bioactive properties. Food Chemistry 25, 3132-138.

Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., Núñez, M. J. 2005. Effect of Solvent, Temperature, and Solvent-to-Solid Ratio on the Total Phenolic Content and Antiradical Activity of Extracts from Different Components of Grape Pomace. Journal of agricultural and food chemistry 53(6), 2111-2117.

Ribéreau-Gayon, P., Stonestreet, E. 1966. Dosage des tanins du vin rouge et détermination de leur structure. Chimie Analytique 48(4), 188-196.

Shrikhande, A. J. 2000. Wine by-products with health benefits. Food Research International 33(6), 469-474.

Routes to recycle food byproducts into new food chains

Vera Lavelli[a]

[a] *DeFENS, Department of Food, Environmental and Nutritional Sciences, University of Milan, 20133 Milan, Italy, vera.lavelli@unimi.it*

ABSTRACT

The bioeconomy approach calls for a transition of food production processes from linear systems to circular systems, that mimic natural cycles and have the capacity to utilize by-products as resources. The present report aims to illustrate three strategies to use food by-products as raw materials for food value chains. Winemaking by-products are considered as a case-study. These latter mainly consist of grape marc (skins and seeds). According to the FAO, ~ 80 Mt of grapes are produced throughout the world annually, most of which are used in winemaking, generating approximately 3.4 Mt of grape marc.

In the first recycling route, grape marc is processed to obtain health-promoting food ingredients to be used in functional cereal-based, dairy, and fruit-based products that can be applied as a dietary intervention to prevent major chronic diseases, such as cardiovascular disease and diabetes.

A second route is to process grape marc into "clean label" food additives with multifunctional properties such as antioxidant, colorant and antimicrobial agents that replace the synthetic ones in meat-, fish- and fruit-based products.

The third route aims at a sustainable production of food, thus addressing the needs of populations with endemic nutritional deficiencies. Grape marc is therefore used as a growth substrate for the basidiomycetes belonging to the genus *Pleurotus*, which possesses a complex enzymatic system that converts lignocellulosic materials into a fungal biomass that is rich in proteins as well as in different bioactive compounds.

Keywords: by-product; waste; antioxidant dietary fibre; phenolics; food protein

INTRODUCTION

Food supply chains have significant environmental impacts due to their use of resources and production of emissions, effluents and waste materials. The "costs of non-recycling" encompass both environmental pollution and economic and social impacts, including employment rate and nutrition security. The primary solid residue of winemaking, i.e., grape marc (otherwise referred to as grape pomace) is traditionally distilled to produce different types of "wine alcohol**"** or used as fertilizer or animal feed. Red grape skins are also used for the production of the colorant "enocyanine", while grape seed are extracted to obtain grape seed oil and grape seed proanthocyanidins. However, these alternatives lack economic and social benefits. The interest of the scientific community and producers has recently directed to more profitable and so-called sustainable options that are based on the recovery of nutritionally valuable fractions (Garcia-Lomillo, Gonzalez-SanJosè, 2016; Lavelli et al., 2016a).

In the first recycling route, grape pomace is processed to obtain health-promoting food ingredients. Dietary fibre is the main component of grape pomace, ranging between 43% and 75% d.w. (Garcia-Lomillo, Gonzalez-SanJosè, 2016). Its peculiar feature is to be associated to a large fraction of insoluble proanthocyanidins, thus it is generally referred to as "antioxidant dietary fibre" (ADF) (Bravo, Saura-Calixto, 1998). To recover ADF, winemaking byproducts are dried at temperatures below 60 °C, milled and sieved to specific particle sizes depending on the application. An *in vivo* study was performed with grape pomace ADF involving both normocholesterolemic and hypercholesterolemic adults, supplemented with 7.5 g/d of ADF, containing 5.25 g of dietary fibre and 1.4 g of polyphenols for 16 weeks. Grape pomace ADF was more effective than other fibre sources in improving lipid profiles and blood pressure (Perez-Jimenez et al., 2008). This latter study has raised an increasing interest in the use of grape ADF as an ingredient in new functional foods.

A second route is to process grape marc into "clean label" food additives, based on the multifunctional properties of grape phenolics, such as antioxidant, colorant or antimicrobial agents. The soluble phenolic fraction is also relevant in grape pomace. The content of soluble proanthocyanidins ranges between 1.16 and 44.6 g/kg d.w. in the skin and between 23.1 and 68.5 g/kg d.w. in the seeds; total anthocyanin content of red grape skins is in the range of 2.5–132 g/kg d.w.; the total flavonol content is in the range

of 0.3–2.6 g/kg d.w. of grape skins but less than 0.1 g/kg d.w. in grape seeds (Lavelli et al., 2016a). Solvents used to recover the soluble phenolic fraction include water, ethanol:water mixtures and aqueous solutions of organic acids. Extraction techniques include conventional solid-liquid extraction, ultrasound-assisted and microwave-assisted extraction and high pressure extraction. The crude extract is generally dried to obtain a powder. In a few cases, using a tailored approach that takes into account the final target application, the addition of suitable carrier materials can be exploited to increase and modify the stability and solubility of phenolic compounds (Lavelli et al., 2017).
The third recycling route aims at a sustainable production of nutrients, especially proteins, through the use of winemaking byproducts as growing substrate for *Pleurotus* spp. In fact, *Pleurotus* spp. are a fast yielding and nutritious source of food that can be prepared with limited capital investment and technical skill from various agricultural waste materials, including winemaking byproducts. Overall, 28 different industrial by-products were tested as a growth substrate for the *Pleurotus* genus, resulting in a protein content in the range 9.3–37.4 g/100g d.w. Use of winemaking byproducts as growth substrate led to a protein content of 27.8 g/kg d.w.. Furthermore, the protein of *Pleurotus* generally meets the essential amino acid scoring patterns recommended for children, adolescents and adults (Lavelli et al., 2018). Conversely, the amount of protein in grape pomace is in the range 11–14% d.w. and their digestibility is approximately 20%, probably due to proanthocyanidin-protein complexes (Bravo et al., 1998). Additionally, *Pleurotus* spp. is a good source of dietary fibre with unique structural features (branched ß-glucans) and low-molecular weight bioactive compounds, vitamins and minerals. Indeed, investigations on the nutritional and therapeutic uses of the genus *Pleurotus* have impressively accelerated during the last ten years (Correa et al., 2016). The present report aims to illustrate various food applications that have been developed in the framework of the three strategies above described.

MATERIALS & METHODS

The articles published in the last 20 years reporting food applications of winemaking byproducts were selected from Scopus and Web of Science database using the above-indicated keywords.

RESULTS & DISCUSSION

One route to recycle winemaking byproducts is to use grape pomace, grape skins or grape seeds ADF for food fortification. To get health benefits from ADF intake, there is practical significance in identifying the maximal amount of ADF that can be incorporated in foods without affecting the consumers' liking. Representative applications of ADF are shown in Table 1.

Table 1. Use of antioxidant dietary fibre (ADF) from grape pomace, grape skin and grape seeds for food fortification.

ADF source and particle size	Addition level[a]	Target food
Skin ADF < 0.250 mm	8–24 g ADF/kg (8 g ADF/kg)	Cheese (Torri et al., 2016)
Pomace ADF < 0.18 mm	10–30 g ADF/kg (10 g ADF/kg)	Yogurt (Marchiani et al., 2016)
Pomace ADF < 0.500 mm	5–20 g ADF/kg (20 g ADF/kg)	Chicken (Sáyago-Ayerdi et al., 2009)
Skin ADF < 0.250 mm	20 g ADF/kg	Beef (García-Lomillo et al., 2016)
Skin ADF < 1 mm	30 g ADF/kg (30 g ADF/kg)	Fish (Ribeiro et al., 2013)
Skin ADF 0.125 – 0.500 mm	30 g ADF/kg	Tomato puree (Lavelli et al., 2014)
Pomace ADF < 0.811 mm	25–75 g ADF/kg (25 g ADF/kg)	Wheat pasta (Sant'Anna et al., 2014)
Skin ADF < 0.150 mm	100 g ADF/kg	Bread (Lavelli et al., 2016b)
Seed ADF < 0.150 mm	25–100 g ADF/kg (75 g ADF/kg)	Bread (Hoye, Ross, 2011)
Pomace ADF < 0.150 mm	100–300 g ADF/kg (200 g ADF/kg)	Muffins (Mildner-Szkudlarz et al., 2015)

[a]The optimal amount is indicated in brackets.

In general, the particle size of ADF played a major role in its chemico-physical properties and consumers' acceptability (Lavelli et al., 2014). Indeed, the sensory effects elicited by ADF addition in

foods are related to the perception of texture, even if color, taste, flavor and odor attributes are also elicited. The amount of ADF incorporated into foods was approximately 10g/kg in yogurt and cheese (Marchiani et al., 2016; Torri et al., 2016), 20–30 g/kg in meat- and fish-based products (Sáyago-Ayerdi et al., 2009; García-Lomillo et al., 2016), vegetable purees (Lavelli et al., 2014) and pasta (Sant'Anna et al., 2014), 75–200 g/kg in bread and bakery products (Hoye, Ross, 2011; Mildner-Szkudlarz et al., 2015; Lavelli et al., 2016b). Hence, in most foods except for dairy products, ADF was added at levels that were proven to have a healthy effect as observed by Perez-Jimenez et al. (2008). In thermally treated foods, ADF inhibits the formation of harmful advanced glycation end-products (Mildner-Szkudlarz et al., 2015). ADF also delivers phenolics that are inhibitors of mammalian α-glucosidase and α-amylase, hence in starch-based foods it can potentially address the needs of diabetic people (Lavelli et al., 2016b). Another recycling route is to use winemaking by-products as a source of "clean label" food additives (Table 2). The powdered phenolic extracts have been applied as antioxidants in meat- and fish-based products, at a concentration of 0.05–1.3 g/kg (Rojas, Brewer, 2007; Carpenter, 2007; Ozen, 2011; Selani et al., 2011). Applications of phenolic extract powder as antimicrobials have also been proposed. In meat inoculated with *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Aeromonas hydrophila*, significant reductions in the population of these species were observed during storage at 4 ºC (Ahn et al., 2007), but the amount of grape seed phenolics necessary for this effect was high, i.e., 8.5 g/kg. However, in refrigerated meat not challenged with pathogen microorganisms, low addition of pehnolics, i.e., 373 mg/kg led to a decrease of total viable counts, lactic acid bacteria, *Pseudomonas* and psychotropic aerobic bacteria (Lorenzo et al., 2014). Powdered and liquid phenolic extracts have also been applied as colorants in gelatin and pectin gel, yogurt and biscuits in the concentration range 58–315 mg/kg (Maier et al., 2009; Karaaslan et al., 2011; Pasqualone et al., 2014).

Table 2. Use of phenolic extracts from grape pomace, grape skin and grape seeds as a "clean label" food additive.

Extract source and state	Addition level[a]	Target food and functionality
Seed extract powder	0.1–0.2 g TP (F) /kg	Meat/antioxidant (Rojas, Brewer, 2007)
Seed extract powder	0.05–1 g TP (GAE)/kg	Meat/antioxidant (Carpenter et al., 2007)
Pomace water extract	0.06 g TP (GAE)/kg	Meat/antioxidant (Selani et al., 2011)
Seed extract powder	1.32 g TP (GAE)/kg	Fish/antioxidant (Ozen et al., 2011)
Pomace phenolics in O/W[b]	3 mg TP(GAE)/kg	Hazelnut paste/antioxidant (Spigno et al., 2013)
Seed extract powder	8.5 g TP (GAE)/kg	Meat/antimicrobial (Ahn et al., 2007)
Seed extract powder	373 mg TP (GAE)/kg	Meat/antimicrobial (Lorenzo et al., 2014)
Pomace extract powder	190 mg ACN/kg	Gelatin and pectin gels/colorant (Maier et al., 2009)
Skin water extract	58 mg ACN/L	Yogurt/colorant (Karaaslan et al., 2011)
Skin phenolics in MD[c]	100 mg ACN/kg	Apple puree/colorant (Lavelli et al., 2016c)
Pomace extract[d]	315 mg ACN/kg	Biscuits/colorant (Pasqualone et al., 2014)

[a]TP: total phenolics, expressed as: F: flavanols or GAE: gallic acid equivalents. ACN: anthocyanins. [b]O/W: oil-in- water nanoemulsion. [c]MD: maltodextrin-encapsulated. [d]Solvent was 85:15 ethanol/1 M citric acid, pH 4.

Some studies have pointed out that the properties of grape phenolics could be lower in foods than in model systems. The low solubility of certain polyphenols in food systems limits their efficacy (Shan et al., 2011). Furthermore, phenolic activity is decreased by phenolic interaction with the food matrix, especially with proteins (Lavelli et al., 2016b). The aforementioned observations highlight the importance of applying novel formulation strategies. To increase the antioxidant effect in the heterogeneous food matrix, grape pomace phenolics were formulated in oil/water nanoemulsion, and proved to be effective in an hazelnut paste at 3 mg/kg (Spigno et al., 2016). Encapsulation of grape skin phenolics in maltodextrin was effective for their use as colorant in beverages (Lavelli et al., 2016c).
A third recycling approach is to convert winemaking byproducts into a protein-rich biomass through solid state fermentation by *Pleurotus*. Efforts have been made to use dried *Pleurotus* spp. to increase protein content of cereal-based or tapioca-based foods (Table 3). In wheat bread and tapioca cracker the increase in protein content upon addition of 20% and 25% of *Pleurotus* powder was from 7.96 to 14.21 g/100 g f.w. and from 0.47 to 3.88 g/100 g f.w, respectively (Okafor et al., 2012; Yahya et al., 2017). Another approach was to use *Pleurotus* powder in processed meat and poultry products to or to replace

high-cost proteins (Wan Rosli et al., 2011; Wan Rosli, Solihah, 2017). Beside protein, *Pleurotus* addition to foods could have a great impact on micronutrient content. However, knowledge on the effect of growth substrate on the level of vitamins, mineral and bioactive compounds in *Pleurotus* spp. is still lacking (Lavelli et al., 2018).

Table 3. Use of dried *Pleurotus* spp. as food ingredient to increase protein content or to replace high-cost protein.

***Pleurotus* species**	**Addition level**	**Target food and functionality**
P. pulmonarius	5–25%	Bread/increase in protein (Okafor et al., 2012)
P. sajor-caju	5–20%	Tapioca cracker/increase in protein (Yahya et al., 2017)
P. sajor-caju	25–50%	Chicken patty/replace of high-cost protein (Wan Rosli et al., 2011)
P. sajor-caju	25–50%	Beef patty/replace of high-cost protein (Wan Rosli, Solihah, 2017)

CONCLUSION

The ADF recovered from winemaking byproducts can be incorporated in various foods without negative impact on consumers' liking, at levels that have been proven to have healthy effects in hypercholesterolemic adults. Hence, the recovered ADF could be used to implement an everyday dietary intervention over a long-term period.

The use of phenolic fractions recovered from winemaking byproducts as clean label antioxidant additives seem to be promising, while testing new formulation strategies (encapsulation or nanoemulsion) is necessary to improve their efficacy as antimicrobials and colorants. Indeed, phenolic interaction with the food matrix could reduce their solubility, stability and overall efficacy.

The use of winemaking byproducts as growth substrate for *Pleurotus* spp. can lead to a sustainable production of protein-rich foods. At the same time, to take advantage of the great potential of *Pleurotus* spp., a major knowledge on its micronutrients and bioactive compounds is needed.

ACKNOWLEDGEMENTS

Research funded by Ager Foundation, Project Valorvitis 2.0 - La ricerca e l'innovazione incontrano il mercato and Cariplo Foundation, Project ReMarcForFood n. 2016-0740.

REFERENCES

Ahn J., Grün I.U., Mustapha A. 2007. Effects of plant extracts on microbial growth, color change, and lipid oxidation in cooked beef. Food Microbiology 24(1), 7-14.

Bravo L., Saura-Calixto F. 1998. Characterization of dietary fiber and the in vitro indigestible fraction of grape pomace. American Journal of Enology and Viticulture 49(2), 135-141.

Carpenter R., O'Grady M.N., O'Callaghan Y.C., O'Brien N.M., Kerry J.P. 2007. Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. Meat Science 76(4), 604-610.

Corrêa R. C. G., Brugnari T., Bracht A., Peralta R. M., Ferreira I. C. F. R. 2016. Biotechnological, nutritional and therapeutic uses of *Pleurotus* spp. (Oyster mushroom) related with its chemical composition: A review on the past decade findings. Trends in Food Science and Technology 50, 103–117.

García-Lomillo J., González-SanJosé M.L., Skibsted L.H., Jongbergb S. 2016. Effect of skin wine pomace and sulfite on protein oxidation in beef patties during high-oxygen atmosphere storage. Food and Bioprocess Technology 9(3), 532-542.

Garcıa-Lomillo J., Gonzalez-SanJosé M.L. 2017. Applications of wine pomace in the food industry: approaches and functions. Comprehensive Reviews in Food Science and Food Safety 16(1), 3–22.

Hoye C., Ross C.F. 2011. Total phenolic content, consumer acceptance, and instrumental analysis of bread made with grape seed flour. Journal of Food Science 76(7), S428–S436.

Karaaslan M., Ozden M., Vardin H., Turkoglu H. 2011. Phenolic fortification of yogurt using grape and callus extracts. LWT - Food Science and Technology 44(4), 1065-1072.

Lavelli V., Sri Harsha P.S.C., Torri L., Zeppa G. 2014. Use of winemaking by-products as an ingredient for tomato puree: The effect of particle size on product quality. Food Chemistry 152, 162-168.

Lavelli V., Torri L., Zeppa G., Fiori L., Spigno G. 2016a. Recovery of winemaking by-products for innovative food applications – A review. Italian Journal of Food Science, 28, 542-564.
Lavelli V., Sri Harsha P.S.C., Ferranti P., Scarafoni A., Iametti S. 2016b. Grape skin phenolics as inhibitors of mammalian α-glucosidase and α-amylase – Effect of food matrix and processing on efficacy. Food & Function 7(3), 1655-1663.
Lavelli V., Sri Harsha P.S.C., Spigno G. 2016c. Modelling the stability of maltodextrin-encapsulated grape skin phenolics used as a new ingredient in apple puree. Food Chemistry 209, 323-331.
Lavelli V., Sri Harsha P.S.C., Laureati M., Pagliarini E. 2017. Degradation kinetics of encapsulated grape skin phenolics and micronized grape skins in various water activity environments and criteria to develop wide-ranging and tailor-made food applications. Innovative Food Science and Emerging Technology 39, 156-164.
Lavelli V., Proserpio C., Gallotti F., Laureati M., Pagliarini E. 2018. Circular reuse of bio-resources: the role of *Pleurotus* spp. in the development of functional foods. Food & Function 9, 1353–1372.
Lorenzo J.M., Sineiro J., Amado I.R., Franco D. 2014. Influence of natural extracts on the shelf life of modified atmosphere-packaged pork patties. Meat Science 96(1), 526-534.
Maier T., Fromm M., Schieber A., Kammerer D.R., Carle R. 2009. Process and storage stability of anthocyanins and non-anthocyanin phenolics in pectin and gelatin gels enriched with grape pomace extracts. European Food Research and Technology 229(6), 949-960.
Marchiani R., Bertolino M., Belviso S., Giordano M., Ghirardello D., Torri L., Piochi M., Zeppa G. 2016. Yogurt enrichment with grape pomace: effect of grape cultivar on physicochemical, microbiological and sensory properties. Journal of Food Quality 39(2), 77-89.
Mildner-Szkudlarz S., Siger A., Szwengiel A., Bajerska J. 2015. Natural compounds from grape by-products enhance nutritive value and reduce formation of CML in model muffins. Food Chemistry 172, 78–85.
Okafor J. N. C., Okafor G. I., Ozumba A. U., Elemo G. N. 2012. Quality characteristics of bread made from wheat and Nigerian oyster mushroom (Pleurotus plumonarius) powder. Pakistan Journal of Nutrition 11, 5-10.
Ozen B.O., Eren M., Pala A., Ozmen I. Soyer A. 2011. Effect of plant extracts on lipid oxidation during frozen storage of minced fish muscle. International Journal of Food Science and Technology 46(4), 724–731.
Pasqualone A., Bianco A.M., Paradiso V.M., Summo C., Gambacorta G., Caponio F. 2014. Physico-chemical, sensory and volatile profiles of biscuits enriched with grape marc extract. Food Research International 65, 385-393.
Perez-Jimenez J., Serrano J., Tabernero M., Diaz-Rubio M.E., Arranz S., Garcia-Diz L., Goni I., Saura-Calixto F. 2008. Effects of grape antioxidant dietary fiber in cardiovascular disease risk factors. Nutrition 24(7-8), 646-653.
Rojas M.C., Brewer M.S. 2007. Effect of natural antioxidants on oxidative stability of cooked, refrigerated beef and pork. Journal of Food Science 72(4), S282-S288.
Sant'Anna V., Christiano F.D.P., Marczak L.D.F., Tessaro I.C., Thys R.C.S. 2014. The effect of the incorporation of grape marc powder in fettuccini pasta properties. LWT - Food Science and Technology 58(2), 497-501.
Sáyago-Ayerdi S.G., Brenes A., Goñi I. 2009. Effect of grape antioxidant dietary fiber on the lipid oxidation of raw and cooked chicken hamburgers. LWT - Food Science and Technology 42(5), 971-976.
Selani M.M., Contreras-Castillo C.J., Shirahigue, L.D., Gallo C.R., Plata-Oviedo M., Montes-Villanueva N.D. 2011. Wine industry residues extracts as natural antioxidants in raw and cooked chicken meat during frozen storage. Meat Science 88(3), 397-403.
Shan B, Cai Y.Z., Brooks J.D., Corke H. 2011. Potential application of spice and herb extracts as natural preservatives in cheese. Journal of Medicinal Food 14(3), 284-290.
Spigno G., Donsì F., Amendola D., Sessa M., Ferrari G., De Faveri D.M. 2013. Nanoencapsulation systems to improve solubility and antioxidant efficiency of a grape marc extract into hazelnut paste. Journal of Food Engineering 114(2), 207-214.
Torri L., Piochi M., Marchiani R., Zeppa G., Dinnella C., Monteleone E. 2016. A sensory- and consumer-based approach to optimize cheese enrichment with grape skin powders. Journal of Dairy Science 99(1), 194-204.
Wan Rosli W. I., Solihah M. A., Mohsin S. S. J. 2011. On the ability of oyster mushroom (*Pleurotus sajor-caju*) conferring changes in proximate composition and sensory evaluation of chicken patty. International Food Research Journal 18, 1463-1469.
Wan Rosli W. I., Solihah M. A. 2012. Effect on the addition of *Pleurotus sajor-caju* (PSC) on physical and sensorial properties of beef patty. International Food Research Journal 19, 993-999.
Yahya F., Yusof N. N. M., Chen C. K. 2017. Effect of varying ratios of oyster mushroom powder to tapioca flour on the physicochemical properties and sensory acceptability of fried mushroom crackers. Malaysian Applied Biology 46, 57-62.

Development and characterization of edible films based on pectin from sugar beet pulp

Monika Ostrman, Aleksandra Vojvodić Cebin, Arijana Bušić, Sonja Vrtar, Draženka Komes

Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia (mostrman@pbf.hr)

ABSTRACT

Nowadays, there is a global interest in the technological use of agro-industrial residues as a renewable source of food or food supplements. In order to improve valorisation of sugar beet pulp as a pectin-rich secondary raw material, the aim of this study was to investigate the delivery potential of pectin extracted from sugar beet pulp (SBP) in the formulation of functional edible films. Beside pectin, new developed edible films, based on yellow tea as a source of bioactive compounds, were additionally enriched with fenugreek and rice proteins. In order to remove the hydrophilic compounds of low molecular weight, extraction of SBP pectin was performed using 70 % ethanolic solution, and afterwards pectin from alcohol insoluble residue was extracted by acid hydrolysis, followed by dialysis, evaporation and freeze drying. Physical properties of proteins and isolated SBP pectin, as well as physico-chemical, mechanical, bioactive and sensory properties of the formulated films were determined. Based on the obtained results, the highest content of total polyphenols (106.22 mg GAE/g film) and individual bioactive compounds (gallic acid, catechin, epigallocatechin and caffeine), as well as the highest value of antioxidant capacity (1.89 mmol Trolox/g film) were determined in the film enriched with rice proteins. Although yellow tea markedly improved the functional potential of all formulated films, pectin film without proteins exhibited the best mechanical and sensory properties, in terms of tensile strength, elasticity, acceptability, colour, transparency and appearance.

Keywords: agrowaste; bioactive compounds; edible films; pectin; sugar beet pulp

INTRODUCTION

The food industry generates a large amount of waste that is often discarded in the environment or used as animal feed. Recently, due to excessive accumulation of agrowaste, there is a growing need for its utilization as a source of various functional ingredients. In this sense, sugar beet pulp as an agro waste from sugar industry could be used as a source of pectin for developing functional, environmentally safe and sustainable edible films.

Edible films are thin layer of edible material that directly coats the food, produced by edible polymers (polysaccharides or proteins and their combination) and food grade additives without changing the original ingredients or processing method of the product. The main tasks of edible films are to extend the limited shelf life of food products, to reduce the oxidation reactions such as degradation, enzymatic browning and oxidative rancidity, and to improve the gas and moisture barriers, but also nowadays it is highly important to enrich food products with the bioactive compounds and to improve their functional and sensory properties (Chiralt et al., 2018).

The most often used biodegradable film-forming biopolymers are pectin, alginate, chitosan, carrageenan and gum arabic. Pectin-based films have lots of benefits for their use in food industry, including low cost, biodegradability, "green-packaging" material characteristics and GRAS status of pectin (FDA, 2013). Pectin films are characterized by crystalline and/or amorphous regions, which are suitable for implementation of bioactive compounds, providing them antimicrobial, nutritive, and antioxidant properties (De'Nobili et al., 2013).

Beside polysaccharides, for the formulation of edible films proteins deriving from different animal and plant sources, including animal tissues, milk, eggs, grains, and oilseeds, due to their availability and low cost are often used. The most commonly used proteins for the formulation of edible films are collagen, gelatin, caseins, whey proteins, corn zein, wheat gluten, soy protein, egg white protein etc. The addition of protein to edible films exhibit valuable characteristics, such as transparency and great barrier properties, but it also contributes to better incorporation of bioactive compounds such as nutrients, antioxidants, flavours etc. (Krochta, 2002).

In order to combine more functional ingredients, the objective of this study was to develop edible films based on pectin extracted from sugar beet pulp, additionally enriched with bioactive compounds from

yellow tea extract and proteins. Also, physico-chemical, mechanical, bioactive and sensory properties of some ingredients and new developed films were evaluated.

MATERIALS & METHODS

Materials

Pectin was extracted from sugar beet pulp, while fenugreek (*Trigonella foenum graecum* L.) and rice (*Oryza sativa* L.) proteins were purchased from specialised food market.

Pectin extraction

Alcohol soluble compounds were extracted from previously chopped and degreased sugar beet pulp using 70 % ethanolic solution, and afterwards obtained residue was used for acidic hydrolysis (HCl, pH 1.5, 2 h, 90 °C). The obtained extracts were precipitated using 96 % ethanol during the night at 4 °C, followed by dialysis, evaporation and freeze drying.

Pectin characterization

The equivalent (molar) weight (EW) and degree of esterification (DE) were determined according to the modified method of Aina et al. (2012). The content of galacturonic acid was detected by spectrophotometric method according to the modified procedure of Melton et al. (2001).

Physical properties of proteins

Foaming capacity (FC) and foaming stability (FS) of proteins were determined according to Kempka et al. (2015), using Ultraturax IKA-T18 basic device for stirring the samples. Electrical conductivity (EC) of protein solutions was determined using SevenCompact conductometer (Mettler Toledo, Switzerland). Water holding capacity (WHC) and fat absorption capacity (FAC) were measured according to the modified method of Stone et al. (2015), while determination of emulsion activity index (EAI) and emulsion stability index (ESI) were carried out according to the modified method of Belščak-Cvitanović et al. (2018).

Formulation of pectin-based edible films

Edible films were prepared by combination of pectin and protein solutions (1:1), both previously prepared in yellow tea extract using conventional technique, with the addition of glycerol to ensure film plasticization (Table 1). All films were prepared in duplicate.

Table 1. Composition of formulated edible films

Sample	Content of pectin (%)	Content of proteins (%)
F1	100	-
F2	50	50 (fenugreek proteins)
F3	50	50 (rice proteins)

Physico-chemical and mechanical properties of formulated films

Dry matter content of produced edible films was determined according to AOAC method (1990). The thickness of films (n=6) was measured using a digital hand-held micrometer (Digital Micrometer, Type 40 EX, Germany) and the results were expressed in μm. Texture analysis of films was carried out using a TA.HDPlus texture analyser (Stable Micro Systems, Godalming, Great Britain). The maximum force (N), needed for compression, represented the hardness of the samples, while elasticity of samples was measured as a ratio of probe travel distance and expressed in mm. Determination of sample colour was performed using a colorimeter and the readings of L* (lightness), a* (redness) and b*(yellowness) were recorderd (Konica Minolta, Sensing, CM-700d, CM-A177, Japan).

Total polyphenolic content and antioxidant capacity

The total polyphenolic content of film solutions (5 g film was diluted in 30 mL of 10 % etanolic solution) was determined spectrophotometrically by the Folin-Ciocalteau colorimetric assay, according to a modified method of Singleton and Rossi (1965), while flavan-3-ols was determined by the vanillin assay (Di Stefano, 1989). All measurements were carried out in duplicates and the results were expressed as mg of gallic acid equivalents (GAE)/g of film. Antioxidant capacity of films was determined by two commonly used assays: ABTS (Re et al., 1999) and DPPH (Brand-Williams et al., 1995), and expressed as mmol of Trolox/g of film.

HPLC analysis

The HPLC method was conducted using Aglient 1200 Series HPLC device (Aglient, Sant Clara, USA) and a Photo Array Detector (Aglient, Santa Clara, USA) with a reversed-phase ACE C-18 column (Aglient, Santa Clara, USA) (250 x 4.6 mm, 5 μm i.d.). Methanol (solvent A) and 2 % formic acid (solvent B) were used as mobile phases and the eluation was performed with a gradient starting at 98 % A, duration of method was 35 minutes, with 5 minutes post time. The injection volume was 10 μL, and flow was 1 mL/min (Belščak-Cvitanović et al., 2012). For the HPLC analysis, all film solutions were filtered through 0.45 μm filter (Nylon Membranes, Supelco, Bellefonte, USA) before injection. For quantification purposes, chromatograms were recorded and detection of the phenolic compounds was carried out at 278 nm. Peak identification was achieved by comparison of both the retention time and UV absorption spectrum with those obtained with standards.

Sensory analysis of formulated edible films

For the sensory evaluation of edible films, a hedonistic score scale of 1 to 9 was used, where 9 indicates a highly desirable quality and 1 meant a defective product. The sensory characteristics of edible films were evaluated in terms of appearance, colour, transparency, elasticity and acceptability.

RESULTS & DISCUSSION

In this study, extracted pectin from sugar beet pulp and selected proteins were characterized and employed for preparation of functional edible films. The sugar beet pulp pectin was characterized in terms of degree of esterification (DE), equimolar weight (EW), pH value and galacturonic acid content (GalUA) (Table 2). The obtained value for DE of pectin was 74.52±0.21 %, indicating a low methoxy pectin, while the EW was 1086.96±1.02 g/mol. The GalUA in sugar beet pulp pectin was 84.04±0.18 %. Similar result was obtained in the study of Phatak et al. (2006), who determined galacturonic acid content of 81.80 % in the extracted pectin from sugar beet pulp.

Table 2. Characterization of extracted sugar beet pulp pectin

Sample	DE (%)	EW (g/mol)	pH	GaIUA (%)
Sugar beet pulp pectin	74.52±0.21	1086.96±1.02	3.12±0.00	84.04±0.18

Examined characteristics of fenugreek (F_P) and rice (R_P) proteins are presented in Table 3. As can be seen, higher values of foaming capacity (FC), foaming stability (FS) and electrical conductivity (EC) were determined for fenugreek proteins, while, in terms of WHC and FAC, as well as of ESI and EAI there were no marked difference between evaluated proteins.

Table 3. Characterization of fenugreek (F_P) and rice (R_P) proteins

Sample	FC (%)	FS (%)	EC (μs/cm)	WHC (mL/g)	FAC (mL/g)	EAI (m^2/g)	ESI (%)
F_P	20.21±0.24	10.17±0.22	462.23±2.56	37.80±0.23	38.40±0.27	30.46±0.47	97.78±1.04
R_P	6.66±0.01	2.22±0.00	87.80±0.22	37.72±1.14	35.21±0.35	27.94±0.91	93.85±1.17

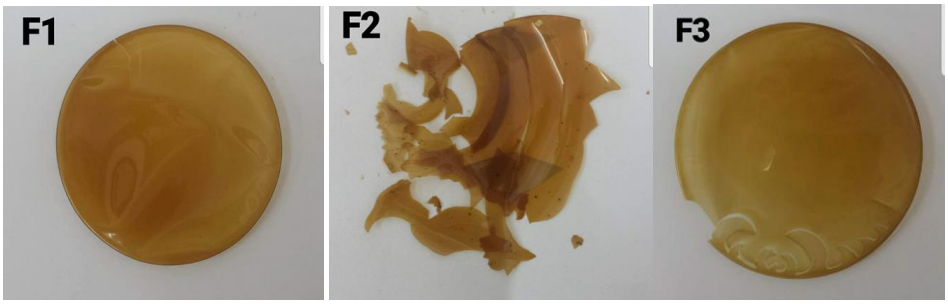


Figure 1. Formulated films: plain pectin film - control film (F1), pectin with addition of fenugreek proteins (F2) and rice proteins (F3)

Physico-chemical and mechanical properties of the formulated films are shown in Table 4. Addition of both type of proteins to film resulted with the higher content (~12 % higher) of dry matter in F2 and F3 films, what reflected on tensile strength and elasticity, as two important properties of packaging materials. Namely, compared to the control film (F1), incorporation of proteins into pectin-based films decreased tensile strength and elasticity and both parameters were not measurable due to the films breaking (Figure 1). Compared to the control film (F1), the addition of proteins into film slightly increased its lightness (higher L* values). F1 and F2 films were slightly more reddish (higher a* values), while F3 was more yellowish (~40 % lower a* value). The highest b* value (green component) had the control film (F1). According to the results for the total colour difference (ΔE), film produced by a combination of pectin and rice proteins showed slightly higher total colour difference (38.31±1.53).

Table 4. Physico-chemical and mechanical characteristics of formulated films

Sample	Dry matter (%)	Thickness (µm)	Tensile strength (N)	Elasticity (%)	L*	a*	b*	ΔE
F1	81.99±0.01	96.50±2.28	3.64±0.83	18.02±2.40	21.42±2.72	2.24±0.47	6.33±0.91	-
F2	94.45±0.56	97.13±9.11	-	-	26.64±1.53	2.16±0.31	5.71±0.36	34.79±2.09
F3	94.11±0.07	96.60±4.94	-	-	25.43±2.83	1.36±0.31	5.43±1.77	38.31±1.53

Pectin-protein films exhibited a higher content of total polyphenolic compounds compared to the plain pectin film (Figure 2), and among all samples, pectin-based film formulated with addition of rice proteins showed the highest content of polyphenolic compounds (106.22 mg of GAE/g). Among pectin films formulated with addition of proteins, rice proteins also showed better function for incorporation of flavan-3-ols than film prepared with fenugreek proteins.

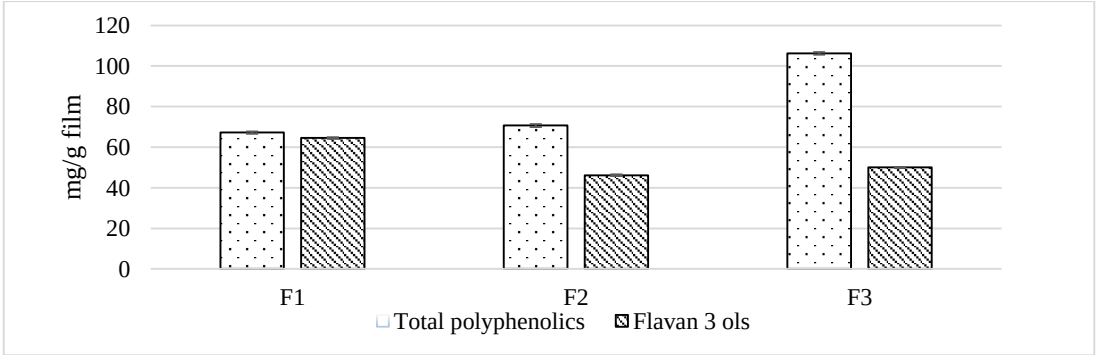


Figure 2. Total polyphenols (mg of GAE/g film) and flavan-3-ols content (mg of catechin/g film) in formulated films. Results are shown as mean ± SD.

According to the results of antioxidant capacity, presented on Figure 3, it can be seen that among prepared edible films, film F3 was characterized with the highest antioxidant capacity determined by ABTS assay, confirming previously presented results of total polyphenolic content.

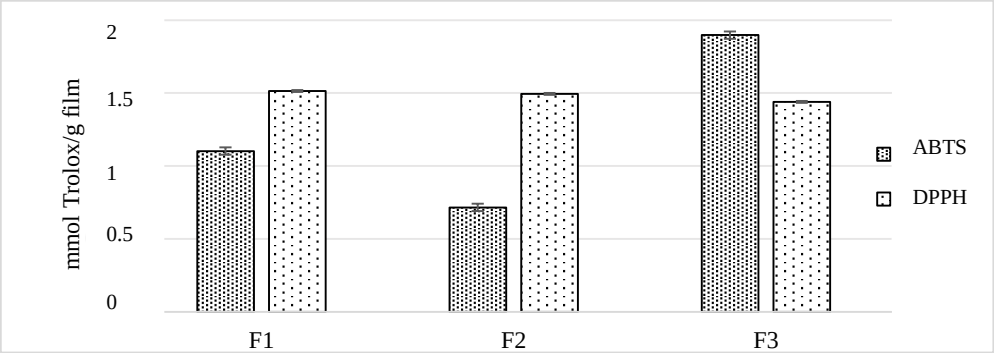


Figure 3. Antioxidant capacity (mmol Trolox/g film) of formulated films determined by ABTS and DPPH assays. Results are shown as mean ± SD.

Table 5 summarizes specific bioactive compounds (polyphenols and methylxanthines) detected in formulated edible films. Epigallocatechin gallate (EGCG), as a representative polyphenolic compound of yellow tea, was determined in the highest content in all films, ranging from 25.15 to 45.47 mg/g. Beside polyphenolic compounds, all films contained also high content of methylxanthine caffeine (from 32.87 to 50.90 mg/g). Among all prepared films, sample F3 was characterized with the highest content of bioactive compounds which could be attributed to a good ability of rice protein for incorporation of yellow tea bioactives into this film. All identified compounds of edible films are well-known antioxidants which contributes to functional properties of edible films with potential beneficial effects on human health (Gramza-Michalowska et al., 2015; Kujawska et al., 2016).

Table 5. The content of bioactive compounds in formulated films

Compound / Sample	Gallic acid	EGCG	Epigallocatechin	Catechin	Caffeine
			mg/g		
F1	1.38±0.04	45.47±0.31	5.41±0.04	6.13±0.28	32.87±0.28
F2	1.91±0.08	25.15±0.69	2.65±0.12	3.59±0.09	40.23±0.00
F3	2.79±0.08	44.49±0.49	7.83±0.10	8.88±0.01	50.90±0.14

According to the results of sensory evaluation, all evaluated properties of control film (F1) were scored with the highest marks compared to the films formulated with protein addition (Figure 4). Among samples prepared with proteins, film with rice proteins (F3) showed better appearance, acceptability, transparency and colour.

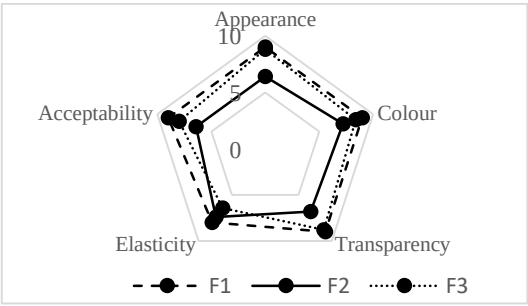


Figure 4. Sensory analysis of pectin-based edible films

CONCLUSION

Extracted pectin from sugar beet pulp, consisting of 84.04 % galacturonic acid, showed good ability for formulation of edible films based on yellow tea extract. Among the formulated films, the pectin-based film with addition of rice proteins exhibited the highest total polyphenolic content, as well as antioxidant capacity, but on the other hand, plain pectin film showed better mechanical and sensory properties. Although some further improvements in the film formulations are needed, based on the obtained results it can be concluded that combination of extracted pectin, proteins and yellow tea extract showed a good potential for formulation of functional edible films.

REFERENCES

Aina V.O., Barau M.M., Mamman O.A., Zakari A., Haruna H., Hauwa Umar M.S., Abba Y.B. 2012. Extraction and Characterization of Pectin from Peels of Lemon (*Citrus limon*), Grape Fruit (*Citrus paradisi*) and Sweet Orange (*Citrus sinensis*). British Journal of Pharmacology and Toxicology. 3, 259-262.

Belščak-Cvitanovi, A., Komes D., Benković M., Karlović S., Hečimović I., Jeţek D., Bauman I. 2012. Innovative formulation of chocolates enriched with plant polyphenols from *Rubus idaeus* L. Leaves and charazterization of their physical, bioactive and sensory properties. Food Research International. 48, 820-830.

Belščak-Cvitanović A., Vojvodić A., Bušić A., Keppler J., Steffen-Heins A., Komes D. 2018. Encapsulation templated approach to valorization of cocoa husk, poppy and hemp macrostructural and bioactive constituents. Industrial Crops & Products. 112, 402–411.

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

Chiralt A., Gonzalez-Martinez C., Vargas M., Atares L. 2018. Edible films and coatings from proteins. Proteins in Food Processing. 477-500.

De'Nobili M., P´erez C., Navarro D., Stortz C., Rojas A. 2013. Hydrolytic stability of l-(+)-ascorbic acid in low methoxyl pectin films with potential antioxidant activity at food interfaces. Food Bioproducts Processing. 6(1), 186–97.

Di Stefano R., Cravero M., Gentilini N. 1989. Methods for studying polyphenols in wine. L'Enotecnica. 25:83–9.

FDA. 2013. Direct food substances affirmed as generally recognized as safe. Subpart B–Listing of Specific Substances Affirmed as GRAS. Section 184, 1588.

Gramza-Michalowska A., Sidor A., Regula J., Kulczynski B. 2015. PCL assay application in superoxide anion-radical scavenging capacity of tea Camellia sinensis extracts. Acta Scientiarum Polonorum - Technologia Alimentaria. 14, 331-341.

Kempka A.P., Horvath F.J., Fagundes P., Prestes R.C. 2015. Foaming and emulsifying capacity, foam and emulsion stability of proteins of porcine blood: determination at different values of pH and concentrations. Revista Brasileira de Tecnologia Agroindustrial, 1798-1800.

Krochta J.M., 2002. Proteins as raw materials for films and coatings: Definitions, current status and opportunities. In: Protein-Based Films and Coatings, A. Gennadios (edi.), CRC Press, Boca Raton, Florida, 672.

Kujawska M., Ewertowska M., Adamska T., Jodynis-Liebert J., Ignatowicz E., GramzaMichalowska A. 2016. Protective effect of yellow tea extract on Nnitrosodiethylamine-induced liver carcinogenesis. Pharmaceutical Biology. 54(9), 1891-1900.

Melton D.L., Smith B.G. 2001. Determination of the Uronic Acid Content of Plant Cell Walls Using a Colorimetric Assay. Current Protocols in Food Analytical Chemistry. 736-739.

Phatak L., Chang K.C., Brown G. 2006. Isolation and Characterization of Pectin in Sugar-Beet Pulp. Journal of Food Science 53(3), 830–833.

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 & Medicine. 26, 1231-1237.

Singleton V.L. Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent, American Journal of Enology and Viticulture, 37, 144-158.

Stone A. K., Karalash A., Tyler R. T., Warkentin T. D., Nickerson M. D. 2015. Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. Food Research International. 76, 31–38.

Onion peel as a source of pectin and quercetin: extraction, characterization and fabrication of encapsulation systems

Monika Ostrman[a], Marija Soldo[a], Arijana Bušić[a], Aleksandra Vojvodić Cebin[a], Nataša Poklar Ulrih[b], Draženka Komes[a]

Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia (mostrman@pbf.hr)
[b]Biotechnical Faculty, University of Ljubljana, Slovenia

ABSTRACT
The study deals with the evaluation of the onion peel potential, as a source of functional compounds (pectin and quercetin) for the formulation of polysaccharide-based delivery systems and innovative food products. In the first step, quercetin and its derivates, as representative lipophilic polyphenolic antioxidants, were extracted from onion peel by conventional (20 °C and 80 °C) and ultrasound assisted techniques using 70 % aqueous ethanol solution. Afterwards, the obtained quercetin-rich extracts were encapsulated in form of hydrogel particles by ionic gelation, using alginate and pectin as carriers. For that purpose pectin was previously extracted from onion peel using acid hydrolysis, followed by dialysis, evaporation and freeze drying. Pectin was combined with alginate (20:80, 30:70 and 40:60, v/v) for the formulation of quercetin-rich hydrogel particles. The physico-chemical and morphological properties, encapsulation efficiency of polyphenols and quercetin release profile from obtained particles in simulated gastric and intestinal fluids were examined. Although there were no marked differences between encapsulation efficiency (EE_{TPC}>93 %) in developed delivery systems, the highest content of quercetin and its derivatives was determined in alginate-pectin (80:20) particles. The obtained results confirmed a good application potential of quercetin and pectin, both derived from onion peel as compounds for encapsulation-enrichment of food products.
Keywords: encapsulation; ionic gelation; onion peel; pectin; quercetin

INTRODUCTION

One of the greatest challenges for modern food industry is to find solutions for disposal of agrowaste generated during the food production. The reduction of waste throughout any part of food chain is very important for sustainability and economics in food, pharmaceutical or cosmetic industry. Considering the great onion production (the global production of onion in 2016 was around 93.4 million tonnes), onion peel, as secondary raw material, represent widely available and potentially rich source of different functional ingredients for utilization of agrowaste.

Onion peel contains various bioactive compounds like flavonoids (especially quercetin and kaempferol), phenolic acids (caffeic, sinapic and *p*-coumaric), saponins, sterols, and volatile oils (Shafig et al., 2017). Numerous studies have confirmed beneficial effects of onion peel bioactives on human health. It was found that onion peel extract exhibits antiplatelet, antioxidant, antibacterial, anti-inflammatory, antitumor, hypolipidemic and antidiabetic potentials due to available polyphenolic compounds, especially quercetin (Nishijima et al., 2015; Zhu et al., 2018).

On the other hand, onion peel is also a great source of pectin, water-soluble fibre distributed in the cell walls of fruits and vegetables. It prolongs gastric removal and alters intestinal microflora, also lowering of some risk factors of cardiovascular disease, and promoting satiety and weight loss thus preventing obesity (Pokusaeva et al. 2011). Due to its pronounced gelling properties, pectin is frequently used as a carrier material for encapsulation of bioactive compounds. Either as a single encapsulant or prepared with addition of other biopolymers (e.g. alginate), pectin can also serve as a good alternative to syntetic polymeric materials, due to its barrier and nutritional properties (Drusch, 2007).

Encapsulation is the technology of packaging solids, liquids and gaseous materials in small capsules, which are able to release their content at controlled rates under specific conditions (Desai and Park, 2005). Among various encapsulation techniques, ionic gelation is one of the most commonly used, which involves extrusion of a polymer solution through a syringe needle, in which the active compound is dissolved or dispersed. Droplets are received in a dispersant phase and are transformed, after reaction, into spherical gel particles (Munin and Edwards-Levy, 2011).

In the present study, the potential of onion peel as a source of functional compounds (quercetin and pectin) was evaluated, with the aim of its efficient utilization in the food industry. The obtained pectin and quercetin were used as carrier/encapsulant supplements. Alginate-based beads were studied in terms of physico-chemical and morphological properties, encapsulation efficiency of polyphenols (total

polyphenols) and retained antioxidant capacity. Release profile of quercetin and antioxidant capacity from hydrogels were determined in simulated gastrointestinal conditions.

MATERIALS & METHODS

Materials

All chemicals used for the experimental procedures were analytical or HPLC-grade. Pectin was extracted from onion peel, which was collected in the household, while sodium alginate was purchased from Sigma-Aldrich (USA).

Pectin and quercetin extraction

Alcohol soluble compounds (quercetin) were extracted from previously chopped and degreased onion peel using 70 % ethanolic solution by conventional extractions (CE) at 20 °C and 80 °C and ultrasound assisted extraction (UAE). The obtained quercetin rich extracts were analysed and employed for encapsulation purposes, while, residue obtained after extraction was used for extraction of pectin (HCl, pH 1.5, 2h, 90 °C). The obtained extracts were precipitated using 96 % ethanol during the night at 4 °C, followed by dialysis, evaporation and freeze drying.

Extract characterization

The HPLC analysis of bioactive compounds in onion peel extracts was conducted according to the method of Belščak-Cvitanović et al. (2016) using an Aglient 1200 Series HPLC device (Aglient, Santa Clara, USA) and a Photodiode Array detector (Aglient, Santa Clara, USA) using a reversed-phase ACE C-18 column (250 x 4.6 mm, 5 μm i.d.). The antioxidant capacity was measured according to methods of Brand-Williams et al. (1995) - DPPH and Re et al. (1999) - ABTS.

Pectin characterization

The equivalent (molar) weight (EW) and degree of esterification (DE) was determined according to the modified method of Aina et al. (2012), while determination of galacturonic acid content was performed by spectrophotometric method according to the modified procedure of Melton et al. (2001).

Encapsulation of alginate-based delivery systems

Alginate-A (4 % solution), pectin-P (4 % solution) and cross-linking solution consisting of 3 % (w/v) calcium chloride ($CaCl_2$) were prepared in onion peel extracts obtained by ultrasound assisted extraction. The beads were formulated from plain alginate and alginate-pectin solutions (80:20, 70:30 and 60:40). Preparation of alginate-based particles was carried out by ionic gelation using dropwise addition of prepared carrier solutions into a calcium chloride solution.

Physio-chemical properties of produced beads

The water content of produced hydrogel beads was determined according to AOAC method (1990). Particle size of randomly selected hydrogel beads was examined by recording the beads (n=10) with a digital camera on the Dino-Lite calibration plate (minimal distance = 0.2 mm), and afterwards reading the particle size from the captured images. Instrumental texture analysis of hydrogel beads was carried out using a TA.HDPlus texture analyser (Stable Micro Systems, Godalming, Great Britain). Compression test was performed using a 4mm metal (stainless steel) cylindrical probe with flat bottom and a 5 kg load cell, as described by Belščak-Cvitanović et al. (2015). Each analysis was executed five-fold. The maximum force (N) needed for compression (maximal resistance of the surface to compression of probe) represented the hardness of the samples, while elasticity of samples was measured as a ratio of probe travel distance and was expressed in mm. Determination of colour was performed by diffuse reflecting spectrometry on a colorimeter (Konica Minolta, Sensing, CM-700d, CM-A177, Japan).

Encapsulation efficiencies of entrapped bioactives and determination of specific bioactive compounds in hydrogel beads

The percentage of loading efficiency (%) of total polyphenols (Singleton and Rossi, 1965) and retained antioxidant capacity (DPPH and ABTS, according to methods specified in the section ''*Extract characterization*'') was calculated as the ratio between the total polyphenols or antioxidant capacity in the solution of disintegrated beads and their respective content in the initial encapsulant solution. All

analyses were carried out in triplicates. Also, the HPLC analysis of bioactives in hydrogel beads was conducted according to previously mentioned method.

Release profiles of entrapped bioactives

The release profile of quercetin and antioxidant capacity from the obtained beads in simulated gastric and intestinal fluids (SGF and SIF) was performed by evaluating the quercetin content using HPLC analysis and antioxidant capacity using ABTS radical scavenging assay. SGF consisted of sodium chloride (NaCl) dissolved in hydrochloric acid (HCl) solution (pH=1.2), while SIF consisted of phosphate buffer (pH=7.4), proposed by the European Pharmacopeia 7.0. (2010). All analysis were carried out in triplicate.

RESULTS & DISCUSSION

Extracted pectin from onion peel was characterized by the following parameters: degree of esterification - 53.81±1.24 %, equimolar weight - 741.04±14.42 g/mol, pH value - 3.18±0.00 and content of galacturonic acid - 58.21±0.54 % (not shown). According to the degree of esterification, the extracted onion peel pectin is classified as highly metoxyl pectin. The obtained content of galacturonic acid in onion peel was 15 % lower than the one in the study of Babbar et al. (2016), what could be attributed to differences in parameters and solvents used during pectin extraction.

Beside pectin, onion peel was used also as a source of polyphenolic compounds, especially quercetin and its derivates. The content of specific bioactive compounds in onion peel extracts was determined by HPLC analysis and the obtained values are presented in Table 1. Detected polyphenolic compounds belong to flavonoids (quercetin, quercetin-3-glucoside, quercetin derivatives and luteolin) and phenolic acids (protocatechuic, *p*-hydroxybenzoic and vanillic). Quercetin and its derivatives are the most abundant flavonoids, characteristic for onion peel extract and known as strong antioxidants (Nishijima et al., 2015). Consequently, obtained sum of their concentrations (quercetin, quercetin-3-glucoside and quercetin derivatives) was the highest compared to other group of compounds, e.g. 17.55 mg/g in UAE sample. In the case of phenolic acids, protocatechuic acid was dominant, and again it was determined in the highest content in sample UAE (2.19±0.04 mg/g). The highest content of identified compounds was determined in onion peel extract obtained by UAE, followed by CE at 80 °C and 20 °C.

Table 1. The content of specific bioactive compounds (mg/g) in onion peel extract obtained by different extraction techniques

Bioactive compound /Sample	Protocatechuic acid	*p*-hydroxybenzoic acid	Vanillic acid	Quercetin-3-glucoside	Quercetin	Quercetin derivatives	Luteolin
	mg/g						
CE 20 °C	0.91±0.00	0.01±0.00	0.02±0.00	0.09±0.00	1.56±0.01	8.28±0.04	0.04±0.00
CE 80 °C	0.98±0.01	0.02±0.00	0.01±0.00	0.09±0.00	1.68±0.04	9.39±0.15	0.05±0.00
UAE	2.19±0.04	0.04±0.00	0.05±0.00	0.13±0.01	1.62±0.05	15.80±0.74	0.05±0.00

Beside specific bioactive composition, the antioxidant capacity of extracts was also measured. As can be seen (Table 2), the results of antioxidant capacity were in line with the HPLC results, and the highest values of antioxidant capacity were determined (by both methods) in extract prepared by UAE, followed by CE at 80 °C and CE at 20 °C. Therefore, based on the obtained results, onion peel extract prepared by UAE was encapsulated by ionic gelation using alginate and pectin as carriers.

Table 2. Antioxidant capacity of onion peel extract

Sample	ABTS	DPPH
	(mmol Trolox/g)	
CE 20 °C	0.07±0.01	0.05±0.00
CE 80 °C	0.07±0.01	0.05±0.01
UAE	0.20±0.02	0.09±0.01

The influence of carrier's formulations on water content, particle size, texture parameters (hardness, elasticity) of formulated alginate-based hydrogel beads, as well as on colour properties were evaluated and presented in Table 3. In general, alginate-pectin particles exhibited higher water content than plain alginate particles, with the highest detected in AP_60:40 particles. As can be seen, the particle size of formulated alginate-based hydrogel beads encapsulating onion peel polyphenols was ranging from 2.34 to 2.48 mm, what is consistent to other studies which reported that the size of calcium alginate beads produced by simple extrusion dripping is usually larger than 1 mm (Lee et al., 2013), and can even go up to 6 mm (Burey et al., 2008). According to the obtained results, plain alginate beads encapsulating onion peel extract were screened as largest, while the addition of pectin reduced particle size of beads. Also, the addition of pectin to alginate resulted with softer and less elastic beads structure, exhibiting the lowest values in beads with the highest pectin content (AP_60:40). In terms of colour properties, these particles were the darkest, respectively they had the lowest L* value, as well as a* (less reddish) and b* (less yellowish) values. The highest total colour difference (8.19) was determined between A and AP_60:40 samples.

Table 3. Physico-chemical properties of formulated beads

Sample	Water content (%)	Particle size (mm)	Hardness (N)	Elasticity (%)	L*	a*	b*	ΔE
A	92.15±2.67	2.48±0.24	0.18±0.01	0.30±0.02	32.87±0.06	15.74±0.15	23.30±0.08	-
AP_80:20	92.37±0.40	2.47±0.22	0.18±0.01	0.29±0.01	28.16±0.04	13.50±0.08	17.39±0.06	5.21±0.20
AP_70:30	92.76±0.39	2.40±0.17	0.16±0.00	0.29±0.02	26.36±0.14	12.31±0.23	15.82±0.39	7.35±0.55
AP_60:40	93.72±0.24	2.36±0.16	0.07±0.01	0.11±0.01	24.67±0.11	11.99±0.17	13.99±0.21	8.19±0.21

According to determined encapsulation efficiencies (Table 4), total polyphenolic content (TPC) was successfully retained in the hydrogel beads matrix. For plain alginate particles, EE_{TPC} (loading capacities) was around 98 % and it slightly increased with the addition of pectin (AP_70:30, AP_60:40), except for sample AP_80:20, where lower EE_{TPC} was observed. It can be observed that among particles with pectin, ones with lower pectin content had lower EE_{TPC} (AP_80:20<AP_70:30<AP_60:40). In the case of retained antioxidant capacity measured by ABTS and DPPH assays, the encapsulation efficiency was also very high, in the range from 76.70 to 95.31 %. According to observed results, there was good correlation between EE_{TPC} and antioxidant capacity (for EE $r_{TPC/DPPH}$ - 0.98).

Table 4. Encapsulation efficiencies (EE %) of total polyphenols content (TPC) and antioxidant capacity of onion peel extract measured by ABTS and DPPH methods

Sample	EE_{TPC} (%)	EE_{ABTS} (%)	EE_{DPPH} (%)
A	98.58±3.54	76.08±1.65	95.31±1.09
AP_80:20	93.77±5.19	76.70±1.29	84.49±7.04
AP_70:30	98.79±2.12	82.11±2.31	94.46±0.11
AP_60:40	99.08±4.95	77.38±0.12	94.12±2.09

Among investigated compounds, the highest content obtained after crushing of particles was recorded for quercetin derivatives (0.517 - 0.596 mg/g), which are characteristic compounds of onion peel extract (Table 5). In the term of phenolic acids, protocatechuic adic was detected as most abundant (0.494 - 0.663 mg/g), while *p*-hydroxybenzoic acid was detected in a much lower content (0.003 to 0.006 mg/g). The highest content of bioactives was determined in plain alginate particles, followed by lower contents in alginate-pectin beads. Those results indicate that the content of individual bioactive compounds is dependent on employed carrier systems. Low methoxyl pectins (DE<50 %) are known as better bioactives delivery vehicle, since they can form calcium gels with good characteristic in comparison with high metoxyl pectins (Morris et al., 2010). Onion peel pectin which was used for formulation of alginate pectin particles was characterized as high metoxyl pectin, so obtained results could be expected.

Table 5. The content of specific bioactives of onion peel extract retain in broken hydrogel particles (mg/g particle)

Bioactive compound/ Sample	Protocatechuic acid	*p*-hydroxybenzoic acid	Quercetin	Quercetin derivatives
	mg/g			
A	0.663±0.010	0.006±0.000	0.432±0.010	0.596±0.041
AP_80:20	0.547±0.011	0.003±0.000	0.350±0.032	0.583±0.037
AP_70:30	0.510±0.002	0.003±0.000	0.370±0.015	0.524±0.018
AP_60:40	0.494±0.051	0.003±0.000	0.336±0.072	0.517±0.020

The release patterns of quercetin (Figure 1a) and antioxidant capacity (Figure 1b) from the formulated particles in simulated gastric and intestinal fluids showed better delivery performances for alginate-pectin systems, especially in the case of lower pectin content (80:20). Namely, the release profiles of quercetin revealed its continuous release in simulated gastric fluid from samples A and AP_80:20, while the other two systems showed minor fluctuations. Extension of continuous release of quercetin in simulated intestinal fluid was recorded for all particles, except for plain alginate. The analysis showed that AP_60:40 system released the highest content of quercetin (~0.2 mg/g beads). The release patterns for antioxidant capacity did not differ markedly between formulated particles in the gastric conditions, since almost no release of antioxidant capacity was detected, while in simulated intestinal fluid sharper and prolonged release was observed. In the case of plain alginate particles, the release profiles of antioxidant capacity was prolonged up to 150 min. Thus, this indicates that the release properties of beads could be also effected by their particle size (A- the largest beads), since it was reported that release of bioactives and consequently antioxidant activity from delivery system is prolonged when the particle size of bead is larger (Stojanovic et al., 2012; Bušić et al., 2018). Also, alginate beads are characterized as stable in acidic solutions and degrade under higher pH value (Li et al., 2016), what was also confirmed in this study, since after placing alginate beads from gastric (pH=1.2) to intestinal fluid (pH=7.4) they were totally degraded with time (after 140/150 min) and quercetin was fully released.

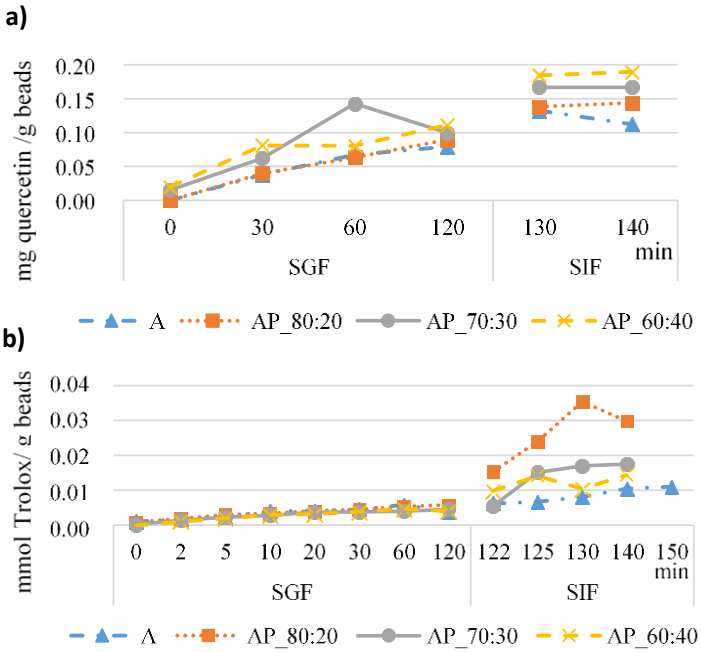


Figure 1. Release profiles of quercetin **(a)** and antioxidant capacity obtained by ABTS assay **(b)** from particles encapsulating onion peel extract in simulated gastric (SGF) and intestinal fluids (SIF)

CONCLUSION

In the present study, onion peel was used as a source of pectin (additional carrier) and quercetin (encapsulant) for fabrication of hydrogels with high encapsulation efficiency of bioactives and its desirable release properties. Considering the physico-chemical properties of prepared beads, the

composition of delivery systems influenced texture parameters, since the beads prepared with higher content of pectin had lower hardness and elasticity. Contrary to the results obtained for encapsulation efficiency, where bigger beads were able to entrap higher amount of polyphenols, favourable extended release of quercetin from beads in simulated gastrointestinal fluids was observed from smaller beads (ones prepared with pectin). The obtained results were good example of onion peel utilization and could be suitable for design of efficient delivery systems applicable in food and/or pharmacy industry.

REFERENCES

Aina V.O., Barau M.M., Mamman O.A., Zakari A., Haruna H., Hauwa Umar M.S., Abba Y.B. 2012. Extraction and Characterization of Pectin from Peels of Lemon (*Citrus limon*), Grape Fruit (*Citrus paradisi*) and Sweet Orange (*Citrus sinensis*). British Journal of Pharmacology and Toxicology, 3, 259-262.

AOAC. 1990. Moisture in tobacco (966.02.). In: K. Helrich (Ed.), AOAC: Official Methods of Analysis Arlington: Association of Official Analytical Chemists Inc., 64.

Babbar N., Baldassarre S., Maesen M., Prandi B., Dejonghe W., Sforza S., Elst K. 2016. Enzymatic production of pectic oligosaccharides from onion skins. Carbohydrate Polymers. 146, 245-252.

Belščak-Cvitanović A., Komes D., Karlović S., Djaković S., Špoljarić I., Mršić G., Ježek D. 2015. Improving the controlled delivery formulations of caffeine in alginate hydrogel beads combined with pectin, carrageenan, chitosan and psyllium. Food Chemistry, 167, 378–386.

Belščak-Cvitanović A., Bušić A., Barišić L., Vrsaljko D., Karlović S., Špoljarić I., Vojvodić A., Mršić G., Komes D. 2016. Emulsion templated microencapsulation of dandelion (*Taraxacum officinale* L.) polyphenols and *ß*-carotene by ionotropic gelation of alginate and pectin, Food Hydrocolloids, 57, 139-152.

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

Burey P., Bhandari B. R., Howes T., Gidley M. J. 2008. Hydrocolloid gel particles: formation, characterization, and application. Critical Reviews in Food Science and Nutrition, 48(5), 361–377.

Bušić A., Belščak-Cvitanović A., Vojvodić Cebin A., Karlović S., Kovač V., Špoljarić I., Mršić G., Komes D. 2018. Structuring new alginate network aimed for delivery of dandelion (*Taraxacum officinale* L.) polyphenols using ionic gelation and new filler materials. Food Research International, 111, 244-255.

Desai, K. G. H., Park H. J. 2005. Recent developments in microencapsulation of food ingredients. Drying Technology, 23, 1361–1394.

Drusch, S. 2007. Sugar beet pectin: A novel emulsifying wall component for microencapsulation of lipophilic food ingredients by spray-drying. Food Hydrocolloids, 21, 1223-1228.

Lee B. B., Ravindra P., Chan E. S. 2013. Size and shape of calcium alginate beads produced by extrusion dripping. Chemical & Engineering Technology, 36(10), 1627–1642.

Li J., Kim S. Y., Chen X., Park H. J. 2016. Calcium-alginate beads loaded with gallic acid: preparation and characterization. LWT – Food Science and Technology, 68, 667–673.

Melton D.L., Smith B.G. 2001. Determination of the Uronic Acid Content of Plant Cell Walls Using a Colorimetric Assay. Current Protocols in Food Analytical Chemistry, 736-739.

Morris G., Kök S., Harding S., Adams G. 2010. Polysaccharide drug delivery systems based on pectin and chitosan. Biotechnology and Genetic Engineering Reviews, 27, 257-84.

Munin A., Edwards-Lévy 2011. Encapsulation of Natural Polyphenolic Compounds; a Review. Pharmaceutics, 3, 793-829.

Nishijima T., Takida Y., Saito Y., Ikeda T., Iwai K. 2015. Simultaneous ingestion of high-methoxy pectin from apple can enhance absorption of quercetin in human subjects. British Journal of Nutrition, 113, 1531–1538.

Pokusaeva K., Fitzgerald G.F., van Sinderen D. 2011. Carbohydrate metabolism in Bifidobacteria. Generation Nutrition, 6(3), 285–306.

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 & Medicine, 26, 1231-1237.

Shafiq S., Shakir M., Ali Q. 2017. Medicinal uses of Onion (*Allium cepa* L.): An Overview. Life Science Journal, 14, 100-107.

Singleton V.L., Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent, American Journal of Enology and Viticulture, 37, 144-158.

Stojanović Z., Marković S. 2012. Determination of Particle Size Distributions by Laser Diffraction. Technics-New Materials, 21, 11-20.

Zhu D. Y., Ma Y. L., Thakur K., Wang C. H., Wang H., Ren Y. F., Zhang J. G., Wei Z. J. 2018. Effects of extraction methods on the rheological properties of polysaccharides from onion (*Allium cepa* L.). International Journal of Biological Macromolecules, 112, 22-32.

Antioxidant capacity of fruit species characteristic for gardens in Istria (Croatia)

Josipa Bilić[a], Marina Svorcina[b], Danijela Poljuha[b]

[a] *Materials Research Centre METRIS, Zagrebačka 30, 52100 Pula, Croatia (josipa.bilic@centarmetris.hr)*
[b] *Institute of Agriculture and Tourism, Karla Huguesa 8, 52440 Poreč, Croatia (danijela@iptpo.hr)*

ABSTRACT
The present study was funded by the Istria Tourist Board, and performed in order to provide an overview of the antioxidant capacity of various typical foods and food products in Istria as well as to compare the obtained values with the available databases. A special emphasis throughout the screening process has been put on here presented ˝old, forgotten˝ fruit species, common in public and private gardens in Istrian region, such as black elder (*Sambucus nigra* L.), loquat (*Eriobotrya japonica* (Thunb.) Lindl.), jujube (*Ziziphus jujuba* Mill.), strawberry tree (*Arbutus unedo* L.), blackberry (*Rubus fruticosus* L.), black mulberry (*Morus nigra* L.), autochthonous cultivars of fig (*Ficus carica* L.), etc. Among the 42 investigated samples of 14 different fruit species/cultivars extracted in water/methanol, blackberry, strawberry tree and black elder fruits proved to have the highest antioxidant capacity (6.33±0.07; 5.04±0.07; 4.46±0.1 mmol $FeSO_4^+$/100 g FW, respectively) according to a modified FRAP assay. The comparison of obtained values with the results in available databases was possible only in a few cases of matching species, but in most cases our results were within the range specified for corresponding sample types. The scientific findings of this study will not only help in the gourmet promotion of the Istrian region, but will assist in the revitalisation of neglected fruit species and old cultivars, that are potentially suitable for organic farming purposes and as such represent a valuable element of sustainable development and biodiversity conservation.

Keywords: autochthonous cultivars; antioxidants; gardens; FRAP; neglected fruits

INTRODUCTION

In the promotion of Istrian region as an attractive tourist destination, Istrian gastronomy is increasingly used as an important segment of the tourist offer and as a shaping tool for Istrian culture identity in which preservation of the tradition of growing old autochthonous plant cultivars and species is one of the main goals (Oliva et al., 2012). High antioxidant capacity is an important characteristic and a scientific foundation for the promotion of Istrian diet which is based on high quality, ecologically grown food products with high nutritional values and positive health effects. Fruit species like mulberry, black elder or strawberry tree are found all over the Istrian County's public and private gardens (Turalija et al., 2017), but often get neglected in cuisine even though they are rich in flavonoids and other antioxidants (Zhishen et al., 1999; Atkinson, Atkinson, 2002; Roschek et al., 2009; Mendes et al., 2011; Miguel et al., 2014; Miljković et al. 2015). The aim of this study was to provide a scientific background for the revitalisation of common Istrian garden fruits using simple screening method for antioxidant capacity measurement.

MATERIALS & METHODS

Plant material
Total of 42 fruit samples of 14 different species/cultivars (three per species/cultivar), were collected at the peak of their ripeness from the public and private gardens in Istrian County (Table 1) during 2017. As soon as they were collected they were pitted, weighed and then freeze-dryed (ScanVac, CoolSafe 55-4, LaboGene Aps., Denmark) at -50 °C, from 24 to 96 hours. Samples were then stored at -20 °C until pulverization in kitchen blender (Moulinex 243 Illico). Prepared samples were then stored in aliquots at +4 °C until analyses were performed.

Chemicals
All reagents and solvents used were of adequate analytical grade. HPLC grade methanol was obtained from J.T. Baker® (Avantor Performance Materials S.A., Arnhem, The Netherlands). 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) and sodium acetate trihydrate were purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Ferric (III) chloride hexahydrate and glacial acetic acid were

purchased from Sigma-Aldrich Chemie GmbH (Merck & Co., Inc., Taufkirchen, Germany). Hydrochloric acid was obtained from Kemika d.d. (Zagreb, Croatia).

Extraction

Extraction was carried out using protocols of Harlvorsen et al. (2002) and Carlsen et al. (2010). Three replicates were weighed out for each sample (0.2±0.01 g). All samples were extracted in water/methanol. Briefly, 1 mL of water was added to each replicate and then 9 mL of methanol was added after homogenisation. The extracts were then mixed, sonicated in ice water bath for 15 min and then kept overnight (15-16 h) at +4 °C under vigorous stirring in a becker of adequate size. Next, extracts were mixed once more and then centrifuged at 12402 g for 2 min at +4 °C. The concentration of antioxidants in supernatant was measured in triplicate.

Determination of antioxidant capacity

Antioxidant capacity (AC) of extracts was determined by FRAP assay of Benzie and Strain (1996) with minor modifications. The measurements of absorption changes that appear when the Fe^{3+}-TPTZ complex reduces to the Fe^{2+}-TPTZ form in the presence of antioxidants was performed at 593 nm after 4 min incubation (NanoPhotometer Implen P300, Implen GmbH, München, Germany). The results were calculated according to the calibration curve for ferrous sulphate heptahydrate (100-1000 μM) ($y = 0.5235x$, y = absorbance at 593 nm, x = concentration of $FeSO_4^+$ in mM, $R^2 = 0.9949$) and expressed as reducing ability equivalent to 1 mmol $FeSO_4^+$ per 100 g of DW (plant dry weight), LW (liophilized weight) and recalculated as FW (plant fresh weight). Each AC determination was performed in triplicate, and the results are expressed as mean values ±SD.

RESULTS & DISCUSSION

The focus of our study was the investigation of antioxidant capacity of autochthonous fruit species/cultivars common in Istrian region (Table 1). FRAP assay was chosen regarding the only available reference table/list of foods and food produces (Harlvorsen et al., 2002; Carlsen et al., 2010) and the results are presented in Table 2. Strawberry tree and black elder fruit proved to have the highest antioxidant capacity regarding the lyophilised (17.78±0.25 and 17.73±0.39 mmol $FeSO_4^+$/100 g LW respectively) and dry weight (20.43±0.33 and 19.90±0.44 mmol $FeSO_4^+$/100 g DW), but when recalculated in fresh weight, blackberry fruit proved to have the highest value (6.33±0.07 mmol $FeSO_4^+$/100 g FW). Fig cultivars 'Zamorčica', 'Bjelica' and 'Petrovača bijela' showed the lowest values among all the investigated samples (0.68±0.04, 0.27±0.02 and 0.06±0.01 $FeSO_4^+$/100 g FW respectively). The comparison of obtained values with the results in available databases was possible only in a few cases of matching species, but in most cases our results were within the range specified for corresponding sample types (Halvorsen et al., 2006). In respect to The Antioxidant Food Table (Carlsen et al., 2010), fig cultivars and black elder berries fall into the range of values given in the table, while redcurrant analysed in our study proved to have higher values than the ones stated in the paper with value of 2.64±0.15 mmol $FeSO_4^+$/100 g FW. However, these differences cannot be accounted as significant, as the total antioxidant capacity is influenced by various factors that make the comparison difficult, such as different analysed cultivars, harvesting time, degree of ripeness, agricultural practice, soil characteristics, etc. Also, the "ranking" approach must be considered with caution, in terms of nutritional information, since it can induce consumers' preferences based only on the higher antioxidant content of particular food. In that regard, we should have in mind that healthy nutrition implies a nutritionally adequate diet, consistent with a comprehensive lifestyle, and not the consumption of particular foods. Moreover, since we here consider a regional gourmet promotion, we have to highlight the influence of culinary methods (thermal treatments, interaction of different ingredients in particular dish) and storage that affect the antioxidant content and represent the important factors related to this issue. Besides, the overall obtained AC values refer to *in vitro* tests, and have not been confirmed *in vivo*. Therefore, one of the future steps in our research will be the analysis of biological activity of our extracts.

Table 1. List of samples analysed in the study.

Sample/Cultivar	Sampling location
Cherry plum (*Prunus cerasifera* Ehrh.)	Poreč and surroundings/ local population
Black elder fruit (*Sambucus nigra* L.)	Poreč and surroundings/ local population
Mulberry (*Morus nigra* L.)	Pula and surroundings/ local population
Blackberry (*Rubus fruticosus* L.)	Poreč and surroundings/ local population
Raspberry (*Rubus idaeus* L.)	Poreč and surroundings/ local population
Loquat (*Eriobotrya japonica* (Thunb.) Lindl.)	Pula and surroundings/ local population
Service tree (*Sorbus domestica* L.)	Kaštelir/local population
Red currant (*Ribes rubrum* L.)	Pula and surroundings/ local population
Blackthorne (*Prunus spinosa* L.)	Pula and surroundings/ local population
Jujube (*Ziziphus jujuba* Mill.)	Poreč and surroundings/ local population
Strawberry tree (*Arbutus unedo* L.)	Poreč and surroundings/ local population
Fig (*Ficus carica* L.)/cv. Zamorčica	Collection[+]/local cultivar
Fig (*Ficus carica* L.)/cv. Petrovača bijela	Collection[+]/local cultivar
Fig (*Ficus carica* L.)/cv. Bjelica	Collection[+]/local cultivar

[+]*Collection of Institute of Agriculture and Tourism in Poreč*

Table 2. Results of FRAP assay, ranged according to the obtained values expressed per FW.

Sample/Cultivar	mmol $FeSO_4^+$/ 100 g LW[*] (± SD)	mmol $FeSO_4^+$/ 100 g DW[**] (± SD)	mmol $FeSO_4^+$/ 100 g FW[***] (± SD)
Blackberry (*Rubus fruticosus* L.)	17.135 (± 0.198)	18.630 (± 0.216)	6.331 (± 0.073)
Strawberry tree (*Arbutus unedo* L.)	17.782 (± 0.250)	20.426 (± 0.333)	5.044 (± 0.071)
Black elder fruit (*Sambucus nigra* L.)	17.728 (± 0.390)	19.901 (± 0.437)	4.456 (± 0.098)
Blackthorne (*Prunus spinosa* L.)	9.598 (± 0.305)	10.746 (± 0.342)	4.305 (± 0.137)
Jujube (*Ziziphus jujuba* Mill.)	16.471 (± 0.369)	18.377 (± 0.412)	4.176 (± 0.094)
Red currant (*Ribes rubrum* L.)	15.296 (± 0.842)	17.318 (± 0.954)	2.635 (± 0.145)
Raspberry (*Rubus idaeus* L.)	14.505 (± 0.300)	16.326 (± 0.337)	2.336 (± 0.048)

Service tree (*Sorbus domestica* L.)	5.451 (± 0.407)	5.872 (± 0.438)	2,294 (± 0.171)
Mulberry (*Morus nigra* L.)	8.941 (± 0.491)	11.100 (± 0.981)	2.028 (± 0.111)
Loquat (*Eriobotrya japonica* (Thunb.) Lindl.)	15.125 (± 0.431)	17.916 (± 0.510)	1.759 (± 0.050)
Cherry plum (*Prunus cerasifera* Ehrh.)	10.743 (± 0.244)	12.298 (± 0.280)	1.543 (± 0.035)
Fig (*Ficus carica* L.)/cv. 'Zamorčica'	2.136 (± 0.126)	2.697 (± 0.162)	0.677 (± 0.040)
Fig (*Ficus carica* L.)/cv. 'Bjelica'	1.454 (± 0.097)	1.895 (± 0.131)	0.271 (± 0.018)
Fig (*Ficus carica* L.)/cv. 'Petrovača bijela'	1.812 (± 0.219)	2.451 (± 0.263)	0.062 (± 0.007)

[*] *LW (Lyophilised Weight) - all the samples were lyophilised*
[**] *DW (Dry Weight) - one gram of every sample was taken to be analysed for dry weight (105 °C until constant mass, minimum 4 h)*
[***] *FW (Fresh Weight) - recalculated using the weight before lyophilisation*

CONCLUSION

The total of 42 samples of 14 different "forgotten" fruit species/cultivars collected from public and private gardens in Istrian County were lyophilised, extracted in water/methanol (1:9) and subjected to FRAP, antioxidant capacity assay in order to provide scientific background for the promotion of investigated species in Istrian gastronomy. Among the investigated samples blackberry, strawberry tree and black elder fruits proved to have the highest antioxidant capacity. Modified FRAP assay proved to be a simple screening method for AC analysis of foods and food products, and the results of this study will contribute to the revitalisation of neglected fruit species commonly found all over Istrian region.

ACKNOWLEDGEMENTS

The authors thank the Istria Tourist Board for the financial support of this research project.

REFERENCES

Atkinson M.D., Atkinson E. 2002. *Sambucus nigra* L. J. Ecol. 90, 895-923.
Benzie I.F, Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239(1), 70-76.
Carlsen M.H., Halvorsen B.L., Holte K., Bøhn S.K., Dragland S., Sampson L., Willey C., Senoo H., Umezono Y., Sanada C., Barikmo I., Berhe N., Willett W.C., Phillips K.M., Jacobs D.R. Jr., Blomhoff R. 2010. The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. J. Nutr. 9:3.
Halvorsen B.L., Carlsen M.H., Phillips K.M., Bøhn S.K., Holte K., Jacobs D.R. Jr., Blomhoff R. 2006. Content of redox-active compounds (ie, antioxidants) in foods consumed in the United States. Am. J. Clin. Nutr. 84, 95-135.
Mendes L., de Freitas V., Baptista P., Carvalho M. 2011. Comparative antihemolytic and radical scaveging activities of strawberry tree (*Arbutus unedo* L.) leaf and fruit. Food Chem. Toxicol. 49(9), 2285-2291.
Miguel M.G., Faleiro M.L., Guerreiro A.C., Antunes M.D. 2014. *Arbutus unedo* L.: chemical and biological properties. Molecules. 19(10), 15799-15823.
Miljković V., Nikolić Lj., Radulović N., Arsić B., Nikolić G., Kostić D., Bojanić Z., Zvezdanović J. 2015. Flavonoids in mulberry fruit. Identification of nonanthocyanin phenolics in some mulberry fruit species (*Morus alba* L., *Morus rubra* L. and *Morus nigra* L.). Agro Food Industry Hi Tech. 26(3), 38-42.
Oliva E., Paliaga M., Božac Marijanović D. 2012. Agricultural produce of Istria used in regional branding-strategic concept. In: Marketing challenges in new economy. Juraj Dobrila University of Pula. ISBN 978-953-7498-57-3

Roschek B. Jr., Fink R.C., McMichael M.D., Li D., Alberte R.S. 2009. Elderberry flavonoids bind to prevent H1N1 infection *in vitro*. Phytochemistry. 70, 1255-1261.
Turalija A., Perković A., Vizentaner J., Avdić J., Jukić V., Horvat D. 2017. A fruit tree as an identification element of ornamental gardens shaping in Croatia-Istria ornamental gardens case. BulletinUASVM Horticulture. 74(1), 51-60.
Zhishen J., Mengcheng T., Jianming W. 1999. Research on antioxidant activity of flavonoids from natural materials. Food Chem. 64, 555-559.

Stability of health-promoting compounds of carrots during drying process

Marijana Ceilinger[1], Ivona Elez Garofulić[2], Sanja Lončarić[2], Sunčica Čoklica[3], Svjetlana Matotan[3], Valerija Bukvić[3], Lenkica Penava[1], Jasmina Ranilović[3], Predrag Vujević[4], Verica Dragović-Uzelac[2]

[1]*Belupo, Pharmaceuticals & Cosmetics Inc., Koprivnica, Croatia*
[2]*Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia*
[3]*Podravka Inc., Research & Development, Koprivnica, Croatia*
[4]*Croatian Center for Agriculture, Food and Rural affairs, Zagreb, Croatia*

ABSTRACT

Stability of bioactive compounds (phenols and carotenoids) during drying process and the antioxidant capacities of two different coloured carrot varieties (domestic yellow and conventional purple carrot) were examined.

Sliced carrots were dehydrated either by using conventional or freeze drying process. In fresh and dried samples total phenolic content was determined by the Folin-Ciocalteu method and antioxidant capacity according to the DPPH radical scavenging method. Four types of carotenoids were determined by the use of HPLC methods. Purple carrot contained 5.9 fold total phenols (1235 mg GAE /100 g d.w.) than domestic yellow carrot and remarkable higher antioxidant capacity (4155 μmol in purple and 213 μmol TE/100 g d.w. in yellow).The most common carotenoid was lutein in amount of 6.18 mg in purple and 4.00 mg/100 g d.w. in yellow carrot. Yellow carrot contained slightly more β-carotene (2.60 mg/100 g d.w.). Lycopene was not detected and α-carotene was detected only in freeze dried sample of purple carrot. Drying process did not affect remarkable on antioxidant capacity and on total phenolic content, but the amount of carotenoids decreased remarkably in conventionally dried yellow carrot sample. This study highlights purple carrot as a rich source of polyphenol with very high antioxidant capacity. Dried carrots, especially purple carrot, could presents very valuable source of health-promoting compounds in diet.

Keywords: Polyphenols, carotenoids, antioxidant capacity, carrots, drying

INTRODUCTION

Carrots *(Daucus carota L.)* are very widespread and represent a very important horticultural crop. In Croatia 18225 tons of carrots were produced in the year 2016 (*S*tatistical reports of Croatian Bureau of Statistics, 2017).There are different varieties of carrots depending on the colour such as orange, yellow, red, purple and white, which originate from the carotenoids and polyphenols. Orange carrots are the most popular, while other species are much less represented in the diet. Purple carrot has been grown and consumed for thousands of year in Asia, but in Europe is still not so popular. However, due to its bluish-purple colour with high level of anthocyanins, it can be used as a natural food colorant (Kamiloglu et al., 2018). Numerous epidemiologic studies emphasize the importance of nutrition rich in fruit and vegetables in combating disease and carrots of many colours could provide basic nutrition and bioavailable phytochemicals acting as a functional food (Arscott,Tanmihardio, 2010). Carrots contain α-carotene, β-carotene, lutein and lycopene, depending on colour varieties. Orange carrots are well known as „good for the eyes“ due to their high content of α- and β-carotene which are vitamin A precursors, whereas lutein is an important pigment in yellow carrots (Arscott, Tanmihardio, 2010). Lutein has a role in eye health and in protection from age-related macular degeneration (Ellison et al., 2017). Lycopene is the carotenoid primarily responsible for colour of red carrots. Purple or black carrots have attracted the attention in nutrition due to their phenolic compounds content, including anthocyanins compounds, which contribute significantly to the antioxidant capacity and are well known for their role in health promotion and prevention of chronic diseases (Algarra et al., 2014; Kamiloglu et al., 2018). Content of carotenoids, polyphenols and antioxidant capacity can vary with cultivar, season, environmental conditions and maturity.

Since carotenoids and polyphenols are sensitive on high temperature, oxygen and light exposure it is important to choose adequate drying technique and adjust the conditions in order to maximally protect this bioactive compounds. According to Witrowa-Rajchert et al. (2009), content of polyphenols and antioxidant capacity is reduced during drying process up to 10 or 35% for freeze drying and up to 30 or 50% for convection drying for purple carrots, depending of tested cultivars. Demiray and Tulek (2017) conclude that drying temperature showed a significant effect on the degradation of β-carotene in carrot during convective drying.

The objectives of this research were: (i) to evaluate the mass fraction of polyphenols and carotenoids, and the antioxidant capacity in two locally grown varieties, domestic yellow carrot and conventional purple carrot; (ii) to determined stability of polyphenols and carotenoids depend on applied drying process (conventional and freeze drying), in order to evaluate their possible potential in the production of new, nutritionally rich food products based on local raw materials.

MATERIALS & METHODS

Domestic yellow carrot grown in Petrijanec near Varaždin, and conventional purple carrot grown in Velika Gorica (Croatia), were obtained from a local producer and the samples were harvested in the phase of technological maturity during September and October 2015. After primary treatment carrots were sliced into rings 4 mm thick and then were conventionally dried (M. Bucher AG Typ 20 dryer) at a temperature of 42°C in order to achieve the dry matter of about 90%. Freeze drying process (Martin Christ alpha 1-4 LSC plus freeze-dryer) was done in two phases, in the 1st phase the samples were frozen to -60°C, after that primary drying was carried out in condition of a vacuum of 0,110-0,13hPa and a temperature of -30°C to 0°C for 16 hours, and isothermal desorption at 21°C for 4-8 hours. Dry matter was determined by drying to a constant mass at 105°C.

Total phenol content was assessed by the use of Folin-Ciocalteu spectrophotometric method, according to the previously described method by Palma et al. (2013). Absorbance was measured at 765 nm (spectrophotometer VWR UV-1600PC) and the results were expressed as galic acid equivalents (mg of GAE/100g on dry matter basis).

The antioxidant capacity was estimated by the DPPH radical scavenging method by the use of 2,2-diphenyl-1-picrylhydrazyl free radical according to the method described by Brand-Williams et al. (1995) with some modifications. The results were expressed as the Trolox equivalent (mg GAE /100 g d.w), and derived from the calibration curves.

Carotenoids: α-carotene, β-carotene, lutein and lycopene were determined by the use of internal HPLC method (Podravka). Carotenoids were extracted with tetrahydrofuran, diluted with the mobile phase and determined by the UV-VIS detector at 450 nm with the HPLC chromatography system. All obtained results are expressed as the mean value ± SD of two determinations.

RESULTS & DISCUSSION

The dry matter of fresh domestic yellow and purple carrot before drying were 11.10% and 12.50%, respectively. Samples of both cultivars were dried by using conventional and freeze drying process. In freeze dried and conventional dried carrots, the dry matter of domestic yellow carrot was 89.66±0.18 % and 90.05±0.83%, respectively, and in commercial purple carrot 87.40±0.53% and 86.7±0.72, respectively.

The amounts of carotenoids in fresh and dried samples of carrots are shown in Table 1. The most common carotenoid is lutein with 6.18±0.23 mg in purple and 4.00±0.28 mg/100g d.w. in yellow carrot. The lutein content for both varieties of fresh carrots are similar to the results described by Surles et al. (2004). The yellow carrot contained more β-carotene (2.60±1.67 mg/100g d.w.) compare to purple carrot (0.62±0.28 mg /100g d.w). According to the previously research (Surles et al., 2004; Sun et al., 2009) β-carotene was higher in purple than in yellow carrot, but in our research it has been detected differently (0.62 mg in purple and 2.60 mg/100g d.w. in yellow carrot). α-carotene has been detected only in the freeze dried sample of purple carrot (0.73 mg/100g d.w.) while Surles et al. (2004) found higher concentrations in purple carrots. Lycopene has not been detected.

The fact that some compounds were detected only in dried samples, but not in fresh (e.g. α-carotene in the conventionally dried sample of purple carrot) or that higher quantities of some carotenoids were found in dried samples could be explained by easier detection in this type of samples because the content is more homogenous and concentrated.
The highest loss of carotenoids was detected in the conventionally dried yellow carrot (15% measured for β-carotene and 46% for lutein in regard to the initial value, but in all the other samples drying process did not affect much on the carotenoids content or in some case even higher concentrations were recorded than in fresh carrots (Figure 4).
Total carotenoids present the sum of detected values for α-carotene, β-carotene, lutein and lycopene. According to this it is also clear that the stability of carotenoids was affected much by drying process only in the case of conventionally dried yellow carrot.

Table 1. Concentrations of carotenoids in fresh and dried samples of domestic yellow carrot and purple carrot

	mg/100g of d.w.				
	α-carotene	**β-carotene**	**lutein**	**lycopene**	**total carotenoids***
D. yellow carrot F	n.d.	2.60 ± 1.67	4.00 ± 0.28	n.d.	6.60
D. yellow carrot DC	n.d.	0.38 ± 0.03	1.82 ± 0.12	n.d.	2.20
D. yellow carrot DF	n.d.	2.36 ± 0.36	7.89 ± 1.21	n.d.	10.25
Purple carrot F	n.d.	0.62 ± 0.28	6.18 ± 0.23	n.d.	6.80
Purple carrot DC	0.73 ± 0.06	2.21 ± 0.14	5.27 ± 0.16	n.d.	8.21
Purple carrot DF	n.d.	0.75 ± 0.14	7.19 ± 0.17	n.d.	7.94

D.yellow carrot = Domestic yellow carrot; F = fresh, DC = conventionally dried, DF = freeze dried;
n.d. = not detected
*sum of α-carotene, β-carotene, lutein and lycopene

The results for the total phenols and the antioxidant capacity are shown in Figure 1 and 2. Purple carrot contained 5.9 fold total phenols (1235±41 mg GAE /100 g d.w.) than domestic yellow carrot (208±4 mg GAE /100 g d.w.). These results are consistent with the literature, purple carrots contain higher amounts of phenols than yellow (Sun et al., 2009). Purple carrots possess much higher antioxidant capacity (4155±115 µmol in purple and 213±7 µmol TE/100g d.w.in yellow carrot). The drying process did not affect much on the antioxidant capacity and the total phenolic content. The largest loss of total phenol content was detected in the freeze dried sample of purple carrot (73% measured in regard to the initial value in fresh carrot), while the losses of the antioxidant capacity and the total phenols in all the other samples were much less expressed (Figure 3).

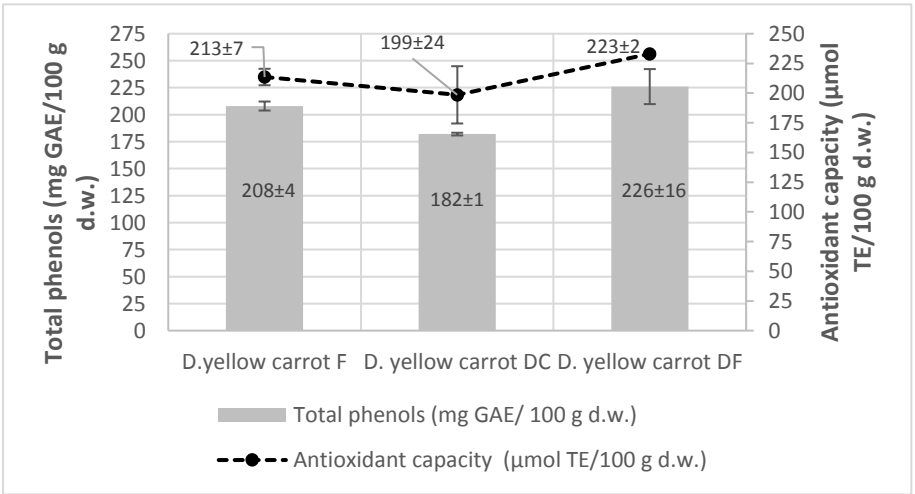


Figure 1. Total phenols and antioxidant capacity of fresh and dried samples of domestic yellow carrot.
F=fresh, DC= conventionally dried, DF= freeze dried

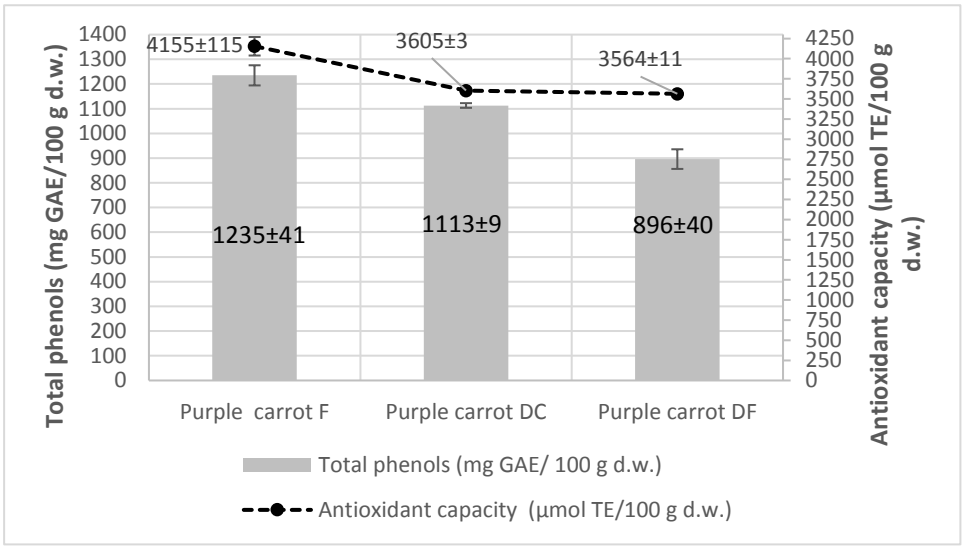


Figure 2. Total phenols and antioxidant capacity of fresh and dried samples of purple carrot.
F = fresh, DC = conventionally dried, DF = freeze dried

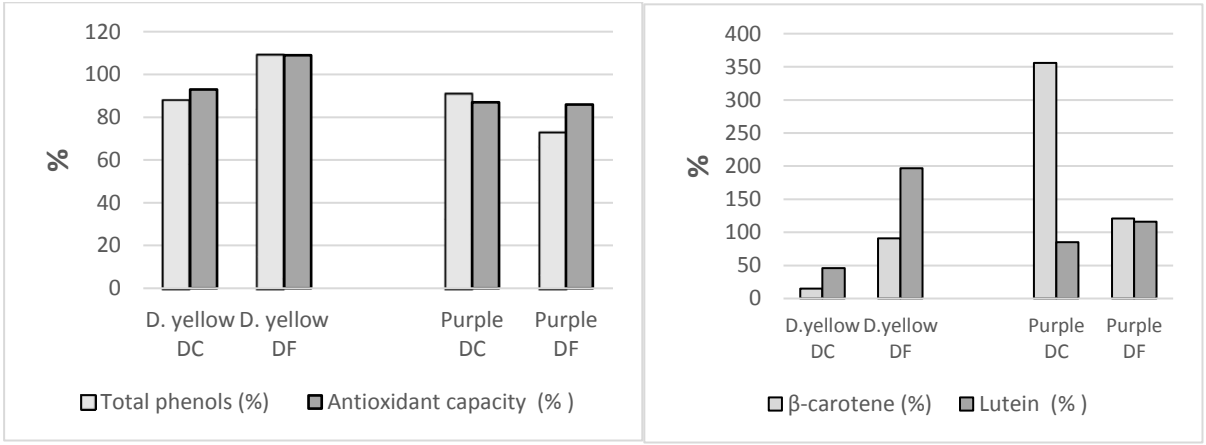


Figure 3. and 4. Changes in the total phenols content and the antioxidant capacity, and in β-carotene and lutein in domestic yellow and purple carrots during drying process compared to the content in fresh carrot samples (value for fresh carrot = 100%).
DC= conventionally dried, DF= freeze dried

CONCLUSION

Purple carrot is a rich source of polyphenol and it possesses a very high antioxidant capacity. Both researched carrots, purple and yellow contains remarkable amounts of carotenoids, especially β-carotene and lutein. Drying process was not remarkable influenced on the content of the researched bioactive compounds (phenols and carotenoids), except on the amount of carotenoids at conventionally dried yellow carrot sample. Dried carrots, especially purple carrot, could represents a very valuable source of

health-promoting compounds in diet and have potential as a raw material for production of new, nutritionally rich food products, especially in the segment of dried products.

REFERENCES

1. Algarra M., Fernandes A., Mateus N., de Freitas V., Esteves da Silva, J.C.G., Casado, J. 2014. Anthocyanin profile and antioxidant capacity of black carrots (Daucus carota L. ssp. sativus var. atrorubens Alef.) from Cuevas Bajas, Spain. Journal of Food Composition and Analysis 33,71–76.
2. Arscott S.A., Tanumihardjo S.A. 2010. Carrots of many colors providebasic nutrition and bioavailable phytochemicals acting as afunctional food. Comprehensive Reviews in Food Science and Food Safety 9, 223–239.
3. Braca, A., De Tommasi, N., Di Bari, L., Pizza, C., Politi, M., Morelli, I. 2001. Antioxidant principles from Bauhinia tarapotensis. Journal of Natural Products 64(7), 892-895.
4. Brand Williams, W., Cuvelier, M.E., Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. Lebensmittel Wissenschaft und Technologie 28, 25–30.
5. Ellison, S., Iorrizo, M., Senalik, D., Simon, P.W. 2017. The next generation of carotenoid studies in carrot (Daucus carota L.). Acta Horticulturae 1153, 93-100. doi: 10.17660/ActaHortic.2017.1153.14.
6. Engin Demiray, E., Tulek Y. 2017. Degradation kinetics of β-carotene in carrot slices during convective drying. International Journal of Food Properties 20(1), 151-156. DOI: 10.1080/10942912.2016.1147460
7. Grauwet, T., Kebede, B. T., Delgado, R., Lemmens, L., Manzoni, F., Vervoort, L., Hendrickx, M., Elmore, J. S. and Van Loey, A. 2015. Evaluating the potential of high pressure high temperature and thermal processing on volatile compounds, nutritional and structural properties of orange and yellow carrots. European Food Research and Technology 240 (1), 183-198.
8. Kamiloglu, S.,Van Camp, J., Capanoglu, E. 2018. Black carrot polyphenols: effect of processing, storage and digestion—an overview. Phytochemistry Reviews 17, 379–395.
9. Ostroški Lj. (Ur.). 2017. Statistička izvješća 1588/2017. Poljoprivredna proizvodnja u Hrvatskoj u 2016. Državni zavod za statistiku. https://www.dzs.hr/Hrv_Eng/publication/2017/SI-1588.pdf
10. Palma, M., Barbero, G. F., Pineiro, Z., Liazid, A., Barroso, C. G., Rostagno, M. A., Prado, J. M., Meireles, M. A. A. 2013. Extraction of Natural Products: Principles and Fundamental Aspects. in: Natural Product Extraction Principles and Application (Rostagno, M. A., Prado, J. M., ed.) RSC Publishing, Cambridge, 58-88.
11. Prior, R.L., Wu, X.L., Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53(10), 4290-4302.
12. Shortle, E., O'Grady, M.N., Gilroy, D., Furey, A., Quinn, N., Kerry, J.P. 2014. Influence of extraction technique on the anti-oxidative potential of hawthorn (*Crataegus monogyna*) extracts in bovine muscle homogenates. Meat Science 98(4), 828-834.
13. Sun, T., Simon, P.W. and Tamumuhardjo, S.A. 2009. Antioxidant Phytochemicals and Antioxidant Capacity of Biofortified Carrots (Daucus carota L.) of Various Colors. Journal of Agricultural and Food Chemistry 57, 4142-4147.
14. Surles, R.L., Weng, N., Simon, P.W., Tanumihardjo, S.A. 2004. Carotenoid Profiles and Consumer Sensory Evaluation of Specialty Carrots (Daucus carota L.) of Various Colors. Journal of Agricultural and Food Chemistry 52 (11), 3417-3421.
15. Witrowa-Rajchert, D., Bawoł, A., Czapski J., Kidoń M. 2009. Studies on Drying of Purple Carrot Roots. Drying Technology: An International Journal, 27(12), 1325-1331. DOI: 10.1080/07373930903226043

Influence of early and late fruit zone leaf removal on the dynamics of anthocyanin accumulation in cv. Merlot in North Dalmatia

Marina Pavlović[1], Zoran Zorić[2], Sandra Pedisić[2], Darko Preiner[3,4]

[1]*Department of Ecology, Agronomy and Aquaculture; University of Zadar, Trg kneza Višeslava 9, 23000 Zadar (mpavlov@unizd.hr)*
[2]*Faculty of Food Technology and Biotechnology; University of Zagreb; Petra Kasandrića 3, 23000 Zadar*
[3]*University of Zagreb Faculty of Agriculture, Department of Viticulture and Enology, Svetošimunska 25, 10000 Zagreb*
[4]*Center of Excellence for Biodiversity and Molecular Plant Breeding (CroP-BioDiv), Svetošimunska 25, 10000 Zagreb*

ABSTRACT

Biosynthesis of anthocyanins is one of the most important biochemical processes, which occur during the growth and development of grape of red cultivars. Anthocyanins are water-soluble pigments present in the vacuoles of the skin cells responsible for the red colour of berry and wines. Increased sunlight exposure enhances the biosynthesis of flavonoids especially anthocyanins during grape ripening. Different timing of partial fruit zone leaf removal can have significant effect on dynamics and total content of anthocyanins in grape. Cv. Merlot was introduced in North Dalmatia by the end of 20th century. This area is characterised by Mediterranean climate, but grapes from some sites with deep fertile soils can result in low level of grape anthocyanins. The aim of this research was to identify the effect of two different timing of fruit zone leaf removal performed (1) during flowering and (2) during veraison on dynamics of anthocyanins accumulation in grapes of Merlot cultivar in comparison to undefoliated control. Berries samples were collected every 10 days starting from veraison to full maturity. Identification and quantification of anthocyanins were conducted by HPLC method. The total anthocyanin content (TAC) ranged from 60.90 to 192.62 mg/100 g berries and from 52.02 to 183.39 mg/100 g berries at the flowering and veraison defoliation, respectively. In control samples TA ranged from 35.12 to 215.14 mg/100 g. The statistical analysis showed that different defoliation time had significant impact on the anthocyanin concentrations of 'Merlot' cultivar grown in North Dalmatia.

Keywords: anthocyanins, Merlot grape, defoliation time

INTRODUCTION

Anthocyanins are one of phenolic compounds, which are present in grape berries, located in the skins, seeds and stems and strongly contribute to the colour and mouthfeel of red wines (Ribereau-Gayon et al., 2000; Kennedy, 2006). The anthocyanin content (AC) in grape depends on numerous of factors such as grape cultivar, ecological conditions, agrotechnical and canopy management in vineyard, and vinifications techniques (Adams, 2006; Downey et al. 2006).
Leaf removal (LR) is a common canopy management method used to alter cluster microclimate, air circulation, sunlight exposure and berry temperature, while reducing *Botrytis* bunch rot infection (Smart et al., 1990). LR can increase soluble solids and TAC and reduce total acidity in grapes (Smart et al., 1990). Some works reported lower AC in grape berries which have been directly exposure to sunlight because higher berry temperature (above 30 °C) inhibited anthocyanins synthesis or even enhanced their degradation (Spayd et al. 2002). A major role in the anthocyanin accumulation in grapes has timing of LR, influence of light exposure and temperature of local growing area (Downey et al 2006; Tarara et al., 2008). Palliotti et al. (2013) have shown that the content of anthocyanin on the grape where late LR (after veraison) was conducted did not significantly differ from the grapes in control treatment (without LR). Last year's most of the researches indicated that early LR during flowering (FLR) enhanced TAC compared VLR, (Sternad Lemut et al. 2013). According to research by Bubola et al. (2014), the application of the early LR on Cabernet Sauvignon in Istria, Croatia, has resulted in a higher content of TAC.

The assessment of LR impact on ripening, is controversial, probably because of the variability in LR timing, the cultivar response and interaction between cultivar and microclimatic conditions. To our knowledge, no data are available in the literature on the anthocyanin accumulation after LR during ripening of red grapes in Dalmatia. Therefore, the aim of this research was to determine impact of early and late LR on anthocyanin content of Merlot grapes from north Dalmatia during ripening.

MATERIALS & METHODS

Plant material and experimental design

Research was conducted on cultivar Merlot during 2015. Vineyard, planted on 2007, located 20 km near Zadar (Baštica, Suhovare), in coastal region of Croatia (lat. 44°06´ N, long. 15°13´ E), vineyard Benkovac-Stankovci. Vine training system was Guyot, with 12-14 buds per plant. Vine spacing was 0.9 m between vines, and 2.8 m between rows (4 100 vines/ha). Vines were grafted on Kober 5BB (*Vitis berlandieri* Planch. x *Vitis riparia* Michx.) rootstock. The experimental field provided no irrigation system, with grass surface between rows. All usual vineyard management practices including disease management and nutrition were conducted. Rainfall and daily mean temperature for 2015 during April – September were obtained from Meteorological and Hydrological Service of Croatia and presented in Table 1.

Table 1. The average temperature and precipitation during the vegetation period (from April through September) in 2015, weather station Benkovac

Month	Average temperature (°C)	Precipitation(mm)
April	12	30
May	18	89.3
June	22.3	16.5
July	26.5	34.3
August	24.8	76.2
September	19.6	75.1
Mean temperature	20.5	
Cumulative rainfall (mm)		321.4

The experiment was completely randomized block design, with three treatments in three replications. Each replication consisted of 15 continuous plants so there were 135 plants in total.

Leaf removal performed during flowering (FLR) and veraison (VLR), while undefoliated plants were control. The veraison was estimated on the colour beginning of the berries. Berries were sampled five times every 10 days starting from veraison (11th August) to full maturity (25th September). In all berry samples HPLC analysis was performed.

Anthocyanins determination

The extraction of phenols from chopped grape berries was performed according to a previously described procedure (Elez Garofulić et al, 2015). The anthocyanins were analysed by a direct injection of the extracts, previously filtered through a 0.45-µm pore size membrane filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Chromatographic separation was performed using HPLC analysis with Agilent 1260 quaternary LC Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with diode array detector (DAD), an automatic injector and ChemStation software. The separation of anthocyanins was performed on a Luna 100-5C18, 5 µm (250×4.6 mm i.d.) column (Phenomenex). Anthocyanin determination was performed using method described previously by Zorić et al. (2014). Detection was carried out at 520 nm. Anthocyanins were characterised on the basis of retention times and UV-VIS spectra of standards and by using literature data (Downey et al, 2008). The quantifications of anthocyanins were made by the external standard method. All anthocyanin standards, delphinidin 3-O-glucoside (Del-3-G), cyanidin-3-glucoside (Cy-3-G), petunidin-3-glucoside (Pet-3-G), peonidin-3-glucoside (Peo-3-G) and malvidin-3-glucoside (Mal-3-G) were prepared as stock solutions in acidified

methanol (1 % of formic acid in methanol, by volume) at a concentration of 100 mg/L. All analyses were done three times and results expressed as mean value.

Statistical analysis
Statistical analysis was carried out using the software Statistica ver. 10.0 (Statsoft Inc, Tulsa, OK, USA). In order to explore the influence of two different timing of fruit zone leaf removal (FLR and VLR) on dynamics of anthocyanins accumulation in Merlot grapes in comparison to undefoliated control, multivariate analysis of variance (MANOVA) was performed and marginal means were compared with Tukey's HSD test. Differences at $p \leq 0.05$ were considered statistically significant.

RESULTS & DISCUSSION

According to literature data, the anthocyanin biosynthesis and content varies with variety, environmental factors, viticultural practices and maturity (Adams, 2006; Downey et al., 2006). Merlot as average ripening variety is known for its relatively high anthocyanin pigment content. The growing season (from April to September) 2015 in North Dalmatia (at the vineyard Benkovac-Stankovci) was without extreme weather conditions: During the vegetation period calculated cumulative rainfall and mean air temperature were 321.4 mm and 20.5 °C, respectively (Table 1). HPLC analyses of all grape berry extracts revealed the presence of 9 anthocyanins: Del-3-G, Cy-3-G, Pet-3-G, Peo-3-G, Malv-3-G, petunidin-3-*p*-acetil-glucoside, peonidin-3-*p*-acetil-glucoside, malvidin-3-*p*-acetil glucoside, malvidin-3-*p*-coumaroyl-glucoside which is in accordance with published data (Drenjančević et al 2017). TAC (as sum of individual anthocyanins) in Merlot grapes after LR (during flowering and veraison phase) and in control untreated samples is shown in Figure 1. Differences in TA content were noted between treatments and sampling time. The malvidin derivatives predominate in analysed samples but the content of other individual anthocyanins increased during ripening (particularly Cy-3-G and Pet-3-G), (data not shown). These results suggesting that LR affect the anthocyanin composition of the Merlot grapes what is consistent with previously reported data (Drenjančević et al., 2017; Downey and Rochfort, 2008).

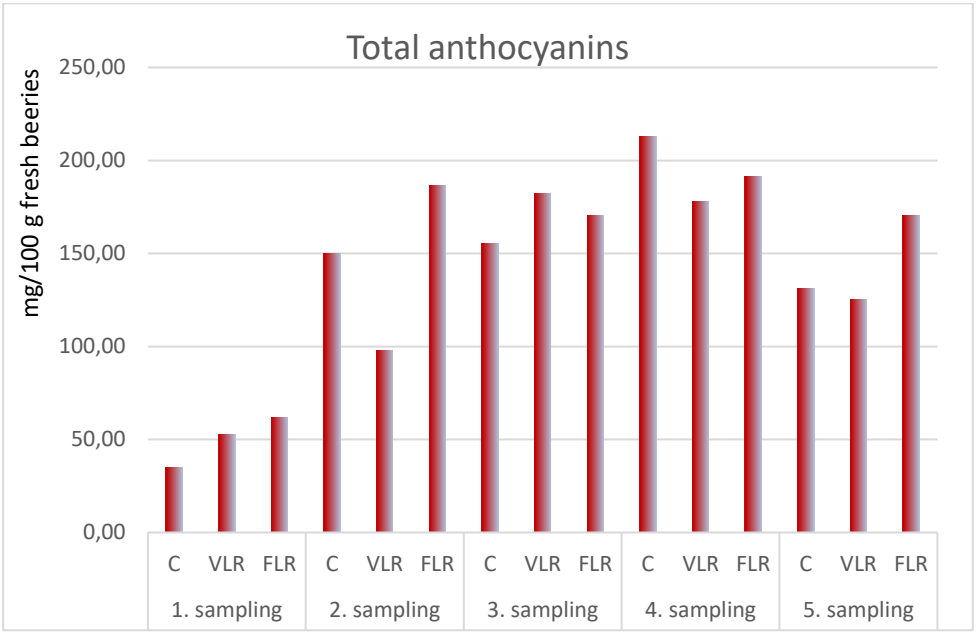


Figure 1. The content of total anthocyanins in Merlot grape cultivar influenced by defoliation period and sampling time

The TA content of 'Merlot' grapes ranged from 35.08 to 213.11 mg/100 g of fresh weigh of berries (fw) in control, 61.64 to 191.5 mg/100 g fw in FLR treatment and from 52.5 to 182.01 mg/100 g fw during VLR, respectively. In comparison to controls, grapes under FLR achieved higher TA content (from

9.57% to 75.71%) during sampling except in 4th sampling time (-10.11%). Our results are in good agreement with some recent investigations on red grape varieties (Merlot, Teran, Cabernet Sauvignon, Plavac mali) grown in the coastal region of Croatia (Bubola et al., 2014; 2017). However, Sivilotti et al., (2016) observed that defoliation in Merlot grapes grown in Udine (Italy), before and after flowering compared to untreated control had no effects on anthocyanin concentrations. The anthocyanin concentration in Pinot noir (grown in Switzerland), was highly increased, irrespectively of the period of LR (Verdenal et al., 2017). Generally, the TA content was lower in VLR grapes in comparison with control and FLR treatments particularly in grapes sampled at later sampling time what is in accordance with data reported by Tardaguila et al., (2010). Probably, VLR seems to be cultivar dependent, since this treatment promoted a general increase in anthocyanin accumulation in Cabernet Sauvignon berries (Tessarin et al., 2014).

The statistical analysis confirmed that FLR and sampling time had significant impact on the TA in Merlot grapes grown in North Dalmatia (Table 2). The TA content in FLR grapes increased and reached a maximum in 4th sampling time (30 days after veraison) and then decreased at full maturity (5th sampling time). The grape samples from VLR treatment showed variations in TA content during ripening. The positive influence of FLR on anthocyanin content in Mediterranean conditions has been observed in grape varieties of Tempranillo, Carignan, Barbera and Lambrusco (Diago et al., 2012; Tardaguila et al., 2010). Some studies reported when air temperature is higher than 30 °C for some hours in a day and for a long period during ripening, skin anthocyanin accumulation can be decreased (Spayd et al. 2002, Chorti et al. 2010). It seems that effect of late VLR in warmer vineyard conditions losing many of its advantages due to the changes of microclimatic conditions. Probably, anthocyanin accumulation or degradation depending on grape sensibility and responses to abiotic stresses (Guan et al. 2016; Pastore et al. 2017).

Table 2. The content of total anthocyanins in Merlot grape cultivar influenced by defoliation period and harvest time

	N	Anthocyanins (mg/100 g)
		$p < 0.05$*
Defoliation		
1 – Control	15	137.16 ± 0.38^{b}
2 – May 2015	15	156.19 ± 0.38^{c}
3 – August 2015	15	127.21 ± 0.38^{a}
Sampling time		
1	9	49.73 ± 0.49^{a}
2	9	145.06 ± 0.49^{c}
3	9	169.55 ± 0.49^{c}
4	9	194.09 ± 0.49^{d}
5	9	142.51 ± 0.49^{b}

Results are expressed as mean ± standard error. Means with the same letter within the column are not significantly different at $p<0.05$ by Tukey's HSD test.
*Statistically significant factor at 95% confidence level

CONCLUSION

Different time of leaf removal had significant impact on the anthocyanin accumulation in Merlot grape during ripening. Early leaf removal during flowering increased anthocyanin content in comparison to leaf removal in veraison and could be used for the production of anthocyanin rich Merlot grape berry in in North Dalmatia.

REFERENCES

1. Adams D.O. (2006). Phenolics and ripening in grape berries. Am. J. Enol. Vitic. 57: 249-256.
2. Bubola, M., (2015). Primjena rane defolijacije u svrhu povećanja kvalitete grožđa i vina, Institut za poljoprivredu i turizam, Poreč.
3. Bubola, M., Sivilotti, P., Diklić, K., Užila, Z., Palčić, I., Plavša, T. (2017). Manipulation of Teran grape composition with severe shoot trimming and cluster thinning. Acta Horticulturae 1188, 91-96.
4. Chorti, E., Guidoni, S., Ferrandino, A., Novello, V., (2010). Effect of different cluster sunlight exposure levels on ripening and anthocyanins accumulation in Nebbiolo grapes. Am. J. Enol. Vitic. 61, 23–3.
5. Diago, M. P., Ayestaran, B., Guadalupe, Z., Poni, S., Taraguila, J. (2012). Impact of prebloom and fruit set basal leaf removal on the flavonol and anthocyanin composition of Tempranillo grapes. Am. J. Enol. Vitic. 63: 367-376.
6. Downey, M. O., Dokoozlian, N. K., Krstic, M. P. (2006). Cultural practice and environmental impacts on the flavonoid composition of grapes and wine. A review. Am. J. Enol. Vitic 57: 257-268
7. Downey, M.O. and Rochfort, S. (2008). Simultaneous separation by reversed-phase high-performance liquid chromatography and mass spectral identification of anthocyanins and flavonols in Shiraz grape skin, J.Chrom. A, **1,** 1, 43-47.
8. Drenjančević, M., Jukić,V.,Zmaić, K., Kujundžić,T., Rastija,V. (2017). Effects of early leaf removal on grape yield,chemical characteristics,and antioxidant activity of grape variety Cabernet sauvignon and wine from eastern Croatia. Acta Agricul. Scandinavica; 67, 8, 705-711.
9. Elez Garofulić, I; Režek Jambrak, A.; Milošević, S.; Dragović-Uzelac, V.; Zorić, Z.; Herceg, Z. (2015) The effect of gas phase plasma treatment on the anthocyanin and phenolic acid content of sour cherry Marasca (Prunus cerasus var. Marasca) juice. LWT-Food Science and Technology. 62, 1; 894-900.
10. Guan L, Dai Z, Wu BH, Wu J, Merlin I, Hilbert G, Renaud C, Gomès E, Edwards E, Li SH, Delrot S. (2016). Anthocyanin biosynthesis is differentially regulated by light in the skin and flesh of white-fleshed and teinturier grape berries. Planta. 243, 23–41.
11. Kennedy, J.A., Saucier, C., Glories, Y., (2006). Grape and wine phenolocs: History and perspective. Am. J. Enol. Vitic. 57: 239-248.
12. Pallioti, A. Panara, F., Silvestroni, O. Lanari, V., Sabbatini, P., Howell, G. S., Gatti, M., Poni, S. (2013). Influence of mehanical postveraison leaf removal apical to the cluster zone on delay of fruit ripening in Sangiovese (Vitis vinifera L.) grapevines. Aust. J. Grape Wine Res.19: 369-377.
13. Pastore C, Allegro G, Valentini G, Muzzi E, Filippetti I. (2017) . Anthocyanin and flavonol composition response to veraison leaf removal on Cabernet Sauvignon, Nero d'Avola, Raboso,Piave and Sangiovese Vitis vinifera L. cultivars. Scientia Hort. 218,147–155.
14. Ribereau - Gayon, P. Glories, Y. Maujean, A., Dubourdieu, D. (2000). Handbook of enology Volume 2. The chemistry of wine stabilization and treatments. John Wiley & sons. Itd, Chichester.
15. Smart, R.E., Dick, J.K., Gravett, I.M., Fisher, B.M., 1990. Canopy management to improve grape yield and wine quality: principles and practices. S. Afr. J. Enol. Vitic.11: 3-17.
16. Spayd, S. E., Tarara J. M., Mee, D. L., Ferguson, J. C. (2002). Separation of sunlight and temperature effects of the composition of Vitis vinifera cv. Merlot berries. Am. J. Enol. Vitic. 53, 171-182.
17. Sternad Lemut M., Trost, K., Sivilotti, P., Arapitsas, P., Vrhovsek, U. (2013). Early versus late leaf removal strategies for Pinot Noir (Vitis vinifera L.): effect on colour-related phenolics in young wines following alcoholic fermentation. J. Sci. Food Agric. 93, 3670–3681.
18. Tarara, J. M., Lee, J., Spayd, S. E., Scagel, C. F. (2008). Berry temperature and solar radiation alter acylation, proportion and contcentration of anthocyanin in Merlot grapes. Am. J. Enol. Vitic. 59: 235-247.
19. Tardaguila, J., Martinez de Toda, F., Poni, S., Diago, M. P. (2010). Impact of early leaf removal on yield and fruit and wine composition of Vitis vinifera L. Graciano and Carignan. Am. J. Enol. Vitic. 61: 372-381.
20. Tessarin, P., Boliani, A.C., Bothelo, r.v., Rusin, C., Versari, A., Parpinello, G.P., Rombola, A.D.(2014). Effects of late defoliations on chemical and sensory characteristics of cv. Uva Longanesi wines. J. Soil Sci. Plant Nut. 14: 1021-1038.
21. Verdenal, T., Zufferey, V., Dienes-Nagy, A., Gindro, K., Belcher, S., Lorenzini, F., Rösti, J., Koestel, C., Spring, J.-L., & Viret, O. (2017). Pre-flowering defoliation affects berry structure and enhances wine sensory parameters. Oeno One, 51(3).
22. Zorić, Z., Dragović-Uzelac, V., Pedisić, S., Kurtanjek, Z., & Elez Garofulic, I. (2014). Kinetics of the degradation of anthocyanins, phenolic acids and flavonols during heat treatments of freeze-dried sour cherry marasca paste. Food Technology and Biotechnology, 52(1), 101-108.

Influence of the chitosan coating type on the cow's yellow cheese storage stability

Jana Simonovska[s], Mishela Temkov[s], Sandra Mojsova[b], Darko Dimitrovski[s], Vesna Rafajlovska[s], Elena Velickova[s]

[a] *Department of Food and Biotechnology, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia (velickova@tmf.ukim.edu.mk)*

[b] *Food Institute, Faculty of Veterinary Medicine Skopje, Ss. Cyril and Methodius University in Skopje, Lazar Pop Trajkov 5-7, 1000 Skopje, Republic of Macedonia (kostova.sandra@fvm.ukim.edu.mk)*

ABSTRACT

Currently, the dairy industry, especially the cheese production sector is trying to overcome the appearance of surface mold and growth of unwanted bacteria strains. Therefore, there is a growing interest for development of packaging materials with antimicrobial and functional properties that will ensure storage stability and good physical characteristics of the cheese.

Chitosan films with oregano extract were applied via laminating, dipping and spraying. The chitosan film without oregano extract was used as a control sample. The cheese removed from its original package was coated with chitosan films before storage. The cheese samples were stored in plastic containers at 4ºC. The weight loss, color and texture retention over period of 2 weeks were examined. The mold growth was inspected visually every day. The total bacterial count was determined once per week.

The results showed that cheese samples lost between 7.4 and 9.5% of their weight during storage depending on the type of the coating. There was no statistically significant difference in the color over time, while the difference among the different samples was significant. The samples coated with dipping and spraying resembled the most the control cheese. The cheese samples laminated with chitosan films, with and without oregano extracts, were darker, compared to dripped and sprayed coatings. All samples were characterized with good texture retention. The microbial infection of the coated samples started after a week, while the control sample showed first signs of mold after the third day of storage.

Keywords: chitosan films; coating type; yellow cheese; shelf-life quality; microbial stability

INTRODUCTION

Cheese represents traditional food product regularly produced in the Balkan countries in varieties such as white brined cheese, yellow cheese and beaten cheese, all belonging to the hard and semi-hard group of cheese according to the standard classification (Youssef et al., 2017). It is usually made by coagulation of the casein in milk and entrapment of the fat globules in the formed coagulum. Cheese manufacture is done in several steps and therefore a strict control of all internal and external factors is needed to avoid defects. After ripening the most common problem during storage is the appearance of surface mold.

Currently, cheese is still mostly packaged in plastic bags or covers that are non-biodegradable and present a big environmental problem everywhere. A new approach is the use of biodegradable edible films and coatings (Velickova et al., 2013). They indicate that the surface of the food is coated with a thin layer of edible biopolymer. The difference between them is that the film is prepared separately by casting and then it is applied on the cheese by wrapping or laminating, while the coating is applied directly on the cheese surface to form a film via dipping, spraying or brushing. Edible coatings can be fortified with plant extracts that show antimicrobial activity to prolong even more the storage stability of the product.

This study aims at using chitosan films and coatings fortified with oregano extracts to improve the storage stability of ripe cheese. Control and coated cheese samples were stored in plastic containers at 4ºC over two week period. The quality was evaluated by determining the weight loss, the color and texture retention and the microbial stability by following the mold growth and the total bacterial count.

MATERIALS & METHODS

2.1. Materials

Chitosan was purchased from Sigma Aldrich (St. Louis, USA), while glycerol, Tween 80 and acetic acid were from Merck, Germany. All chemicals were of analytical grade. The alcoholic oregano extract was obtained with Soxhlet extraction from dried oregano herbs.

2.2. Preparation of the edible films and coatings

Chitosan solution (0.8%, w/v) was prepared by dissolving 0.8 g of chitosan in 100 mL of acetic acid (1%, v/v) with continuous stirring at room temperature (20 ± 2 ºC). After, the complete dissolution of the chitosan, 0.2 g of glycerol and 0.2 g of Tween 80, were added and the solution was stirred for another 30 min. The chitosan films were casted in a plastic casting container and dried at 40 ºC for 48 h. The dried chitosan films were peeled off the container before applying them on cheese surface. The fortified chitosan films and coatings were prepared by mixing 100 ml of 0.8 % chitosan solution with 20 ml oregano extract ($1g_{extract}/5ml_{ethanol}$). The fortified chitosan films were casted and dryed, as described previously.

2.3. Preparation of the samples

Cow's yellow cheese, commercially produced, was bought from the retailers. The plastic package was removed and slices (20x50x3mm) were cut. Untreated slices were used as controls. The chitosan (Ch) and fortified chitosan (O) films were peeled from the containers and cut in the same dimensions to cover the cheese slices. The rest of the fortified chitosan solution was applied by dipping (D) the slices into solution for 20 s and letting the coating dry on the cheese surface. Another applied technique was spraying (S), where the cheese samples were sprayed on both sides with fortified chitosan solution. All samples were stored in plastic containers at 4ºC.

2.4. Quality control of cheese samples

To determine weight loss, the same samples were weighed at the beginning of the experiment after the settling of the coating and thereafter each week during the storage period. Weight loss was expressed as the percentage loss of the initial total weight. Five samples in three repetitions were used to evaluate the weight loss of every group.

The color was evaluated using a Dr. Lange spectro-color colorimeter to obtain L* (lightness), a* (redness) and b* (yellowness) values as mean of 5 readings.

The firmness of the fresh, control, and coated samples was measured at 20ºC using a texture analyzer (TA-XT2i of Stable Micro Systems, Godalming, England) with a 5 kg load cell. The penetration method was used. Crosshead speed was set at 1 mm/s and the penetration depth was 5 mm. A single slice was placed on a hollow platform and a 2 mm needle probe was used to cut through the strawberry tissue from one side to another, penetrating the total slice height. The textural parameter measured on the resulting force-distance curves was firmness and the mean values of 5 replicates, expressed in N, were reported.

2.5. Microbial stability of cheese samples

The slices were examined for mold during storage. Samples from all groups were visually inspected for any signs of mycelia development on the slice surface. Infected cheese samples were characterized as moldy, with green and gray spots. The results were expressed as the percentage of infected samples. Fifteen samples were used for each measurement. Bacteriological analysis was performed according to ISO 21528-1:2004 for *Enterobacteriaceae*, ISO 6888-1/2:1999 for coagulase positive *Staphylococci*, ISO 4833 for aerobic

mesophilic bacteria and ISO 16649-1/2:2001 for *E. coli* (Rulebook on the microbiological safety of foods, 2008).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was performed on the experimental data to evaluate significant differences between the samples at 95% confidence interval according to Tukey's test using Minitab 15 statistical software.

RESULTS & DISCUSSION

Edible films and coatings can be useful as coating material for cow's yellow cheese since it could combine the antimicrobial activity of chitosan and oregano extract with desirable oxygen, carbon dioxide and water vapor permeability. The coating would allow for the movement of water vapor across the film, thus preventing water loss as well as microbial spoilage. The weight loss of the cheese slices during storage is presented in Figure 1. The results showed that cheese samples lost between 7.4 and 9.5% of their weight during storage depending on the type of the coating.

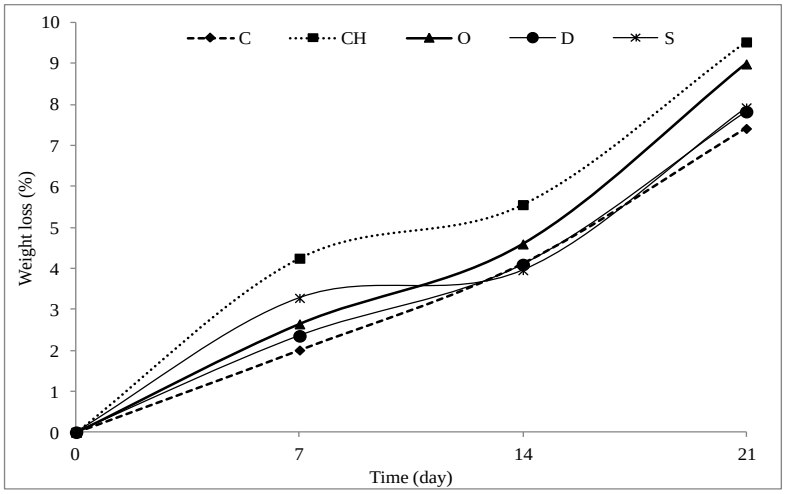


Figure 1. Weight loss in cheese slices during storage

The color and texture parameters of the cheese samples during storage are given in Figure 2. There was no statistically significant difference in the color over time, while the difference among the different samples was significant. The samples coated with dipping and spraying resembled the most the control cheese. The cheese samples laminated with chitosan films, with and without oregano extracts, were darker, compared to dripped and sprayed coatings. All samples were characterized with good texture retention.

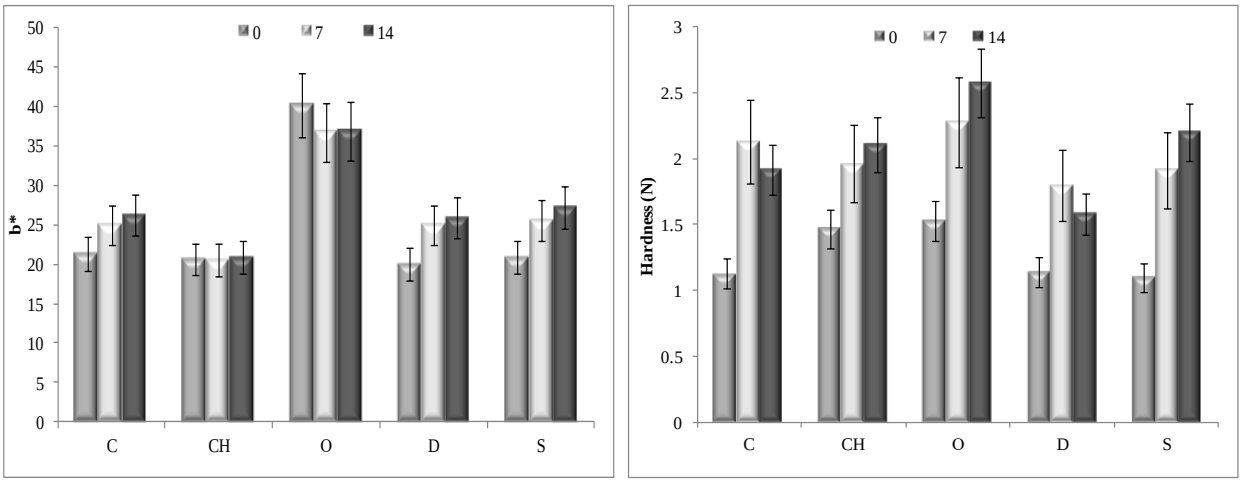


Figure 2. Color and texture parameters of cheese slices during storage

The microbial stability of the samples is presented in Table 1. The microbial infection of the coated samples started after a week, while the control sample showed first signs of mold after the third day of storage. The total bacterial count showed that chitosan films fortified with oregano extract gave the best microbial stability. Coating applied with dipping and spraying enabled better microbial protection in comparison with the standard chitosan film. Good microbial stability of coated cheese was already reported by several authors (Di Pierro et al., 2011, Unalan et al., 2013, Olle Resa et al., 2014).

Table 1. Microbial stability of cheese samples during storage

Cheese samples	Visual mold infection (%)			Total Bacterial Count (log CFU/g cheese)		
	0	7	14	0	7	14
C	0	30±1	65±3	5.5±0.3	6.5±0.4	8.3±0.6
CH	0	15±2	30±2	5.6±0.2	5.9±0.5	7.1±0.7
O	0	10±1	20±2	5.5±0.4	5.6±0.3	6.2±0.5
D	0	9±1	22±1	5.7±0.3	5.7±0.4	6.6±0.3
S	0	11±2	25±2	5.5±0.2	5.8±0.3	6.8±0.4

CONCLUSION

Edible films and coatings prepared from chitosan and fortified with oregano extracts can be used as packaging materials of cheese. The chitosan coated cheese was better preserved than the control, uncoated cheese, but the addition of the oregano extract, further improved the quality characteristics of the samples. Different coating techniques did not significantly affect the storage stability of the cheese.

REFERENCES

Di Pierro P., Sorrentino A., Mariniello L., Giosafatto C.V.L., Porta R. 2011. Chitosan/whey protein film as active coating to extend Ricotta cheese shelf-life. LWT - Food Science and Technology 44, 2324-2327.

Ollé Resa C.P., Gerschenson L.N., Jagus R.J. 2014. Natamycin and nisin supported on starch edible films for controlling mixed culture growth on model systems and Port Salut cheese. Food Control 44, 146-151.

Velickova E., Winkelhausen E., Kuzmanova S., Moldao-Martins M., Alves V.D. 2013. Characterization of multilayered and composite edible films from chitosan and beeswax. Food Science and Technology International 21(2), 83-93.

Youssef A.M., Assem F.M., El-Sayed S.M., Salama H., Abd El-Salam M.H. 2017. Utilization of edible films and coatings as packaging materials for preservation of cheeses. Journal of Packaging Technology and Research 1, 87-99.
Ünalan I.U., Arcan I., Korel F., Yemenicioğlu A. 2013. Application of active zein-based films with controlled release properties to control *Listeria monocytogenes* growth and lipid oxidation in fresh Kashar cheese. Innovative Food Science and Emerging Technologies 20, 208-214.

In situ immobilization of inulinase with aqueous two phase systems onto chitosan beads

Mishela Temkov[a,b], Mariya Brazkova[b], Elena Velickova[a], Albert Krastanov[b]
[a] Department of Food and Biotechnology, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Rudjer Boskovic 16, 1000 Skopje, Republic of Macedonia (mishela@tmf.ukim.edu.mk); (velickova@tmf.ukim.edu.mk)
[b] Department Biotechnology, Faculty of Technology, University of Food Technologies, 26 Mariza Blvd. 4002, Plovdiv, Bulgaria (abt-kr@rocketmail.com); (brazkova.m@gmail.com)

ABSTRACT

Inulinase from *Bacillus* sp 11/3 was purified with aqueous two phase systems (ATP) and two multiple forms of the enzyme were recovered from the polyethylene glycol (PEG) upper phase. The main subject of this research was to immobilize the enzymes on chitosan beads without the purification step from the PEG phase. In addition, the performance of the immobilized enzymes was analyzed. Chitosan beads with diameter of 3-4 mm were produced in 1.5 % acetic acid, and activated with 2% glutaraldehyde overnight. 50 % PEG solution, containing the enzymes was added to the activated support for covalent bonding on the surface of the beads. The immobilized preparations were analyzed for: optimal enzyme load in a relation to support weight and immobilization yield and activity recovery in a relation to time. Moreover, pH, thermal and operational stability were determined as well as the kinetic parameters. All characteristics were calculated on the base of inulinase activity assay in different conditions, using inulin as substrate. Chitosan beads proved to be good, easy to handle and low cost, support for *in situ* immobilization of inulinase from PEG phase with ATP purification.

Keywords: inulinase; immobilization; chitosan; polyethylene glycol; characterization

INTRODUCTION

Fructose and fructo-oligosaccharides (FOS) have reached its potential in the food industry due to their many advantages. They may act as prebiotics, they have low calorie value, contribute for improvement of gastrointestinal system conditions and have the ability to increase calcium and iron absorption in the human body (Apolinario et al., 2014; Yewale et al., 2013). Inulin is a storage carbohydrate polymer, composed of linear chains of fructosyl groups linked by β (2→1) glycosidic bonds, terminating with a glucose molecule at the reducing end (Barclay et al., 2012). Therefore, this biopolymer is recognized as the most suitable source for FOS production. In order to obtain FOS, inulinases (2,1-β-D-fructan fructanohydrolase; EC3.2.1.7) has been widely applied (Yewale et al., 2013). Immobilization of the enzymes is a technique that enables their reusability, enhances their pH and thermal stability, and facilitates their recovery from the product (Karimi *et al.*, 2014). Immobilization of the enzymes could be performed by covalent or non-covalent binding on various organic or inorganic supports (Karimi et al., 2014). Chitosan as a natural glucosamine polymer derived by deacetylation of chitin, a major component of arthropod and crustacean shells has many advantages as carrier for the enzymes (Juang et al., 2002). It is cheap, non-toxic, biocompatible, hydrophobic, and has big adhesion area (Chang, Juang, 2005). Moreover, it possesses hydroxyl (-OH) and amino ($-NH_2$) reactive groups which bind the enzyme. It could be additionally activated with bifunctional agent as glutaraldehyde for cross-linkage with the enzyme.

In our previous study, the inulinase was partially purified by aqueous two phase systems (ATPS), yielding two multiple forms of the enzyme in the PEG rich phase. To overcome the problem existing with recovering the target protein from the phase forming polymer, still avoiding the traditional complex and expensive methods, *in situ* immobilization method was explored by direct immobilization of the enzyme

from the PEG phase onto the support. Purification and *in situ* immobilization of papain with ATPS was reported by Li et al., 2010, resulting in high immobilization yield over 90%.

MATERIALS & METHODS

2.1. Materials

Chitosan was purchased from Sigma Aldrich (St. Louis, USA) and polyethylene glycol (PEG) (M_w = 1000 g/mol) was provided by Merck, Germany. Inulin from Jerusalem artichoke (DP > 25) was generously given as a gift by Beneo, Belgium. 4-Hydroxybenzhydrazide (PAH-BAH) was obtained from Alfa Aesar (Karlsruhe, Germany), while Bradford reagent and bovine serum albumin (BSA) were obtained by Bio-Rad (California, USA). All chemicals were of analytical grade.

2.2 Inulinase production and purification

Extracellular inulinase was obtained by submerged cultivation of *Bacillus sp 11/3* in a liquid medium composed of (g L^{-1}): inulin, 4.0; yeast extract 4.0; peptone 4.0; $MgSO_4$ 0.16; K_2HPO_4 0.8. The fermentation was conducted in flasks on a rotary shaker at pH 8 and temperature of 50 °C for 24 h (Gavrailov, Ivanova, 2014). Then the culture medium was freed from the cells by centrifugation at 14000 rpm for 3 min. The free cell medium was used as a crude enzyme solution. Inulinase from *Bacillus sp.* 11/3 was purified in aqueous two phase systems (ATPS) consisted of 26% PEG 1000 and 26% $MgSO_4$, as it was found to be the most suitable in a previous study (Temkov *et al.,* 2018). Two multiple forms of the inulinase were extracted in the PEG rich phase. The PEG phase, together with the enzymes was than diluted up to 50 %, and as such was used for enzyme immobilization.

2.3. Preparation and activation of chitosan beads

Chitosan beads were prepared according to the method reported by Li *et al*, 2010 (Li *et al.,* 2010). The chitosan powder (1%, w/v) was dissolved in 1.5% (v/v) acetic acid solution at 80 ºC. The obtained chitosan solution was dropped through a syringe into 1 M NaOH 30% (v/v) methanol solution. The produced beads were of uniform shape and diameter of 2.5–3.0 mm and after production were washed with plenty of distilled water. To the solution of 2% (w/v) glutaraldehyde in 0.02 M potassium phosphate buffer (pH 8.0) the beads were added in a ratio 4:1 and stirred (120 rpm) in a rotary shaker at 25 ºC overnight. The activated beads were thoroughly rinsed with distilled water and stored at 4 ºC.

2.4. Immobilization of inulinase onto activated chitosan beads

Chitosan beads in different weights were added to 5 ml enzyme solution (the PEG phase from ATPS) in glass vials and the immobilization was conducted at a room temperature in a rotary shaker and the samples for immobilization activity and efficiency were taken during 48 h in determined intervals. After that, the immobilized preparations (Ch/PEG beads) were washed with distilled water, than rinsed with 1 M NaCl solution in 0.02 M pH 7.0 potassium phosphate buffer (PBS) and stored at 4 ºC. The efficiency of immobilization at different support weights and different time intervals is evaluated by using the following equation:

$$\text{Immobilization yield (\%)} = \left(1 - \frac{\text{Protein concentration of supernatant}}{\text{Protein concentration of PEG phase}}\right) \times 100 \quad (1)$$

$$\text{Activity recovery (\%)} = \frac{\text{Total activity of immobilized enzyme}}{\text{Total activity of enzyme in PEG phase}} \times 100 \quad (2)$$

2.5. Inulinase activity assay

Inulinase activity assay was performed by incubating 0.1 g Ch/PEG beads with 2.5 mL 2% (w/v) inulin prepared in phosphate buffer pH 5.8 at 50 °C for 20 min. The enzyme reaction was stopped by incubating the tubes in boiling water. For estimating the inulinase activity, the produced fructose was determined by a method described by Lever, 1972 (Lever, 1972) with some modifications. 250 μL of the reaction mixture and 1750 μL solution of PAH BAH dissolved in 10 % 0.5 M HCl and 90 % 0.5M NaOH were boiled for

10 min. After cooling the absorbance at 410 nm was measured by using a microplate reader SPECTROSTAR – Nano (BMG LABTECH). The concentration of fructose was calculated using a calibration standard curve. One unit of enzymatic activity was defined as the amount of enzyme needed to liberate 1 μmol of fructose per minute under the assay conditions. Assays were performed in triplicate and average value was used in calculations.

2.6. Protein content assay

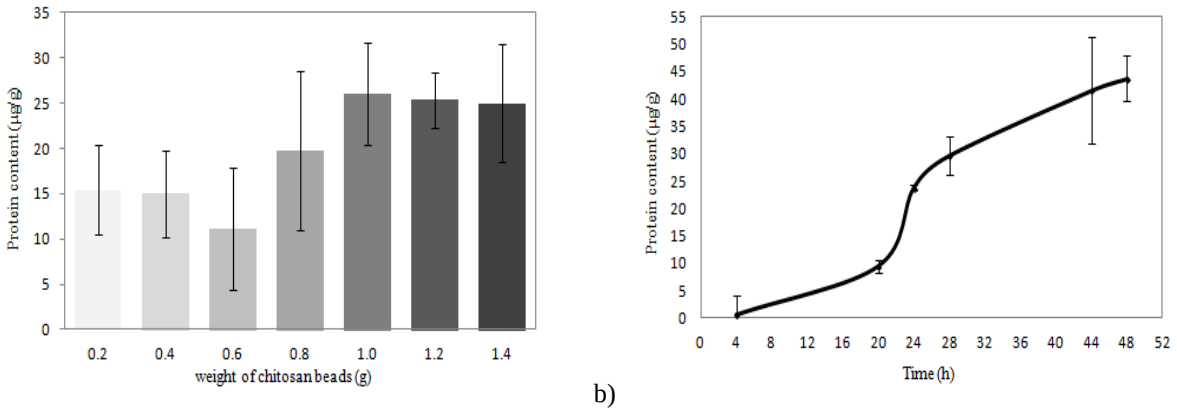
Protein concentration was determined according to Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

2.7. Operational stability of immobilized inulinase enzyme

The reusability of the immobilized inulinase on Ch/PEG beads was estimated as described in the activity assay of inulinase. After each cycle which lasted 1 h, the beads were removed by decantation, washed with distilled water and re-suspended in new portion of 2 % inulin solution for the next cycle. The activity of the immobilized enzyme after first cycle was interpret as the control and taken as a relative activity of 100% (Altun, Cetinus, 2007).

RESULTS & DISCUSSION

The objective of this work is the immobilization of inulinase, previously purified by optimized ATPS composed of 26 % PEG 1000 and 26 % $MgSO_4$ on Ch/PEG beads, which is a two step process Temkov et al., 2018. First, the produced uniform chitosan beads were pre-activated with glutaraldehyde in order to crosslink them for their prolonged exposure in acidic solutions (Çetinus, Öztop, 2003). Second step involves a treatment of pre-activated chitosan beads with 50 % diluted solution of PEG-rich phase after the ATPS, which contained the partially purified inulinase. The mechanism of this covalent bonding is by multipoint or multisubunit attachment of the enzyme on the surface of the beads. The main reaction is between the amino group from the peptide in the enzyme and the free aldehyde group from the glutaraldehyde to produce a Schiff base (Chagas et al., 2015). Due to the multipoint attachment of the enzyme on the chitosan beads their rigidification is increased.



a) b)

Figure 1. Optimisation of immobilization of inulinase on chitosan – PEG beads: a) optimal weight of carrier b) optimal time

On the other hand it generates conformational changes in the structure of the enzyme molecule, which displace the active centre and therefore the enzyme activity is decreased compared to the one of the free inulinase. It was assumed that PEG from the solution is also attached by loose hydrogen bonds between its hydroxyl groups and the amino groups from chitosan.

The immobilization amount of inulinase vs. the amount of the grafted chitosan beads with PEG and the optimal time of immobilization is presented on Figure 1 (a-b). From Fig 1-a , it can be seen as the

amount of the support icreased from 0.2 to 1.0 g, the amount of the protein attached to the chitosan beads inctreased from 15.43 to 25.99 mg/g. The immobilization yield increased from 30.13 % for 0.2 g chitosan beads to 50.75 % for 1.0 g carrier. The further increase of the mass carrier did not lead to greater immobilization yield or larger amount of protein bonded on the surface of the beads. The enzyme recovery was also increased from 10.26 % for 0.2 g chitosan beads to 57.31 % for 1.0 g carrier, while for 1.2 and 1.4 g beads it decreased to 39.28 and 37.58 %. This might happened due to the saturation of the reactive groups whether they come from the chitosan or bifunctional agent, with the enzyme or the polymer. The used PEG in ATP was with molecular mass of 1000 g/mol^{-1}, while the extracted two multiple forms of inulinase in this phase had 24 and 56 kDa (Temkov et al., 2018). The size of the PEG molecules may limit the space for more sites for an attacment and therefore, the maximal yield of immobilization is around 50%. Another important feature of the immobilization process is the course of immobilization i.e. how long does it take for the enzyme to be absorbed on the matrix. Figure 1. b) presents the absorbed protein on the support vs. immobilization time. It can be seen that in the first 4 h there is almost no absorption of the enzyme. From the 4th till the 20th h the immobilization process took place in a slow linear mode, while from 20th to 24th h the absorption of the protein increased rapidly. After 24 h, the attachment of the enzyme to the matrix continued until the 48th h, but with decreased velocit. The difference of absorbed protein and anzyme recovery between 44th h and 48th h was insignificant, therefore it was assumed that the immobilization has finished after 48 h with enzyme recovery of 76.61 %. Li et al., 2010, when immobilized *in situ* papain with PEG, extracted from ATPS, optmized immobilization for 36 h, with ratio of 1.2 g chitosan beads to 5 mL enzyme in PEG solution, immobilization yield and the activity recovery reached to 90.4% and 40.3% respectively.

Operational stability or reusability is an additional benefit of immobilization, as the immobilized preparation can be separated and recovered from the reaction mixture and used many times, which adds value to the overall economy of the process.

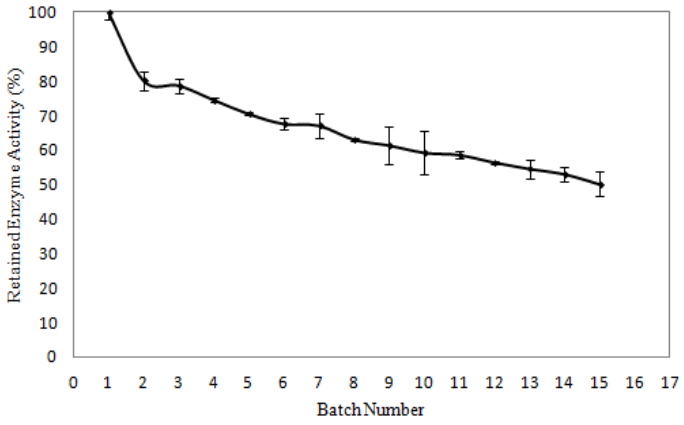


Figure 2. Operational stability of immobilized inulinase on chitosan-PEG beads

For this study 1 g immobilized preparation were added to 10 mL 2 % inulin in pH 5.8 (optimal pH, data not shown) and the batch reaction was set for 1 hour at 30 ºC (optimal temperature, data not shown). The same immobilized beads were used for next cycle. The results are shown in Fig 2, presenting the retained enzyme activity during repeated consecutive batches. The enzyme activity after the first cycle was taken as 100 % and it substantially dropped to 80 % after the second cycle, but afterwards the activity slowly decreased and the immobilized inulinase retained around 50 % of its activity until the 16th cycle. This exhibiting high operational stability might be due to the Schiff's base created by irreversible cross linking of glutaraldehyde. Monier *et al.*, 2010 reported that immobilized horseradish peroxidase retained 65.8 % residual activity after 6 consecutive operations. Our findings are a promising immobilized inulinase for industrial application.

CONCLUSION

In situ immobilization of inulinase on Ch/PEG beads was successful with 50 % immobilization yield and 76 % enzyme recovery after 48 h of immobilization. This technique shows potential as it avoids other purification steps for obtaining pure inulinase from PEG-rich phase when partially purified by ATPS. The immobilized preparation shows good operational stability of 16 consecutive cycles, which gives opportunities for effective industrial application.

REFERENCES

Altun, G.D., Cetinus, S.A., 2007. Immobilization of pepsin on chitosan beads. Food chemistry 100, 964-971.

Apolinario, A.C., de Lima Damasceno, B.P., de Macedo Beltrao, N.E., Pessoa, A., Converti, A., da Silva, J.A., 2014. Inulin-type fructans: a review on different aspects of biochemical and pharmaceutical technology. Carbohydrate polymers 101, 368-378.

Barclay, T., Ginic-Markovic, M., Johnston, M.R., Cooper, P.D., Petrovsky, N., 2012. Analysis of the hydrolysis of inulin using real time 1H NMR spectroscopy. Carbohydrate research 352, 117-125.

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry 72, 248-254.

Çetinus, Ş.A, Öztop, H.N, 2003. Immobilization of catalase into chemically crosslinked chitosan beads. Enzyme and microbial technology 32, 889-894.

Chagas, P.M., Torres, J.A., Silva, M.C., Correa, A.D., 2015. Immobilized soybean hull peroxidase for the oxidation of phenolic compounds in coffee processing wastewater. International journal of biological macromolecules 81, 568-575.

Chang, M.-Y., Juang, R.-S., 2005. Activities, stabilities, and reaction kinetics of three free and chitosan–clay composite immobilized enzymes. Enzyme and microbial technology 36, 75-82.

Gavrailov, S., Ivanova, V., 2014. Isolation and characteristics of a thermophilic *Bacillus* strain, producer of inulinase. Journal of BioScience and Biotechnology, 83-94.

Juang, R.S., Wu, F.-C., Tseng, R.-L., 2002. Use of chemically modified chitosan beads for sorption and enzyme immobilization. Advances in Environmental Research 6, 171-177.

Karimi, M., Chaudhury, I., Jianjun, C., Safari, M., Sadeghi, R., Habibi-Rezaei, M., Kokini, J., 2014. Immobilization of endo-inulinase on non-porous amino functionalized silica nanoparticles. Journal of Molecular Catalysis B: Enzymatic 104, 48-55.

Lever, M., 1972. A new reaction for colorimetric determination of carbohydrates. Analitical Biochemistry 47, 273 - 279.

Li, M., Su, E., You, P., Gong, X., Sun, M., Xu, D., Wei, D., 2010. Purification and in situ immobilization of papain with aqueous two-phase system. PloS one 5, e15168.

Monier, M., Ayad, D.M., Wei, Y., Sarhan, A.A., 2010. Immobilization of horseradish peroxidase on modified chitosan beads. International journal of biological macromolecules 46, 324-330.

Temkov, M., Strinska, H., Dobrev, G., Velickova, E., Muresan, V., Krastanov, A., 2018. Purification of bacterial inulinase in aqueous two-phase systems. Engineering in Life Sciences. doi.org/10.1002/elsc.201700174

Yewale, T., Singhal, R.S., Vaidya, A.A., 2013. Immobilization of inulinase from *Aspergillus niger* NCIM 945 on chitosan and its application in continuous inulin hydrolysis. Biocatalysis and Agricultural Biotechnology 2, 96-101.

Assessment of lunch plate waste and meal acceptance among elementary school children

Karla Ferk[1], Martina Bituh[2], Ružica Brečić[3], Irena Colić Barić[4]

[1]*Gradaute student of Nutrition Science, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, karla.ferk@hotmail.com*
[2]*Laboratory for Food Chemistry and Biochemistry, Department of Food Quality Control, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb*
[3]*Department of Marketing, Faculty of Economic and Business, University of Zagreb, Trg J.F. Kennedyja 6, 10000 Zagreb*
[4]*Laboratory of Nutrition Science, Department of Food Quality Control, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb*

Objective: School meals contribute to the daily energy and nutrient intake of children. Plate waste in schools does not only represent a loss of resources and money, but may also lead to the child's dietary intake being inadequate. The objective of the study was to assess lunch plate waste and meal acceptance among school children, while identifying reasons of food waste.
Methods: The lunch plate waste assessment was conducted among 137 elementary school children. Three randomly chosen portions of the lunch meal were weighted before serving, while after the meal each plate's leftovers were weighted separately. Meal acceptance was assessed with the Taste and Rate method, and the reasons for food waste were determined with a short questionnaire.
Results: On average, 41% of bread, 27% of leek stew, 23% of meat and 7% of pudding was wasted. On a scale from 1 ("I didn't like it at all") to 5 ("I liked it a lot"), the majority of children (69%) graded the stew with high grades, with an average of 4.0 ± 1.2. Among children who did not finish the stew the most common reasons were that they disliked the taste (36%), the portion size was too large (36%) or they did not eat that meal at home (22%).
Conclusion: This study confirms previous findings that food preferences are a good predictor of food consumption, with family habits also influencing school meal acceptance through dietary habits and food exposure. School meals should be revised and adjusted in order to minimize plate waste and meet the dietary needs of schoolchildren.

Keywords: plate waste, elementary school, lunch, preferences

INTRODUCTION

A healthy diet is highly important in childhood, not only because it ensures adequate growth and development by providing enough energy and nutrients, but also because it can protect from obesity and non-communicable chronic diseases later in life (Nicklas and Hayes, 2008). Dietary habits adopted in childhood are often maintained in adulthood (Mikkilä *et al*, 2005), therefore it is important to expose children to foods with a beneficial impact on health. Fruits and vegetables have been shown to have positive health effects (Hartley *et al*, 2013), but children's intake is often inadequate (Kuzman *et al*, 2012). Aside from family, schools also play an important role in food intake because that is where children spend a large part of their day (Buzby and Guthrie, 2004). Children who participate in school nutrition programmes eat at least one meal prepared at school which affects their daily energy and nutrition intake (Smith and Cunningham-Sabo, 2013). However, when children do not finish their meals, they fail to receive all its benefits from a nutrition point of view (Buzby and Guthie, 2004). At the same time, a lot of food is wasted, which represents a needless financial expense and a loss of resources (FAO, 2011). Therefore, the aim of this study was to a) assess plate waste in two elementary schools, b) determine how much the children liked the vegetable meal, and c) identify reasons for plate waste in order to determine possible strategies to increase the school meal consumption.

MATERIALS & METHODS

The study was conducted on the 22nd of March 2017 in two elementary schools in Zagreb among 137 students from 1st to 4th grade. It was part of the Horizon 2020 project, Stenght2Food, and it was approved by the Ministry of science, education and sports of the Republic of Croatia on the condition that the Code of ethical research with children is respected. The participants had their parents' written consent.

All students were served the same meal prepared in the same central kitchen, leek stew with meat, while corn bread and vanilla pudding were optional meal components. Children could not influence the meal size. The meal was served over an A3 size paper plate pad with and identification number on which the children marked how much they enjoyed the meal and why they did not finish it (in case there was leek stew left on the plate). After the meal, plate waste was assessed by weighing leftovers separately with a digital scale (Daewoo, DKS-2055, m ± 1 g). The average servings of leek stew, meat and bread were obtained with three different measurements, while the mass of the pudding was obtained from the label. The total percentage of plate waste for each meal component was calculated as follows:

(Sum of plate waste (g))/(average serving (g) x number of students)) x 100

The school meal was rated with the Taste and Rate method (Wardle *et al*, 2003) with five smiley faces, showing from a happy to a neutral and a frowning expression, followed by ratings from 1 to 5 indicating: 1- "I strongly disliked it", 2- "I disliked it", 3- "It was okay", 4- "I liked it", and 5- "I liked it a lot". After finishing the meal, the students marked how much they liked the leek stew. Furthermore, the children who did not finish the stew had to mark one or more reasons offered: "I disliked the taste", "I disliked the smell/appearance", "I do not eat this meal at home", "I am not hungry", "I cannot eat that much food", and "I did not have enough time".

The data was elaborated with Microsoft Excel (2010). Statistical significance was assessed with the computer program SPSS (version 17.0, Chicago, SPSS Inc.). The correlation between stew plate waste and ratings was assessed with Spearman's correlation coefficient.

RESULTS & DISCUSSION

After lunch, 41.1% of bread, 26.7% of leek stew, 23.4% of meat and 7.1% of pudding served to 137 elementary school students were wasted (Figure 1).

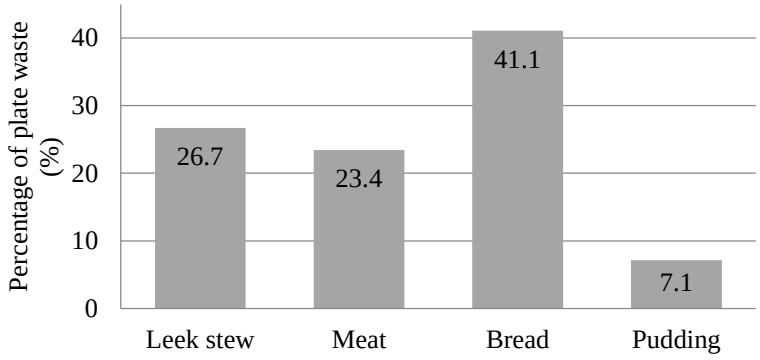


Figure 1. Percentage of leek stew, meat, bread and pudding plate waste (n=137)

Data in literature are inconsistent with some studies reporting that more than half of the vegetables served are being wasted. Cohen *et al* (2014) have assessed by weighting that as much as 60-75% of vegetables served to 3rd to 8th graders are not being consumed, which is a much higher plate waste than shown in the present study. Another study within the same population by Cohen *et al* (2016) reported a plate waste between 53.4% and 65.2%. Similar findings can be seen in the studies by Schwartz *et al* (2015) (46-55%) and Todd *et al* (2017) (58%). Others have reported a lower vegetable plate waste, such as Smith and Cunningham-Sabo (2013) (33.6%) and Niaki *et al* (2017) (26.0-43.4%).

Previous findings have identified fruit and vegetables as the most wasted foods at school meals (Buzby and Guthrie, 2002; Cohen *et al*, 2014; Cohen *et al*, 2016; Merlette *et al*, 2005; Niaki *et al*, 2017), although this study found bread to be the most wasted meal component. These results are consistent with the findings of Smith and Cunningham-Sabo (2013) that measured a 44.6% plate waste of cereal products. In other studies, this plate waste was lower: 13.9-21.5% (Merlette *et al*, 2005), and 18.6-29.0% (Niaki *et al*, 2017). Meat plate waste is consistent with other findings, such as those of Merlette *et al*

(2005) (6.5-25.3%), and of Niaki *et al* (2017) who measured an 18.6-30.9% plate waste of all protein foods (fish, sea products, poultry, eggs, beans, peas, nuts, seeds and soy products).
It should be noted that plate waste can differ among foods in each food group (Niaki *et al*, 2017; Schwartz *et al*, 2015), and it also depends on the preparation method (Merlette *et al*, 2005), which can explain the great variability of literature data.

Judging by the students' rating of the leek stew, it can be concluded that it was well accepted. More than a half of all participants marked that they "liked it a lot" (50.4%) or that they "liked it" (19.0%). A smaller percentage of students "disliked it" (10.2%) or "strongly disliked it" (4.4%), while 16.1% marked "it was okay" (Figure 2).

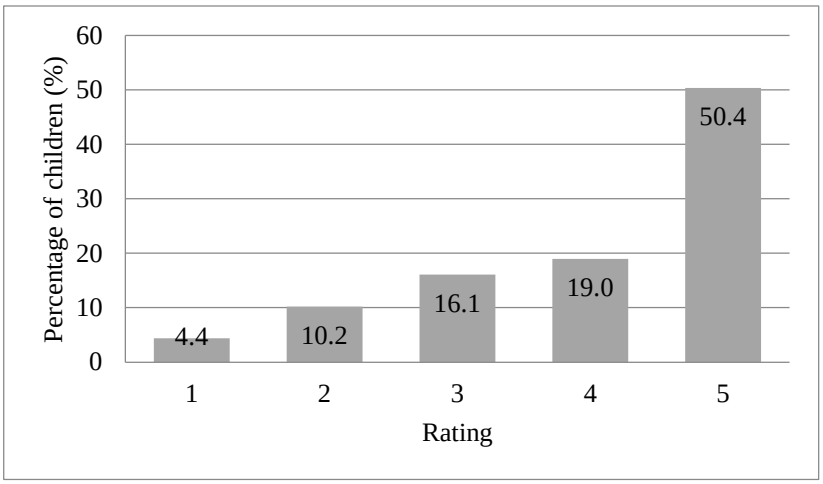


Figure 2. Percentage of children who rated the leek stew with 1 – "I strongly disliked it", 2- "I disliked it", 3- "It was okay", 4 – "I liked it", and 5 "I liked it a lot" (n=137)

The average rating of the leek stew was 4.0 ± 1.2 which indicates that students generally liked the vegetable meal. A similar study was conducted by Baxter and Thompson (2002) who asked 2nd graders if they liked their vegetable meals "a lot", "a little" or "not at all". A larger number of vegetables was rated, therefore, the acceptance varied for different vegetables. The most disliked vegetables (>50% of children stated they didn't like it at all) were broccoli, coleslaw, mixed vegetables and greens, while the most liked vegetables (>50% of children stated they like it a lot) were French fries, mashed potatoes, corn, carrots and celery.

This study also observed a statistically significant ($p<0.001$) negative moderate to good correlation ($R= -0.669$) between stew plate waste and ratings. The more stew was wasted, the lower was the rating. It can be concluded that plate waste is a good indicator of meal acceptance among school children.
Baxter and Thompson (2002) showed similar results when measuring vegetable plate waste and meal liking among 4th graders. They found a strong correlation between consumption and vegetable acceptance, meaning that the more children liked the vegetable the more they ate it.

In this study, if children did not finish their vegetables, it was very important to identify the most common reasons. Answers may point which steps should be carried out to reduce plate waste. Reasons for plate waste among children who did not finish the leek stew are shown in Figure 3. Children were allowed to mark more than one answer. One of the main reasons for plate waste is taste dislike (35.5%). A smaller number of children (14.5%) also found smell or appearance to be unacceptable (14.5%). This further confirms that food preferences are a predictor of plate waste, and interventions targeting food preferences and food presentation can help reduce plate waste (Buzby and Guthrie, 2004). It is important to keep in mind that the first contact between the children and the meal happens at a visual level, therefore school kitchens should make sure that meals look tasty and attractive (USDA, 2016). Involving

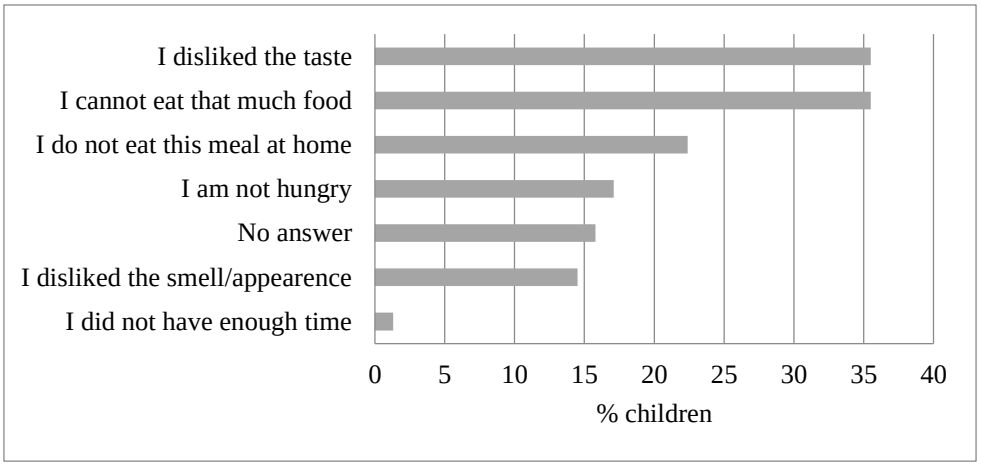


Figure 3. Reasons for plate waste among children that did not finish the leek stew (n= 76)

a professional chef in the preparation and improvement of school meals has shown promising results. In comparison with the control group, students whose school hired a professional chef consumed vegetables more often and in greater quantity (Cohen *et al*, 2012). Zellner and Cobuzzi (2017) noticed that children started liking some previously disliked vegetables after they were prepared by a professional chef. These findings suggest that if children do not like the taste of a certain food, there is no reason to stop offering it. After all, repeated exposure of the disliked vegetable can increase its acceptance (Anzman-Frasca *et al*, 2012).

A different plate waste reduction strategy includes an increase in the variety of daily meal offers, which gives students the opportunity to choose preferred vegetables that they will consume more often (USDA, 2016). However, this might be a challenge for school kitchens that do not have the capacity to prepare a variety of meals.

More than one third of students wasted their stew because they found the portion size to be too large (35.5%). This issue can be approached by tailor-made portions for each student (Buzby and Guthrie, 2004) and by encouraging students to start with smaller portions and take more after finishing the first plate (Engström and Carlsson-Kanyama, 2002).

Some authors have suggested that children are not hungry at lunch time (Niaki *et al*, 2017). In this study, 17.1% of students wasted their stew because they were not hungry. Buzby and Guthrie (2004) highlighted the importance of the meal time, because if it's too close to breakfast, children might still be full from the previous meal, and if it's served too late, children can seek for other food sources in the meantime (i.e. vending machines). Competitive foods can be a great issue, since it leads to children consuming less of their lunch (Merlette *et al*, 2005). Although children cannot be prevented from bringing food from home, access to vending machines can be limited. The timing problem can be solved by organizing lunch between 12:00 and 13:30, as recommended by the Croatian Ministry of health (Capak *et al*, 2013). That can be a challenge for schools with a smaller dining room that host a larger number of students. In any case, it should be further investigated why children lacked appetite during lunch.

CONCLUSION

This study found that the plate waste problem is also present in Croatian elementary schools. It confirms previous findings that food preferences are a good predictor of food consumption: students who ate more leek stew also gave a higher rating. School meals should be revised and adjusted in order to minimize plate waste and meet dietary needs of school lunch consumers. Plate waste caused by large portion sizes can be decreased by simply offering a tailor made size portion. The influence of family habits on meal acceptance can be seen in almost ¼ of plate waste cases. School meal acceptance could be increased with a higher exposure of such meals at home or by offering more often meals that children are more familiar with. It should also be further investigated why some children are not hungry at lunch time.

This project is funded by the European Commission - Horizon 2020 research and innovation programme "Strength2Food" under grant agreement No. 678024.

REFERENCES

Anzman-Frasca, S., Savage, J. S., Marini, M. E., Fisher, J. O., Birch, L. L. 2012. Repeated exposure and associative conditioning promote preschool children's liking of vegetables. Appetite 58(2), 543–553.

Baxter, S. D., Thompson, W. O. 2002. Fourth-Grade Children's Consumption of Fruit and Vegetable Items Available as Part of School Lunches Is Closely Related to Preferences. Journal of Nutrition Education and Behavior 34(3), 166-171.

Buzby, J. C., Guthrie, J. 2002. Plate Waste in School Nutrition Programs: Final report to Congress. Available at: https://www.ers.usda.gov/webdocs/publications/43131/31216_efan02009.pdf?v=41423 (17 June 2018)

Capak, K., Colić Barić, I., Musić Milanović, S., Petrović, G., Pucarin-Cvetković,J., Jureša, V., Pavić Šimetin, I., Pejnović Franelić, I., Pollak, L., Bošnir, J., Pavić, E., Martinis, I., Švenda, I., Krajačić, M., Martinis, O., Gajari, D., Kreškić, V. Horvat Vrabanac, M., Predavec, S., Grgurić-Šimac, V. 2013. Nacionalne smjernice za prehranu učenika u osnovnim školama. Ministarstvo zdravlja Republike Hrvatske, Zagreb.

Cohen, J. F. W., John, J. L., Richardson, S., Cluggish, S. A., Parker, E., Rimm, E. B. 2016. Amount of Time to Eat Lunch Is Associated with Children's Selection and Consumption of School Meal Entrée, Fruits, Vegetables and Milk. Journal of the Academy of Nutrition and Dietetics 116(1), 123-128.

Cohen, J. F. W., Richardson, S., Parker, E., Catalano, P. J., Rimm, E. B. 2014. Impact of the New U.S. Department of Agriculture School Meal Standards on Food Selection, Consumption and Waste. American Journal of Preventive Medicine 46(4), 388-394.

Cohen, J. F.W., Smit, L. A., Parker, E., Austin, S. B., Frazier, A. L., Economos, C. D., Rimm, E. B., 2012. Long-Term Impact of a Chef on School Lunch Consumption: Findings from a 2-Year Pilot Study in Boston Middle Schools. Journal of the Academy of Nutrition and Dietetics 112(6), 927–933.

Engström, R., Carlsson-Kanyama, A. 2004. Food losses in food service institutions, Examples from Sweden. Food Policy 29(3), 203-213.

FAO 2011. Global Food Losses and Food Waste – Extent Causes and Prevention, Food and Agriculture Organisation of the United Nations, Rome.

Hartley, L., Igbinedion, E., Holmes, J., Flowers, N., Thorogood, M., Clarke, A., Strangers, S., Hooper, L., Rees, K. 2013. Increased consumption of fruits and vegetables for the primary prevention of cardiovascular disease. Cohrane database of systematic reviews, DOI: 10.1002/14651858.CD009874.pub2

Kuzman, M., Pavić Šimetin, I., Pejnović Franelić, I. 2012. Ponašanje u vezi sa zdravljem u djece školske dobi 2009/2010. Djeca i mladi u društvenom okruženju. Hrvatski zavod za javno zdravstvo, Zagreb.

Merlette, M. A., Templeton, S. B., Panemangalore, M. 2005. Food Type, Food Preparation, and Competitive Food Purchases Impact School Lunch Plate Waste by Sixth-Grade Students. Journal of the American Dietetic Association 105(11), 179-1782.

Mikkilä, V., Räsänen, L., Raitakari, O. T., Pietinen, P. 2005. Consistent dietary patterns identified from childhood to adulthood: The Cardiovascular Risk in Young Finns Study, British Journal of Nutrition 93(6), 923-931.

Niaki, S. F., Moore, C. E., Chen, T., Weber Cullen, K. 2017. Younger Elementary School Students Waste More School Lunch Food than Older Elementary School Students. Journal of the Academy of Nutrition and Dietetics 117(1), 95-101.

Nicklas, T. A., Hayes, D. 2008. Position of the American Dietetic Association: nutrition guidance for healthy children ages 2 to 11 years. Journal of the American Dietetic Association 104(4):660-677.

Schwartz, M. B., Henderson, K. E., Read, M., Danna, N., Ickovics, J. R. 2015. New School Meal Regulations Increase Fruit Consumption and Do Not Increase Total Plate Waste. Childhood Obesity 11(3), 242-247.

Smith, S., Cunningham-Sabo, L. 2014. Food Choice, plate waste and nutrient intake of elementary- and middle-school students participating in the US National School Lunch Program. Public Health and Nutrition 17(6), 1255-1267.

Todd, L. E., Wells, N. M., Wilkins, J. L., Echon, R. M. 2017. Digital Food Image Analysis as a Measure of Children's Fruit and Vegetable Consumption in the Elementary School Cafeteria: A Description and Critique. Journal of Hunger and Environmental Nutrition 12(4), 516-528.

USDA 2016. Strategies for Successful implementation of the Healthy, Hunger-Free Kids Act. USDA – United States Department of Agriculture. Available at: https://fns-prod.azureedge.net/sites/default/files/ops/HHFKA-PlateWaste.pdf. (17 June 2018)

Wardle, J., Herrera, M-L., Gibson, E. L. 2003. Modifying children's food preference: the effects of exposure and reward on acceptance of an unfamiliar vegetable. European Journal of Clinical Nutrition 57(2), 341-348.

Zellner, D. A., Cobuzzi J. L. 2017. Eat your veggies: A chef-prepared, family style school lunch increases vegetable liking and consumption in elementary school students. Food Quality and Preference 55, 8-15.

Sensory Evaluation of Apple Juices Enriched by Plant Extracts

Danijela Bursać Kovačević[a], Domagoj Gabrić[a], Branka Levaj[a], Verica Dragović-Uzelac[a]

[a] *Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia (dbursac@pbf.hr)*

ABSTRACT

Consumer demand for functional food is constantly growing due to the scientific evidences that support the positive effect of these foods on human health. Moreover, plant extracts, as rich in phytochemicals, are becoming more used as food preservatives and antioxidants from natural sources in foods formulation such as fruit juices and derived products. Apple juice (AJ)A, is one of the most popular fruit juices due to its nutritional and sensory value, could represent a basis for functional food production. Hence, the goal of this study was to characterize sensory profile of apple juice enriched with plant extracts of several medicinal and Mediterranean herbs such as elderberry (*Sambucus nigra*), tilia (*Tilia cordata*), common thyme (*Thymus vulgaris*), Breckland thyme (*Thymus serpyllum*) and lemon balm (*Melissa officinalis*) in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%). A quantitative descriptive analysis (QDA) was used to evaluate following properties: colour (intensity), aroma (apple, plant, cooked, strange), odour (apple, plant, strange), and taste (acidic, sweet, apple, plant, harmony, off-taste). Obtained results showed that AJ with addition of plant extracts in lower concentrations (0.5, 1.0 and 1.5%) were more acceptable than those in higher concentrations (2.0 and 2.5%). Thyme and wild thyme extracts were found to be the most suitable for formulation of functional apple juice due to highest sensory scores for several desirable sensory properties.

Keywords: Apple juice; plant extract; sensory; medicinal and Mediterranean herbs

INTRODUCTION

Human awareness on functional foodstuffs consummation is rapidly growing due to the numerous scientific studies that confirm the positive effect of these foods on human health. Moreover, the consumption of foods or beverages enriched with plant extracts has been reported to have many positive effects on human health, especially concerning prevention of cardiovascular diseases and certain types of cancer [1]. Herbs are good source of phytochemicals and their use in food preservation as a natural preservatives is already known [2, 3]. Aside from the nutritive, biological and preservative impact of plant extract, sensory evaluation represents another important factor that may affect future purchase (choosing) decisions within consumers [4]. Recent study aimed to investigate the influence of green tea extracts (1, 2 and 3 g/L) in cloudy apple juice as an effective anti-browning agent [5]. Authors concluded that sensory perception of all samples were satisfactory. Application of phenolic extracts from three different plants in pineapple juice was previously evaluated. Authors confirmed that the plant extracts can be used to improve the biological quality and safety of foods without significant impact on sensory perception [6]. Another study found the addition of bergamot, which is cultivated only to produce essential oils, is an excellent natural antioxidant for formulation of functional fruit juices. For instance, the addition of bergamot juice (10% or 20%) could replace the synthetic additives usually used in the industrial process (ascorbic acid and citric acid) while a preliminary consumer test encouraged the production of bergamot fortified fruit juices [7]. Apple, blueberry and cranberry juice was enriched with aqueous-extracts of ginger, selected amino acids, vitamins and minerals for creation of a cardio-protective functional beverage [8]. Sensory evaluation was performed to select the best combination of fruit blend as the basis of a functional beverage. Most favourable sensory scores were given to the blend containing 37.5% apple, 50% blueberry and 12.5% cranberry juices. Descriptive analysis of taste and odour of selected fruit juice blend with different levels of ginger (0, 1, 2, and 3%) revealed that up to 2% ginger extract can be used without compromising consumer perception [8].

Apple fruit *(Malus* sp., Rosaceae) is cultivated worldwide and consumed either fresh or processed. In particular, apple juice (AJ) is one of the most popular and widely available fruit juices due to its health benefits and nutritional value [9, 10]. Apples are a rich source of dietary phytochemicals including flavonoids (60% of all polyphenols) and phenolic acids (30% of total polyphenols) therefore represent good basis for functional food production. On the other hand, herbs such as elderberry (*Sambucus nigra*),

tilia (*Tilia cordata*), common thyme (*Thymus vulgaris*), Breckland thyme (*Thymus serpyllum*) and lemon balm (*Melissa officinalis*) are the most commonly growing and using medicinal and Mediterranean Herbs. In a line with this, the aim of the present study was to investigate sensory acceptance of apple juice enriched with above-mentioned plant extracts.

MATERIALS & METHODS

2.1. Plant extracts

Elderberry (*Sambucus nigra*), tilia (*Tilia cordata*), common thyme (*Thymus vulgaris*), Breckland thyme (*Thymus serpyllum*) and lemon balm (*Melissa officinalis*) were obtained from a local producer (Suban Ltd., Strmec, Croatia) and the botanical identification was carried out in cooperation with Faculty of Agriculture, University of Zagreb, Zagreb, Croatia. Prior to the extraction, dried plants were grinded to a fine powder using electrical grinder (Dolcevita CG1, Imetec, Azzano San Paolo, Italy). In case of elderberry and tilia only flowers were used, for common thyme and Breckland thyme flowers and leaves were used and for lemon balm only leaves were used.

5 grams of each herb was weighted, filled in round-bottomed flask and filled up with water to 250 mL and hand-homogenized for few minutes. Extraction was done by reflux heating for 30 minutes. Afterwards, extracts were cooled to room temperature, filtered and used for apple juice enrichment. Each extract was directly mixed into apple juice in concentrations: 0.5, 1.0, 1.5, 2.0 and 2.5%. Apple juice was prepared by dilution from concentrated juice obtained from company Dona d.o.o. (Gornja Stubica, Croatia). Concentrated juice of 65 °Brix was diluted with distilled water to 12 °Brix using a digital handheld refractometer (ATAGO, PAL) and used as basis further for sample preparation. All samples were prepared in triplicate.

2.2. Sensory evaluation and data analysis

Sensory evaluation was based on previously report [11] with minor modifications. A quantitative descriptive analysis (QDA) was used in order to evaluate acceptability and suitability of apple juice enriched with herbal aqueous extracts. Fifteen trained panellists (8 females and 7 males; 22 to 45 years of age) from Faculty of Food Technology and Biotechnology, Zagreb, Croatia were chosen based on their experience, sensory acuity and ability to distinguish small sensory attributes differences. Descriptive terms used were colour (intensity), aroma (apple, plant, cooked, strange), odour (apple, plant, strange), and taste (acidic, sweet, apple, plant, harmony, off-taste). Evaluation was done in two parallels and scoring was on a scale from 0 (having none attribute) to 10 (having highest attribute).

PCA was performed using auto-scaled data to aid interpretation and for rapid screening [12].

RESULTS & DISCUSSION

QDA was performed via 14 sensory descriptors and spider web plots of QDA results were given in Figure 1 A-E. As expected, reference sample (AJ) had the most expressed apple aroma, apple odour and apple taste and the addition of plant extracts resulted in lower expression of these attributes. In particular, the higher concentration of plant extract was added, the lower expression of above-mentioned attributes was observed. Among all AJ with added plant extracts, sample with 0.5% lemon balm extract was recorded as the most expressed for the apple taste, followed by tillia extract (1.0%), elderberry extract (1.5% and 2.0%) and wild thyme extract (2.5%). With respect to apple aroma, addition of elderberry extract was scored highest for all the concentration. Similarly, regardless of added amount, apple odour was the most pronounced in AJ with added wild thyme extract.

Regarding plant aroma, plant odour and plant taste, even at the lowest extract addition of 0.5% was detected by sensory panel. AJ with thyme extracts (0.5-2.5%) was characterized as samples with the most expressed plant odour and plant aroma. With increased concentration of plant extract, plant taste was more expressed whereas AJ with thyme extract at 0.5%, 1.0% and 2.0% were characterized as samples with the highest expressed plant aroma. On the other hand, elderberry plant extract in concentrations of 1.5% and 2.5% was shown as the most expressed in plant taste. Moreover, with no influence of concentration, tillia extracts addition in AJ showed the lowest scores for the plant taste, aroma and odour.

Interestingly, it was found that increased plant extract concentration (0.5% *vs*. 2.5%) resulted in lower expressed acidic taste. In response to sweetness, increasing concentration of plant extract led to

increasing sweet taste perception for AJ with thyme, elderberry and tillia. In the case of lemon balm, higher addition of extract resulted in lower sweetness while for wild thyme it remained unchanged. Several reports confirmed that good ratio between sweetness and acidity is the key parameter in apple juice sensory quality determination [13, 14]. AJ with 1.5% plant extracts (wild thyme, thyme, elderberry) was highly characterized as harmonious, while AJ with wild thyme extract at 0.5% and 1% were recognized as the most harmonious samples. Undesirable descriptors such as: cooked aroma, strange aroma and off-taste were not observed by sensory panel therefore plant extract addition had no considerable influence in terms of unpleasant aroma and taste.

Results of sensory evaluation were summarised and displayed by Principal Component Analysis (PCA) to explore the inter-relationship between all prepared samples and sensory parameters; in other words, which sensory attributes were characteristic in describing the sensory profile of the samples investigated. The variables were 14 sensory attributes and the cases were AJ samples with added plant extracts. The first two factors (PC1 and PC2) represent 63.21 % of the initial data variability (Fig. 2). First principal component separated samples considering the type of plant extract and second principal component separated samples considering the level of plant extract addition. Hence, samples of AJ with added thymus (TH) and wild thymus (WT) extracts were grouped and characterized by colour intensity and sweet taste. The similar results were confirmed by QDA. AJ was positioned strongly on the left and as expected highly characterized by apple aroma, apple odour and apple taste.

Generally, sensory panel was able to detect sensory changes between samples with 0.5, 1.0 and 1.5% plant extract addition. Uneven sensory scores were perceived for AJ with added extracts in higher concentrations (2.0 and 2.5%). Therefore, it can be concluded that addition up to 1.5% of each plant extract to AJ could be considered as an optimal to achieve good sensory properties. Based on all observed, thyme and wild thyme extracts seemed be the best choice for addition in AJ since harmony taste was at highest, apple and plant aroma were good, and taste and odour scores did not significantly differ. Also, wild thyme is a good choice as it was previously shown that extract from Thymus vulgaris L. leaves positively affects human health [15] due to rich polyphenolic profile [16].

CONCLUSION

AJ with addition of plant extracts in concentrations of 0.5, 1.0 and 1.5% were scored as sensory more acceptable than those with plant extracts in concentrations of 2.0 and 2.5%. Thyme and wild thyme extracts found to be the most appropriate for formulation of functional apple juice due to highest sensory scores for several desirable sensory properties. Plant extracts of tilia, elderberry and lemon balm could be used as a valuable addition to apple juice, but only in concentrations above 1.5%.

Acknowledgment: This work was supported by grant from the Croatian Science Foundation: ‘‘Application of innovative technologies for production of plant extracts as ingredients for functional food’’ (HRZZ 3035)’’.

REFERENCES

1. Vauzour, D., et al., *Polyphenols and human health: Prevention of disease and mechanisms of action.* Nutrients, 2010. 2(11). 1106-1131.
2. Gottardi, D., et al., *Beneficial effects of spices in food preservation and safety.* Frontiers in Microbiology, 2016. 7.
3. Tapsell, L.C., et al., *Health benefits of herbs and spices: the past, the present, the future.* The Medical Journal of Australia, 2006. 185(4). 1-24.
4. Piskernik, S., et al., *Control of Alicyclobacillus spp. vegetative cells and spores in apple juice with rosemary extracts.* Food Control, 2016. 60. 205-214.
5. Klimczak, I. and A. Gliszczyńska-Świgło, *Green tea extract as an anti-browning agent for cloudy apple juice.* Journal of the Science of Food and Agriculture, 2017. 97(5). 1420-1426.
6. Arabshahi-Delouee, S. and A. Urooj, *Application of phenolic extracts from selected plants in fruit juice.* International Journal of Food Properties, 2007. 10(3). 479-488.
7. Pernice, R., et al., *Bergamot: A source of natural antioxidants for functionalized fruit juices.* Food Chemistry, 2009. 112(3). 545-550.

8. Gunathilake, K.D.P.P., H.P.V. Rupasinghe, and N.L. Pitts, *Formulation and characterization of a bioactive-enriched fruit beverage designed for cardio-protection.* Food Research International, 2013. 52(2). 535-541.
9. Tu, S.-H., L.-C. Chen, and Y.-S. Ho, *An apple a day to prevent cancer formation: Reducing cancer risk with flavonoids.* Journal of Food and Drug Analysis, 2017. 25(1). 119-124.
10. Candrawinata, V.I., et al., *From apple to juice - The fate of polyphenolic compounds.* Food Reviews International, 2013. 29(3). 276-293.
11. Bursać, D., et al., *The influence of cultivar on sensory profiles of fresh and processed strawberry fruits grown in Croatia.* Flavour and Fragrance Journal, 2007. 22(6). 512-520.
12. Granato, D., et al., *Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: A critical perspective.* Trends in Food Science & Technology, 2018. 72. 83-90.
13. Jaros, D., et al., *Single-cultivar cloudy juice made from table apples: an attempt to identify the driving force for sensory preference.* European Food Research and Technology, 2009. 229(1). 51-61.
14. McLellan, M. and O. Padilla-Zakour, *Juice Processing.* 2004.
15. Pacifico, S., et al., *A polyphenol complex from Thymus vulgaris L. plants cultivated in the Campania Region (Italy): New perspectives against neuroblastoma.* Journal of Functional Foods, 2016. 20. 253-266.
16. Sonmezdag, A.S., H. Kelebek, and S. Selli, *Characterization of aroma-active and phenolic profiles of wild thyme (Thymus serpyllum) by GC-MS-Olfactometry and LC-ESI-MS/MS.* Journal of Food Science and Technology, 2015. 53(4). 1957-1965.

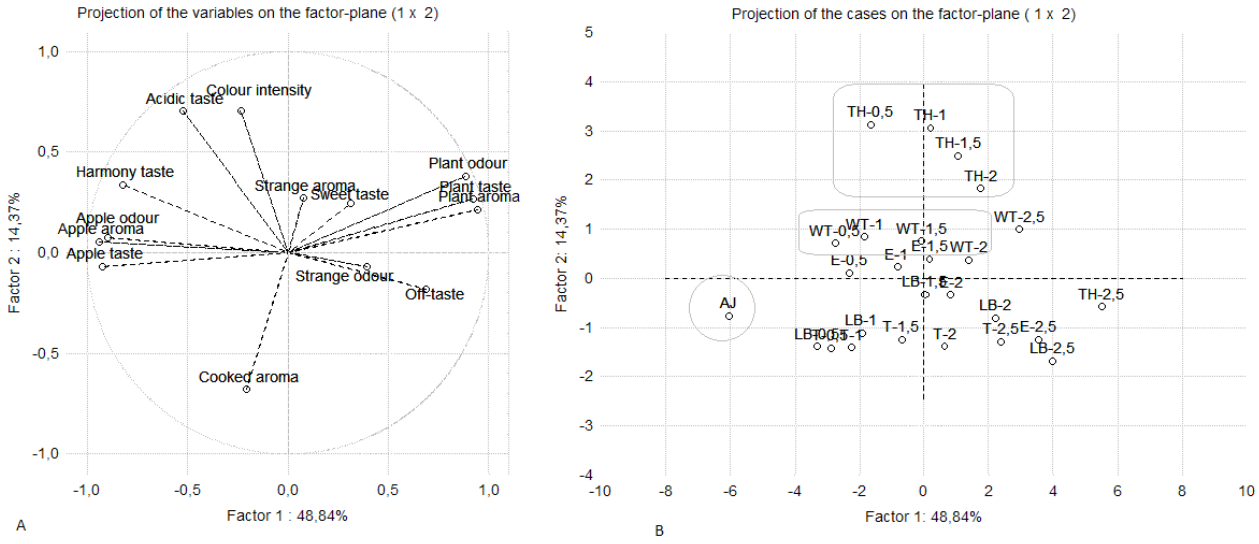


Fig. 2 (A) Principal components analysis (PCI *vs.*PC2) and (B) Loading plot (PCI *vs.* PC2) of all samples and all variables. Sample labels for score plots: AJ, apple juice; 1, 1.5, 2, 2.5, concentrations of added extracts; TH, thymus; WT, wild thymus; T, tilia, LB, lemon balm; E, elderberry

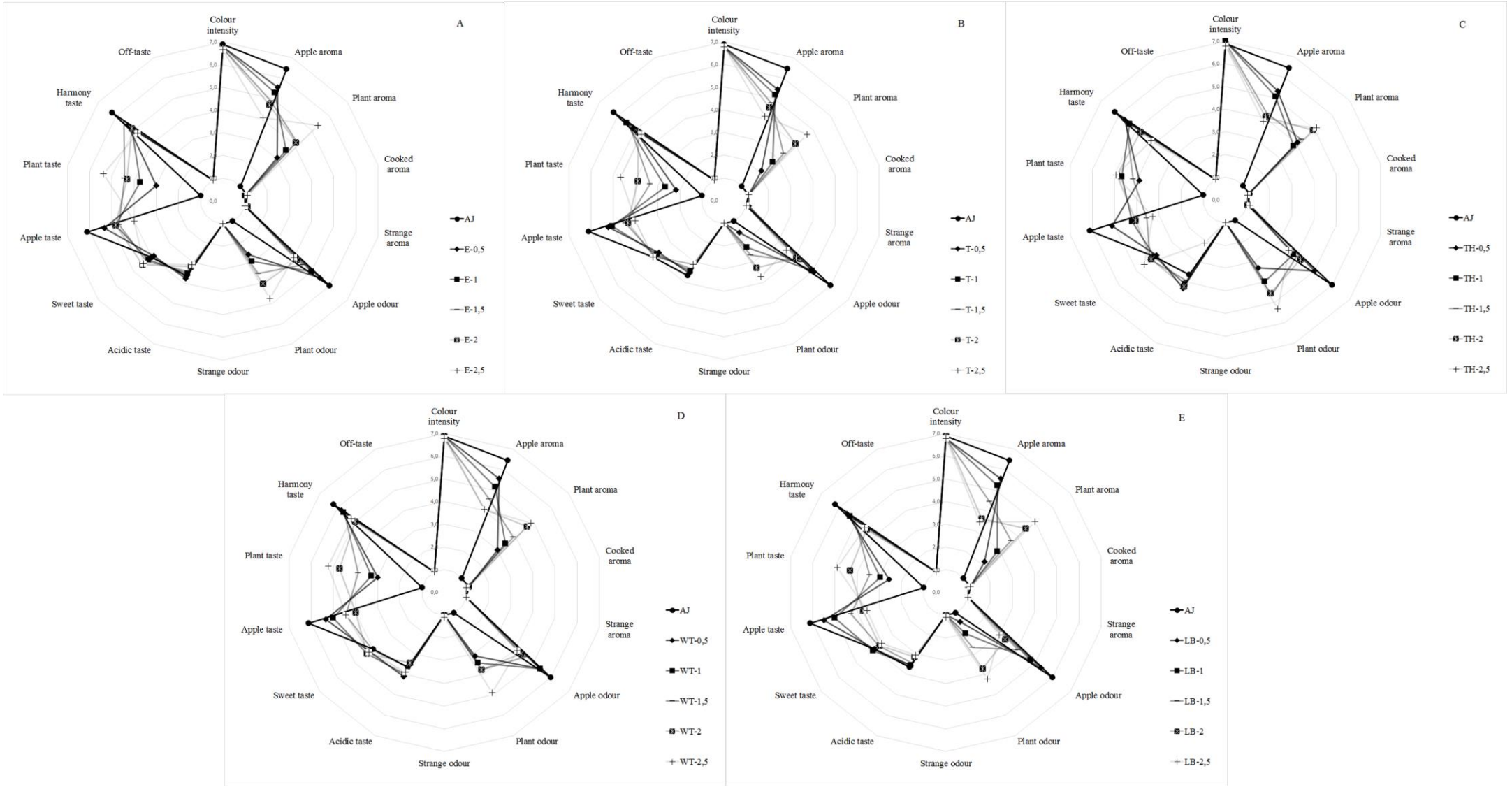


Figure 1. Spider's web plot for apple juices (AJ) with addition of five plant extracts in different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%)

The effect of lipid oxidation on color characteristics of sous-vide cooked mackerel during chilled storage

Janna Cropotova[a], Revilija Mozuraityte[b], Inger Beate Standal[b], Turid Rustad[a]

[a]*Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway (janna.cropotova@ntnu.no, turid.rustad@ntnu.no)*
[b]*SINTEF Ocean, Trondheim, Norway (revilija.mozuraityte@sintef.no)*

ABSTRACT
The aim of the present study was to assess the influence of different sous-vide time-temperature regimes and the use of two types of antioxidants (Fortium TR25 and Fortium RPT40) on lipid oxidation products and their contribution to color parameters of Atlantic mackerel (*Scomber scombrus*) during chilled storage. Atlantic mackerel was sous-vide cooked under four process regimes (for 10 and 20 min at 70°C and 80 °C) and then stored on ice for 1, 3, 9 and 15 days at 0±1°C before analysis. Lipid oxidation products and color parameters of sous-vide cooked mackerel samples were determined. Muscle lipids extracted by chloroform showed an increase in conjugated dienes, trienes and tetraenes, as well as browning development with an increase in temperature of cooking and duration of chilled storage. Sous-vide cooking time had the lowest contribution to the formation of lipid oxidation products, whereas the increase of cooking temperature led to significant accumulation ($p<0.05$) of these products. This parameter also affected color parameters of the fish flesh, leading to an increased yellowness. The redness of the fish flesh was correlated with brown compounds generated from secondary products of lipid oxidation during chilled storage of sous-vide cooked mackerel.
The obtained results have shown that temperature of cooking has the most significant effect on formation of brown compounds from secondary products of lipid oxidation occurring in sous-vide cooked mackerel.

Keywords: lipid oxidation; color parameters; sous-vide cooking; Atlantic mackerel; chilled storage

INTRODUCTION

Fish lipids are known to be rich in essential long-chain omega-3 fatty acids – docosahexaenoic acid (DHA) and eicosapantaenoic acid (EPA) possessing many health benefits, including the prevention of cardiovascular diseases (Kinsella, 1987). However, fish cooking and processing at high temperatures affects polyunsaturated fatty acids, leading to the formation of primary and secondary products of lipid oxidation, which may further generate brown (Pokorny, 1981) and fluorescent compounds (Aubourg, 1998), as well as off-flavour and the loss of essential nutrients (Karre et al., 2013).
Lipid oxidation is a crucial factor limiting both the sensory and nutritional quality and consumer acceptability of fish products. Fish becomes unacceptable to the consumer once a certain degree of rancidity is attained (Larsson and Undeland, 2010). However, lipid oxidation is a complex process, depending on not only the chemical composition of fish, the presence of light and oxygen, but also on the processing and storage conditions (Jacobsen, 1992; Larsson and Undeland, 2010). Lipid oxidation at later stages generates a number of detrimental compounds affecting both the sensory (color, flavor and taste) and nutritional quality of the product (Karre et al., 2013). Nevertheless, it may be reduced or inhibited by the addition of antioxidants, thereby ensuring improved product quality and extended shelf life (Shah et al. 2014).
Another promising approach to retard lipid oxidation in fish products is to use less heat treatment, such as sous-vide cooking. This technology is currently gaining more and more attention from both consumers and catering industries worldwide due to promising quality benefits and increased shelf life of the treated products (Baldwin, 2012). This method implies the use of lower heating temperatures (below 100°C) and cooking in vacuum packaging compared to traditional cooking procedures followed by a rapid cool-down to 0-4°C and subsequent chilled storage. Sous-vide cooking is capable to ensure high quality of the product due to shortage of oxygen inside the vacuum-pack, while retaining more of its nutritional and sensory properties compared to other conventional cooking procedures (Baldwin, 2012).
The main objective of the present study was to investigate the effects of different sous-vide cooking regimes, use of antioxidants and chilled storage on the extent of lipid oxidation in Atlantic mackerel (Scomber scombrus). The study also aimed at establishing a relationship between the changes in

secondary products of lipid oxidation and color parameters of sous-vide cooked mackerel fillets during chilled storage.

MATERIALS & METHODS

Sample preparation and sous-vide cooking

The Atlantic mackerel fillets (*Scomber scombrus*) used as materials in the present study were supplied frozen from Pelagia A.S. (Selje, Norway) in January 2018. The fish fillets were thawed at 0°C overnight prior to the experiment. Two fillets were placed in BST/SR bags and heat-sealed using a vacuum sealing machine (Webomatic Vacuum packaging system, Super max, 3000 sensor). The antioxidant-treated and untreated fish fillets were sous-vide cooked at 70°C and 80°C for 10 and 20 min, followed by a rapid chilling with solid ice and subsequent chilled storage at 0±1°C for 1, 3, 9 and 15 days. Two types of antioxidants were used: Fortium TR25 (rosemary extract) and Fortium RPT40 (ascorbyl palmitate), (Kemin Food Technologies, Herentals, Belgium). In each sampling day, the chilled mackerel samples were taken out of the vacuum packages and analysed. Mackerel fillets treated with Fortium RPT40 and subjected to sous-vide cooking at 70°C and 80°C for 20 min were analysed just in the first and last day of chilled storage.

Measurement of lipid oxidation products

Lipids were extracted from the fish muscle by the Bligh and Dyer (1959) method, and chloroform extracts of lipids were used for determination of lipid oxidation products. A modification of methods by Aubourg (1998) and Mozuraityte et al. (2017) was used for the measurement of conjugated dienes (CD), trienes (CT), tetraenes (CTr) and browning color formation (BCF) compounds. Briefly, 200 uL of the obtained lipid extracts were diluted with the solvent (1:2–1:8, v/v) if the absorbance exceeded 0.8 AU, and absorbance at 233, 268, 315 and 420 nm was measured against the solvent with a plate reader (TECAN, Austria) for determination of CD, CT, CTr and BCF compounds, respectively.

The amount of the lipid oxidation products (CD, CT, CTr and BCF compounds) was calculated according to Aubourg (1998), as follows:

$$CD/CT/CTr/BCF = A \cdot V/w \quad (1)$$

where A is the absorbance reading, V denotes the volume (ml) of the chloroform extract and w is the mass (mg) of the lipid material (oils) in the extract measured.

The results were expressed as CD, CT, CTr and BCF values in ml/mg. The analysis was performed in four replicates for each sample, and the average with standard deviation was calculated.

Color parameters

Color parameters of the fish fillets were measured instrumentally using a Minolta Chroma meter CR-400 (Konica-Minolta, Osaka, Japan). Before starting the analysis, the instrument was calibrated with a standard white plate. The measurements were performed on preselected locations at the surface of each mackerel fillet at room temperature. The data were recorded in color coordinates of a* (redness >0, greenness <0), and b* (yellowness, b* >0, blue <0) according to the Commission Internationale de l'Éclairage (CIE) Lab scale. Color parameters were determined on each samples in three readings and the average was calculated.

RESULTS & DISCUSSION

Lipid oxidation products

Results showing accumulation of CD, CT, CTr and BCF in sous-vide cooked mackerel during chilled storage are displayed in Figures 1-4. Although, the results of conjugated dienes, trienes and brown-forming compounds displayed a large variation of values, there was an insignificant increase ($p<0.05$) in these lipid oxidation products after sous-vide treatment at 80°C in comparison with cooking at 70°C. This tendency may be due to the fact that under elevated temperatures lipids undergo thermo-oxidation, polymerization, hydrolysis, cyclization, and isomerization leading to formation of hydroperoxides and conjugate di-, tri-, and tetradiene groups (Lalas, 2009).

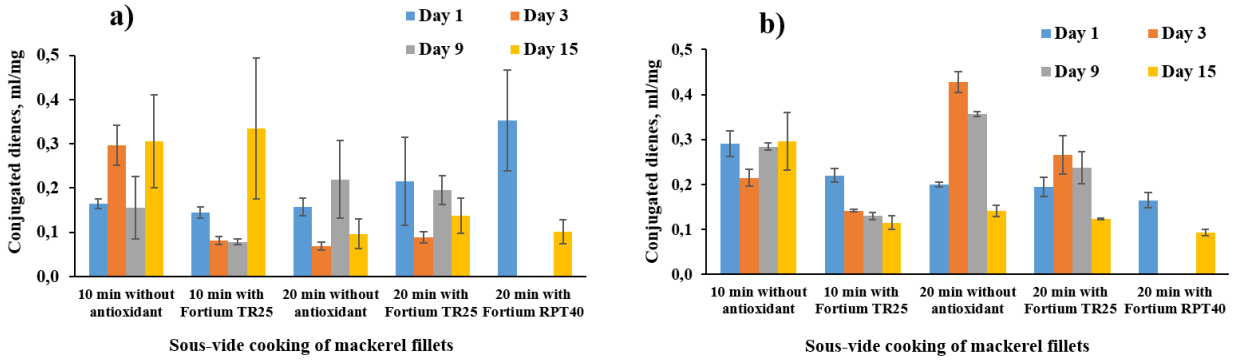


Figure 1. Conjugated dienes formation during sous-vide cooking of Atlantic mackerel at a) 70°C and b) 80°C

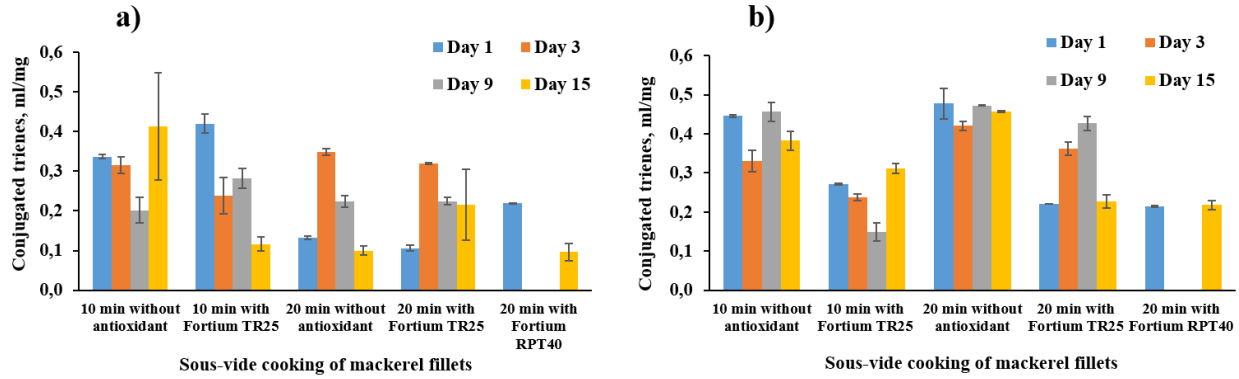


Figure 2. Conjugated trienes formation during sous-vide cooking of Atlantic mackerel at a) 70°C and b) 80°C

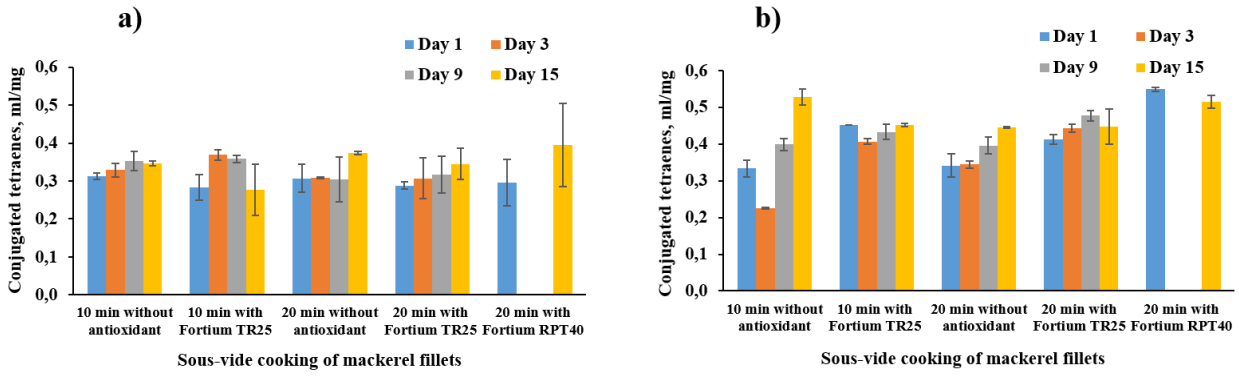


Figure 3. Conjugated tetraenes formation during sous-vide cooking of Atlantic mackerel at a) 70°C and b) 80°C

Conjugated tetraenes gradually increased both with an increase in cooking temperature and duration of subsequent chilled storage for all sous-vide mackerel fillets (Figure 3). According to our hypothesis, chilled storage promoted lipid oxidation at later stages, resulting in the formation of conjugated tetradienes during oxidation of unsaturated fatty acids containing two or more double bonds to achieve a more stable radical (Choe & Min, 2007).

Also, according to Figures 3-4, the highest oxidation rate at latest stages of lipid oxidation is observed for mackerel samples subjected to higher cooking temperature and prolonged chilled storage. The use of antioxidants did not have a significantly positive effect on the decrease of analyzed lipid oxidation products in sous-vide cooked mackerel samples.

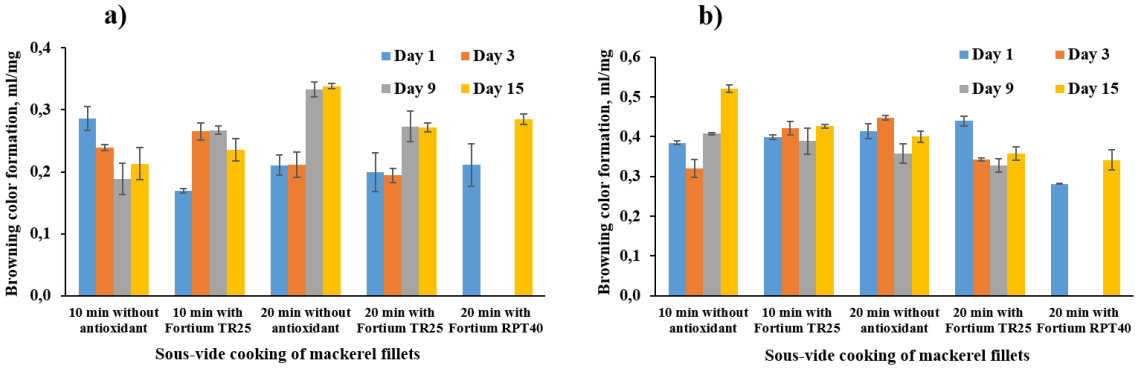


Figure 4. Browning color formation during sous-vide cooking of Atlantic mackerel at a) 70°C and b) 80°C

Color parameters

Yellowness (b*-values) was slightly higher in all sous-vide treated samples throughout the storage period compared to initial raw mackerel fillets (10.04±0.94). This is in agreement with previous study of Karoui and Hassoun, (2017). Due to a high variation of a*-values for sous-vide mackerel fillets among the sampling days (Figure 5), it was impossible to conclude on the tendency.

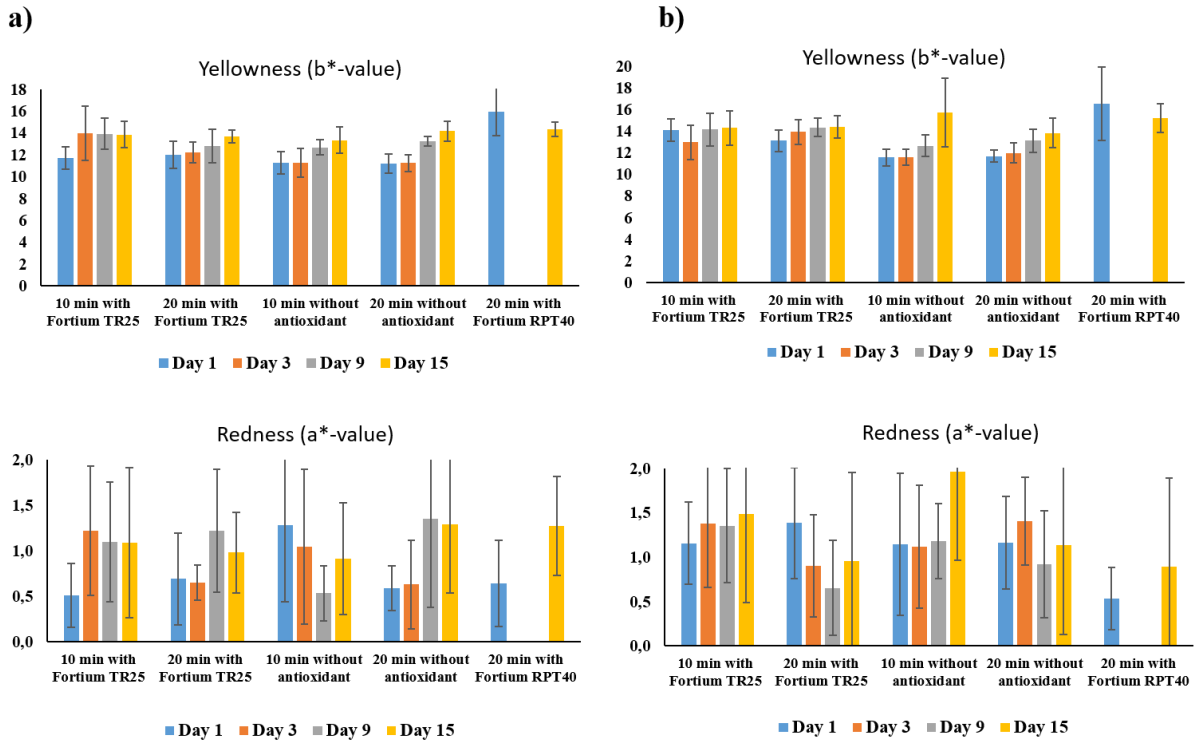


Figure 5. Yellowness and redness of mackerel flesh after sous-vide cooking at a) 70°C and b) 80°C

The increase in yellowness of sous-vide cooked mackerel throughout the storage time is most likely due to accumulation of yellowish-colored compounds generated by decomposition of primary and secondary products of lipid oxidation (Nguyen et al., 2013), giving positive correlation values (R) of 0.859 (Figure 6, a). At the same time, the generation of brown-forming compounds at latest stages of lipid oxidation partially contribute to the redness of the fish tissue (R=0.808), (Figure 6, b).

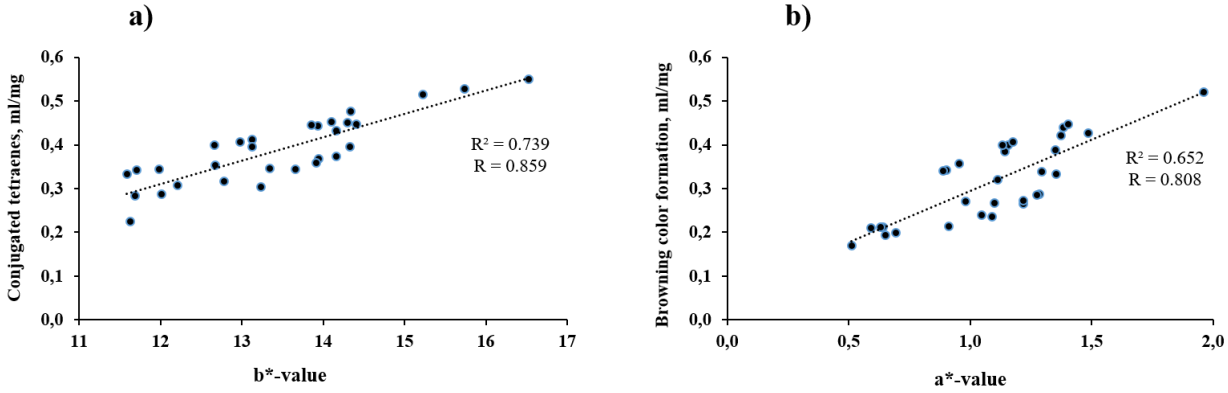


Figure 6. Relationship between a) conjugated tetraenes and yellowness (b*-value) and b) brown-forming compounds of lipid oxidation and redness (a*-value) of sous-vide mackerel fillets during chilled storage

CONCLUSION

The present study demonstrated how different sous-vide regimes, use of antioxidants and chilled storage influenced the generation of lipid oxidation products affecting color parameters of Atlantic mackerel. It was revealed that the addition of antioxidants did not have any significantly positive effect on the decrease in the oxidation development in the cooked fish during chilled storage. Temperature of cooking had the highest influence on accumulation of conjugated dienes, trienes, tetraenes and brown color-forming compounds, whereas heating time had the lowest contribution to lipid oxidation development and color modification in sous-vide cooked mackerel during chilled storage.

REFERENCES

Aubourg S.P. 1998. Lipid changes during long-term storage of canned tuna (Thunnus alalunga). Zeitschrift für Lebensmittel-Untersuchung und -Forschung NLM 206, 33–37.

Baldwin D.E. 2012. Sous Vide cooking: A review. International Journal of Gastronomy and Food Science 1, 15-30.

Bligh E G & Dyer W J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Choe E.& Min D.B. 2007. Chemistry of deep-fat frying oils. Journal of Food Science 72, 77–86.

Jacobsen C. 1992. Sensory impact of lipid oxidation in complex food systems. Lipid 101(12), 484-492.

Karoui, R. & Hassoun A. 2017. Efficiency of rosemary and basil essential oils on the shelf-life extension of Atlantic mackerel (Scomber scombrus) fillets stored at 2°C. Journal of AOAC international 100(2), 335-344.

Karre L., Lopez K., & Getty K.J.K. 2013. Natural antioxidants in meat and poultry products. Meat Science 94, 220-227.

Kinsella J. 1987. Dietary fats and cardiovascular disease. In: Lees R, Karel M (eds) Sea foods and fish oils in human health and disease, Dekker, New York. 1–23.

Lalas S. 2009. Quality of frying oil. In S. Sahin & S.G. Sumnu (Eds.), Advances in deep-fat frying of foods. Boca Raton, Florida: CRC Press. 57–80.

Larsson K. & Undeland I. 2010. Kinetics of oxidation of the lipids and proteins of cod sarcoplasmic reticulum. Journal of the Science of Food and Agriculture 90, 2531–2540.

Mozuraityte, R., Kristinova V., Standal I.B., Evensen H. and Rustad T. (2017). Applicability of traditional and advanced methods for oxidative quality and stability on marine phospholipids. European Journal of Lipid Science and Technology, 119, 1600103.

Nguyen M.V., Arason S., Thorkelsson G., Gudmundsdottir A., Thorarinsdottir K.A. & Vu, B.N. 2013. Effects of added phosphates on lipid stability during salt curing and rehydration of cod (Gadus morhua). Journal of the American Oil Chemists' Society 90(3), 317-326.

Pokorny J. 1981. Browning from lipid-protein interaction, Progress in Food & Nutrition Science, 5, 421-428.

Shah M.A., Bosco S.J.D. & Mir S.A. 2014. Plant extracts as natural antioxidants in meat and meat products. Meat Science 98, 21-33.

Application of The HRMA technique for detecting 9 forbidden animals in halal food products for Halal Food Safety in Thailand

Anat Denyingyhot, Monruedee Khemtham, Vanida Nopponpun and Winai Dahlan

The Halal Science Center, Chulalongkorn University, Bangkok, Thailand (arnat.hsc@gmail.com)

ABSTRACT

In general, food safety is a scientific discipline that aims to detect and prevent the contamination of food with hazards such as physical, chemical and biological substances but for Muslim consumers, surveillance of contamination of prohibited substances under Islamic law, especially forbidden animals is a must as one of the other hazards which directly affect not only to the physical health but also to the spiritual health of the Consumers. Importantly, High resolution melting analysis (HRMA) is a highly molecular technique for the detection of specific types of organisms and quickly, based on the melting temperature (Tm) of the DNA that is specific to the organisms. Hence, this research aims to use a HRMA technique as a tool for halal food safety to detection of contamination of nine forbidden animals in halal foods, namely, pigs, dogs, rats, cats, monkeys, frogs, snakes, crocodiles and donkeys from food samples. The results showed that the HRMA technique was applied to detect the contamination of 300 commercial products of Thailand. It was found that two samples were contaminated with pigs' DNA in the examined products and no DNA of other forbidden animals was detected in any commercial products. Therefore, the HRMA could be used as a high-potential technique for detecting the forbidden animals contaminated in foods and representing the Halal food safety for Muslim consumers.

Keywords: Halal food; Food Safety; Muslim consumer; Forbidden animal; HRMA

INTRODUCTION

Food authenticity is a very important issue, not only for food quality control reason but also for religious reason, especially for halal issue. Halal in an Arabic word means "permit" or "lawful" according to Islamic regulations, not only for food but non-foods and services as well (Eliasi & Dwyer, 2002). Therefore, halal food means any foods permissible to be consumed by Muslims. Currently, the global Muslim population is expected to grow roughly 1.84% per year. It is estimated that the number of Muslims around the world is 2.08 billion people in 2017. Consequently, the halal food market has sharply increased, the trade in halal products is predicted approximately 20% of the global food market and will reach US $ 5.3 trillion by the end of 2016 (Jafari and Scott, 2014). However, Halal food trade is quite strict because there are numerous halal food regulations. For example, halal food cannot contain anything considered unlawful under Islamic law, especially forbidden animals (Regenstein, Chaudry, & Regenstein, 2003)

From Islamic regulations, it indicates that various kinds of forbidden animals would not appear in Halal food such as pigs, dogs, rats, cats, reptiles, and others. The recently survey found that these animals have possibly mix and adulterated with qualified halal food to decrease the processing cost, which has occurred found in Thailand and other countries such as Vietnam, Indonesia and China (Dahlan, 2013; Ali et al., 2015). Despite Thailand amongst the major food producers exporting varieties of food products to the world as well as to Muslim countries, almost all food manufacturing proprietors and producers are non-Muslims. No matter how strict they comply with international food safety standards, the spiritual safety to protect faith and belief of Muslims has yet to be widely and clearly understood and often overlooked, which might result in contaminating of forbidden composition regarding Islamic regulations from misunderstood or intended to produce halal products.

In this study we aim to apply the High resolution melting analysis (HRMA) which is one of DNA-based PCR method that allows genotyping and fingerprinting by discrimination separation DNA sequence variant such as single nucleotide polymorphisms (SNPs) and small insertion and deletions (indels) based on the shape of melting transitions (Tm) of real-time PCR. This method can also be applied for simultaneous detection of the nine forbidden animals composed with pigs, dogs, cats, rats, monkeys, donkeys, frogs, snakes, and crocodiles that have high opportunity to be adulterated in Halal food Safety in Thailand.

MATERIALS & METHODS

Among the nine target forbidden animals pigs (n=4), dogs (n=4), cats (n=4), rats (n=4), monkeys (n=4), donkeys (n=4), frogs (n=4), snakes (n=4), and crocodiles, were collected from various markets, zoological park organization of Thailand and the Faculty of Veterinary Science, Chulalongkorn University. Then, all samples were DNA extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Finally, the extracted DNA concentrations were examined by a Nanodrop 2000 (ThermoScientific, USA). The quantified DNAs were stored at -20 °C until use.

All nine of species-specific primer pairs used in this study are listed in (Table 1). Verification of the specificity of each primer pair was performed using NCBI BLAST (http://www. ncbi.nlm.nih.gov/tools/primer-blast/).To develop technique, HRMA were carried out by using a LightCycler®480 instrument (Roche, Germany). In a total volume of 20 µL containing 20 ng of genomic DNA , 10 µL 2XQIAGEN Multiplex PCR (Qiagen, Hilden, Germany) 0.2 µM of forward and reverse primer, and 1x LightCycler ResoLight dye. The amplification condition was 1 cycle of 5 min at 94°C; 29 cycles of 30 sec at 94°C; 40 sec at 56°C; 1 min at 72°C followed by HRMA ramping from 60°C to 99°C with 50 acquisitions. PCR products were analyzed by QIAxcel Capillary Electrophoresis system (Qiagen, USA).The HRMA developed in this study was validated for its specificity and reliability. In terms of the specificity test, the assay was cross tested with all nine target species and four other animals (horses, chickens, sheep, and goats). Finally, for real-world performance testing, the HRMA was used to test 300 Halal food products, including 140 meat processing products, 65 dairy products, 20 seasonings, 35 snacks, and 40 bakery products.

Table 1. Details of primer sequence, Tm °C

Species	Primer	Sequence (5' to 3')	Tm °C
Pig	PG-F	CCATCCCAATTATAATATCCAACTC	81
	PG-R	TGATTATTTCTTGGCCTGTGTGT	
Dog	DG-F	GAGGTGCGGAAGCGGAGGGGCGGGGGCTCTAGCCGTTCGAT	85
	DG-R	GTGATAAAAGCTGTGGTCG	
Cat	CT-F	TTATGGCTGAATCATCCGATATT	79
	CT-R	CCGTCCCACATGTATGTACAGG	
Rat	RT-F	TAACCACTCCTTCATCGACCTT	83
	RT-R	CCCCGTTGGCGTGTAAATA	
Monkey	MK-F	AGGGGCGGGCCTCCAACAAATACTAGCC	87
	MK-R	TCTATGGCAGAAGGTAGT	
Donkey	DK-F	TTCAAGCTCAACGTCACACA	77
	DK-R	GCTTCTATGTGTCGATAGAATAGTCC	
Frog	FG-F	CACGAAGGTTATACTGTCTCCT	79.5
	FG-R	CTGAGGTAAATGATGATTCTGG	
Snake	SK-F	AAGGTTGCGTAATCATTTG	85.5
	SK-R	GAAAGTGGCCATTATTTGGT	
Crocodile	CD-F	AAAGCATTCTGCCTACACCTGAAA	83.5
	CD-R	TTGTGTTGGCTGCTTTAAGGCCTA	

RESULTS & DISCUSSION

Food safety is one of the important key factors that must be taken into consideration by consumers before buying foods in order to prevent from any risk of biological, chemical and physical foodborne. The contamination of prohibited substances under Islamic law especially forbidden animals is a serious concern for Muslim consumers. Many researches have been reported that the halal food products collected from market surveillance were adulterated with forbidden meats such as pork, dogs, rats, monkeys, and others (Cai et al.,2012 ; Dahlan, 2014; Kitpipit et al., 2014 ; Ali et al., 2015 ; Yanita.I.W et al., 2015). HRMA is a molecular method that allows detecting and differentiating DNA amplicon by discriminating DNA sequence variants such as single nucleotide polymorphisms (SNPs)

and small insertion and deletions (indels) based on the specific melting behaviour of the DNA amplicon (Sakaridis et al., 2013). This method can be applied to detect adulteration of forbidden animals DNA contaminated in food products by evaluating different melting behaviours of each forbidden animal species. HRMA technique provides the same basic principles as a conventional PCR technique, which amplifies a targeted DNA of DNA in vitro. In comparison, PCR with HRMA provides several advantages than conventional PCR. It allows determining the initial number of copies of target, and the accumulation of every amplification product is measured as the reaction progresses in real time. HRMA results with accuracy and high sensitivity. In this study, The HRMA was conducted by employing 52 DNAs from 13 animals (pigs, dogs, cats, rats, monkeys, donkeys, frogs, snakes, crocodiles, horses, chickens, sheep, and goats)

Moreover, for PCR amplification using nine pair of species-specific primers (pigs, dogs, cats, rats, monkeys, donkeys, frogs, snakes, crocodiles) targeting the intra-species conserved and inter-species hyper variable regions of mitochondrial Cytb, ATP6 , ND5 and 16S gene were used to set up HRMA method. Importantly, each set of primers was evaluated the PCR specificity against the targeted DNAs of all animals. And, in each individual assay, each pair of primers was first tested against other nine species of targeted forbidden animals as non-target DNA with the other species, only specific target was testily amplified. In this study, the method highlighted uniquely melting peak that was easily distinguished for each species. To be illustrated, all species were distinguished by their species-specific fragment melting behaviour (Figure 1). Generally, it can be seen that different genotypes have their own unique transitions that are merely shown by their HRMA profile. The results significantly indicated that no cross amplification even on repetition in blind experiment. This revealed that there was high specificity of the established primers.

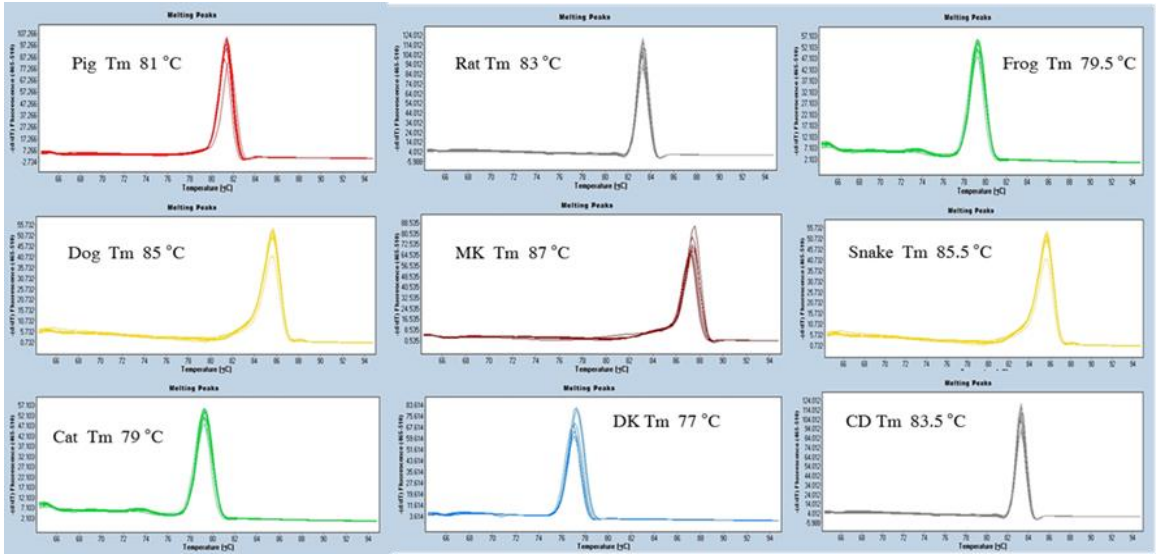


Figure 1. The HRMA profiles of amplified amplicons from species targeted animals: a melting peak

In order to carry out the analysis of commercial food products, 300 commercial food products was used to screen by the developed HRMA. The results indicated that two samples were contaminated with pigs' DNA and no DNA of other forbidden animals was detected in any commercial products (Figure 2)., suggesting its usefulness for detection of 9 forbidden animals. Therefore, the HRMA method could be used as a halal verification technique for detecting aforementioned forbidden animals contaminated in halal food products. Furthermore, the HRMA assay ultimately showed that it is simple, cheap, and rapid method, the cost per sample is comparatively lower than CE analysis (Ulca, 2015; Ali et al., 2015).

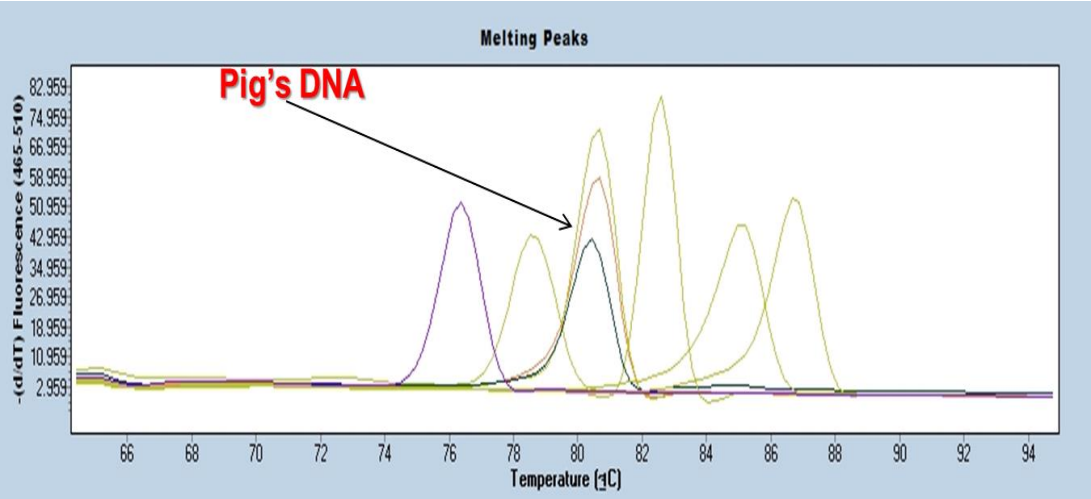


Figure 2. The HRMA profiles that show two samples were contaminated with pigs' DNA in the examined products.

CONCLUSION

The HRM method was scientifically developed in this study and it had been specifically proven in specificity, sensitivity, and reliability for detection of pigs, dogs, cats, rats, monkeys, donkeys, frogs, snakes, crocodiles DNA standards and 300 commercial food products. The assay was also significantly validated to detect these nine forbidden animal species in halal food. Therefore, this method could be potentially applied as a simple and it could be affordable to halal verification technique for detecting the forbidden contamination in halal food manufacturing.

ACKNOWLEDGEMENTS

This work is officially supported by the Halal Science Center, Chulalongkorn University and Research and Development Center, Betagro group

REFERENCES

Eliasi, J. R., & Dwyer, J. T. 2002. Kosher and Halal: religious observances affecting dietary intakes. American Dietetic Association. 101, 911-913.
Jafari, J., & Scott, N. 2014. Muslim world and its tourisms. Annals of Tourism Research, 44, 1-19.
Regenstein, et al., 2003. The Kosher and Halal Food Laws. Comprehensive Reviews in Food Science and Food Safety. 2, 111-117.
Winai Dahlan. 2013. A decade of growth the halal science center.Thana press, Bangkok, 47-59.
Ali, M. E et al., 2015. Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods. Food Chemistry, 177, 214-224
Cai, H et al., 2012. Real-time PCR assays for detection and quantitation of porcine and bovine DNA in gelatin mixtures and gelatin capsules. Journal of Food Composition and Analysis, 25(1), 83-87.
Kitpipit, T et al., 2014. Direct-multiplex PCR assay for meat species identification in food products. Food Chemistry, 163, 77-82.
Sakaridis, I et al., 2013. A fast and accurate method for controlling the correct labeling of products containing buffalo meat using High Resolution Melting (HRM) analysis. Meat Science, 94(1), 84-88.
Yanita I.W et al.,2015. Detection of Rat Meat Adulteration in Meat Ball Formulations Employing Real Time PCR. Asian Journal of Animal Sciences, 9: 460-465.

Rare Earth and Other Trace, Minor and Major Elements in Citrus Leaves

Željka Fiket[a], Martina Furdek Turk[a], Ivica Biljan[b]

[a] *Ruđer Bošković Institute, Division for Marine and Environmental Research, Zagreb, Croatia (zeljka.fiket@irb.hr; mfurdek@irb.hr)*
[b] *University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia (ibiljan@stud.biol.pmf.hr)*

ABSTRACT
In this study, the distribution of rare earth elements and other major and trace elements in leaves of different citruses was investigated. Samples included orange, lemon and tangerine leaves. All samples were analyzed for total concentration of 48 elements by HR-ICP-MS.
Studied citrus leaves showed differences in their element levels, with lemon leaves showing the highest total concentrations of majority of elements, including the highest levels of rare earth elements.

Keywords: rare earth element;, multielement analysis; citrus leaves

INTRODUCTION

Biological samples are subject of research in various environmental and food studies, in which a multiparameter approach is essential for correct assessment of the problem at hand. Namely, emissions from industry, agriculture, mining and urban activities continuously introduce large amounts of potentially toxic elements (Cd, Cr, Cu, Hg, Ni, Pb, Sn, etc.) into the environment, and consequently biota and the food chain (Babula et al., 2008). Thus, among the contaminants present, trace elements are of major environmental as well as health concern (Libes, 1992). However, one of the often overlooked groups of elements in such studies is the group of rare earths (REE).
Unfortunately, an increasing use of REE for industrial and agricultural purposes resulted in their enhanced presence in the environment. Their future application is expected to expand even more, depicting them as emerging pollutants and calling for further research to upgrade our understanding of their chemical behaviour and bioavailability (El-Ramady, 2008). So far, research on biological and toxicological effects of the REEs has been rather limited, and previous investigations suggest their both positive and negative physiological effects.
The capacity of plants or agricultural crops to accumulate REEs depends on the species and the REEs content in the substrate. Among many others, citruses were reported to readily accumulate REE especially given the long-term use of fertilizers enriched in these elements (Turra et al., 2013). So far, limited research has been done with regards to the distribution of rare earth elements in citrus systems (Turra et al., 2013). Therefore, present study aims to investigate the distribution of REEs and other major and trace elements in leaves of three different citrus species.

MATERIALS & METHODS

Sample collection and preparation

Citrus leave samples comprise of three different citrus species: orange, lemon and tangerine. For each species, five leaves were collected from the associated tree. All samples were lyophylized and subsequently stored for multielement analysis.
Digestion protocol was adapted from US EPA Method 3052 (US EPA, 1996). Prior to analysis, subsamples (0.05 g) of samples were subjected to total digestion in the microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) in a one-step procedure consisting of digestion with a mixture of 6 mL nitric acid (HNO_3, 65%, *traceSELECT*, Fluka) and 0.1 mL hydrofluoric acid (HF, 48%, *traceSELECT*, Fluka) (Filipović Marijić and Raspor, 2012).

Multielement analysis

The multi-element analysis was performed by High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS) using an Element 2 instrument (Thermo, Bremen, Germany). The instrument conditions and measurement parameters used throughout the work, as well as the standard preparation procedure have been reported previously by Fiket et al. (2017).

All samples were analyzed for total concentration of 47 elements (Al, As, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Fe, Gd, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nd, Ni, Pb, Pr, Rb, Sb, Sc, Se, Sm, Sn, Sr, Tb, Ti, Tl, Tm, U, V, Y, Yb, Zn and Zr).

Quality control

For quality control purposes, the certified reference material (CRM) Citrus leave (NCS ZC73018, China National Analysis Center for Iron & Steel) was used.

RESULTS & DISCUSSION

Obtained elemental data were validated by comparison with existing certified values for analysed reference material Citrus leave (NCS ZC73018). Reference sheet for NCS ZC73018 lists certified values for all investigated elements, including the rare earths, with exception of Zr.

In NCS ZC73018 average recoveries range from 90% to 106% for all measured elements, with exception of Be (85%) and Sb (88%). For all elements, the obtained recoveries (related to mean ± standard deviation values) displayed partial or complete overlap of the span of recovery values related to certified values for this CRM and their associated uncertainties (Figure 1). The largest standard deviations were observed for Er, Ho, Lu and Tm, elements present in the lowest concentrations (< 0.05 mg kg^{-1}) in this CRM. The certified values for Er, Ho, Lu and Tm have a very wide range of uncertainties, which is why the obtained concentrations for these elements fall in the range of certified values. The similar was noticed for Be and Sb, the elements with certified values that also have a very wide range of uncertainties, for which the obtained concentrations, despite their somewhat lower recoveries, fall in the range of certified values (Figure 1).

The obtained results confirmed the use of HR-ICP-MS as reliable method for multielement analysis of citrus leave samples.

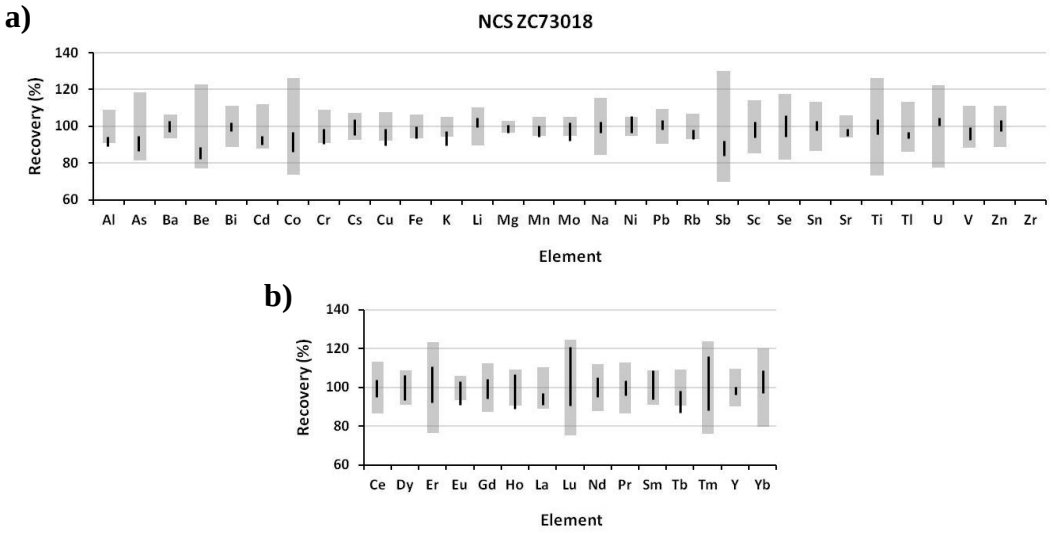


Figure 1. Comparison of measured (indicated by ▌) and certified (indicated by ▒) major, minor and trace (a) and rare earth (b) element concentrations in Citrus leave (NCS ZC73018), presented as recovery ranges related to mean element concentrations including their standard deviations and certified values with associated uncertainties.

The results of measurement of 47 elements in studied citrus leaves are shown in Table 1. The results are reported as average and standard deviation values, calculated from five samples per each species.

Table 2. Measured element concentrations (average and standard deviation), expressed as mg kg^{-1}, in different citrus leaves.

	Orange leave		Lemon leave		Tangerine leave	
Element	**Average**	**STD**	**Average**	**STD**	**Average**	**STD**
Al	132	33	170	9	78.2	6.8
As	0.125	0.035	0.075	0.016	0.050	0.003
Ba	14.9	2.7	18.4	1.8	10.2	0.7
Be	0.006	0.006	0.010	0.003	0.003	0.002
Bi	0.016	0.008	0.010	0.001	0.010	0.001
Ca	33383	4126	31913	4453	22982	908
Cd	0.009	0.002	0.014	0.009	0.009	0.001
Ce	0.145	0.038	0.461	0.051	0.074	0.008
Co	0.039	0.011	0.128	0.009	0.058	0.002
Cr	0.523	0.181	0.356	0.008	0.380	0.030
Cs	0.019	0.003	0.017	0.001	0.024	0.001
Cu	89.3	24.5	38.0	3.7	4.83	0.61
Dy	0.008	0.001	0.014	0.002	0.006	0.001
Er	0.005	0.001	0.006	0.001	0.004	0.001
Eu	0.006	0.002	0.009	0.001	0.004	0.001
Fe	122	20	108	5	114	1
Gd	0.015	0.001	0.019	0.001	0.009	0.001
Ho	0.002	0.001	0.002	0.001	0.001	0.0003
K	14777	1115	4975	751	48298	5484
La	0.082	0.020	0.583	0.070	0.038	0.004
Li	0.487	0.074	1.950	0.768	0.972	0.062
Lu	0.001	0.0003	0.001	0.0003	0.001	0.0003
Mg	1767	209	2186	272	1784	89
Mn	35.9	3.3	121	36	86.4	5.4
Mo	0.133	0.016	0.053	0.004	0.162	0.007
Na	1097	247	389	34	553	35
Nd	0.067	0.020	0.225	0.031	0.038	0.002
Ni	0.338	0.072	1.301	0.064	0.593	0.069
Pb	0.631	0.134	1.26	0.06	0.403	0.047
Pr	0.017	0.005	0.063	0.006	0.009	0.001
Rb	2.78	0.21	1.44	0.09	8.82	1.07
Sb	0.027	0.006	0.020	0.002	0.034	0.001
Sc	0.038	0.024	0.022	0.001	0.012	0.002
Se	0.031	0.015	0.044	0.024	0.032	0.014
Sm	0.013	0.004	0.034	0.003	0.008	0.001
Sn	0.364	0.025	0.591	0.052	0.224	0.030
Sr	128	16	328	36	38.0	2.003
Tb	0.002	0.001	0.003	0.001	0.001	0.0003
Ti	11.7	2.3	9.27	0.58	7.49	0.65
Tl	0.006	0.001	0.016	0.003	0.003	0.001
Tm	0.001	0.0003	0.001	0.0003	0.001	0.0003
U	0.012	0.003	0.024	0.009	0.008	0.001
V	0.217	0.059	0.220	0.019	0.112	0.009
Y	0.058	0.015	0.108	0.013	0.026	0.001
Yb	0.005	0.002	0.005	0.001	0.003	0.001
Zn	43.4	4.4	28.5	2.4	15.5	1.5
Zr	0.311	0.074	0.285	0.011	0.267	0.015

Concentrations of elements in all analysed samples ranged over eight orders of magnitude, from below the detection limit to 50 g kg^{-1} (K). Among the elements, Ca, K, Mg and Na were present at the highest levels in all measured samples (in g kg^{-1}), whereas rare earth elements (Ho, Lu, Tb, and Tm) exhibited the lowest values in all samples (<0.003 mg kg^{-1}). In average, the highest concentrations of majority of elements were observed in lemon leaves, lower in orange leaves and the lowest in tangerine leaves (Table 1).

Compared to the values reported for Citrus leave certified reference material, major and trace element concentrations in three types of citrus leave samples were up to a magnitude lower. Largest discrepancy was observed for Bi (by a factor 15), Cd (by a factor 18), Pb (by a factor 15) and Sn (by a factor 11). Only exceptions were K, Sr and Zn, for which higher concentrations, by a factor 1.2 to 14, were obtained in studied samples compared to those in NCS ZC73018. Overall highest difference (by a factor 14) was observed for Cu.

Nonetheless, order of content of measured major and trace elements corresponds to the general order of content of elements in plants (Kabata-Pendias and Pendias, 1984).

CONCLUSION

Studied citrus leaves showed differences in their element levels, with lemon leaves showing, in average, the highest total concentrations, including the highest levels of REEs. Compared to the certified reference material NCS ZC73018 there are some differences in the measured concentrations, with the highest differences observed for Bi, Cd, Pb and Sn, i.e. elements generally linked to influence of atmospheric deposition and/or vicinity of roads and traffic. Interestingly, Sr, Zn and especially Cu were highest in orange leaves, while K were at the highest in tangerine leaves, suggesting a contribution of liquid fertilizers application to their total concentrations.

REFERENCES

Babula P., Adam V., Opatrilova R., Zehnalek J., Havel L., Kizek R. 2008. Uncommon heavy metals, metalloids and their plant toxicity: a review. Environmental Chemistry Letters 6, 189–213.

El-Ramady H.R. 2008. A contribution on the bio-actions of rare earth elements in the soil/plant environment [Dissertation]. Julius Kühn-Institut, Berlin.

Fiket Ž., Mikac N., Kniewald G., 2017. Mass fractions of forty-six major and trace elements, including rare earth elements, in sediment and soil reference materials used in environmental studies. Geostandards and Geoanalytical Research, 41, 123-135.

Filipović Marijić V., Raspor B., 2012. Site-specific gastrointestinal metal variability in relation to the gut content and fish age of indigenous european chub from the Sava river. Water Air Soil Poll, 223(8), 4769-4783.

Kabata-Pendias A., Pendias. H. 2001. Trace Elements in Soils and Plants. CRC Press, Boca Raton, London, New York, Washington D. C.

Libes S. 1992. An Introduction to Marine Biogeochemistry. John Wiley & Sons, New York, 734 pp.

Merten C., Büchel. G. 2004. Determination of rare Earth elements in acid Mine Drainage by Inductively coupled Plasma Mass spectrometry. Microchimica Acta 148, 163-170.

Turra C., De Nadai Fernandes E. A., Arruda Bacchi M., Sarriés G. A., Barbosa Júnior F., Lai Reyes A. E. 2013. Rare earth elements in citrus production systems, Journal of Plant Nutrition, 36(5), 762-771.

US EPA. 1996. Method 3052: Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices, part of Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response, Washington, D.C., 20pp. Available at: http://www.epa.gov/epaoswer/hazwaste/test/pdfs/3052.pdf

Stability of Mandarin Juice upon Various Storage Temperature during 60 Days

Branka Levaj[a], Maja Repajić[a], Danijela Bursać Kovačević[a], Ivona Elez Garofulić[a], Jasmina Kušan[a], Natalija Jauk[a], Kujtesa Shabanaj[a], Ana Kodrnja[a], Vesna Bolanča[a], Verica Dragović-Uzelac[a]

[a] *Faculty of Food Technology and Biotechnology, Zagreb, Croatia (blevaj@pbf.hr)*
[b] *Second affiliation, City, Country (e-mail)*

ABSTRACT
In Croatia, mandarin is an economically important fruit mostly consumed as fresh without possibility of industrial juice processing. Considering citrus juice should not contain peel component according to legal regulation, it should be processed on specific equipment, which we do not have in Croatia as opposed to apple or other continental fruit processing equipment. The aim of this study was to examine possibility of mandarin juice processing on press usually used for apple pressing. Further, we aimed to monitor juice stability during storage at three temperatures (1 ℃, 22 ℃, 37℃) during 60 days. Therefore, physico – chemical (soluble solids, pH-value and total acidity) and sensory properties (by quantitative descriptive method), total phenolics (by Folin-Ciocalteu reagent), total carotenoids (spectrophotometrically), antioxidant activity (by FRAP method) as well as microbial stability were determined after 60 days. According to sensory evaluation, juice produced by pressing unpeeled mandarin fruit was acceptable but not high quality. Temperature and time of storage affected all investigated parameters as well as sensory properties of juices. However, changes were not very pronounced in the juices stored in 1℃. The rise of storage temperature caused undesirable juice changes. All juices during storage were microbiologically stable.

Keywords: Mandarin juice; processing; storage; temperature; stability

INTRODUCTION

Mandarin as other citrus fruit is very popular fruit due to its delicious and pleasant aroma as well as high nutritional value (Ashurst, 2005). It is well known source of valuable components as vitamin C, carotenoids or phenolics, which have been associated with antioxidant activity and health benefits (Legua et al., 2014). This fruit is mostly consumed fresh, but consumers appreciate mandarin juice, too. Thus, processing into juice may overcome its seasonal character, unsuitability for long term storage and solve the problem with excess of unmarketable fruit. In Croatia, mandarin is an economically very important fruit mostly consumed as fresh without possibility of industrial juice processing (Levaj et. al., 2005). Here, in Croatia, there are only industrial equipment for processing fruit into the juice, primarily based on pressing or pureeing fruit tissue. However, in the manufacturing of citrus juices extraction is the basic technological step. According to Legislative (EU directive, 2012), citrus juice must not contain any peel component, therefore citrus fruit must be processed on specific equipment, which is not available in Croatia. Since, mandarin juice is usually processed via juice extractor equipment, the aim of this study was to examine the possibility of mandarin juice processing by pressing technology usually used for apple pressing. As mandarin juice is very susceptible to browning (Handwerk, Coleman, 1988) and other undesirable changes during storage, the juice stability in the terms of microbial and sensory analyses, determination of total phenolic and total flavonoid content, total carotenoids, colorimetric parameters and antioxidant activity during storage at three temperatures (1℃, 22℃, 37℃) over 60 days was monitored, too.

MATERIALS & METHODS

Material. Mandarin fruit (*Citrus unshiu* Markovich), were harvested in the Neretva Valley and transported to Faculty of Food Technology and Biotechnology in Zagreb. Mandarin fruit (27 kg) was peeled by hand, chopped by sharp knife and juice were produced on small-scale equipment. Pressing was done on hydraulic pack press and pasteurization (90^{o}C/300 sec) (past.) in flow tube heat exchanger (Euclid Ltd., Croatia). Hot juice was filled in sterilized bottles (250 mL), closed and cooling down was

done by immersing bottles in cool water gradually. Juices were stored 60 days on 1°C, 22°C, and 37°C in dark place.

Methods. *Quality parameters and biological active compounds determination*

Soluble solids (SS) were determined by measuring °Brix (Atago refractometer, Tokyo, Japan), total acidity (TA) by titration with 0.1 NaOH (expressed as g citric acid/100 mL juice) and pH-value were determined by pH-meter (S20 SevenEasy, Mettler-Toledo).

Extraction was carried out by two different methods: (i) without hydrolysis with 80 % ethanol (v/v)-ethanolic extract (EE), and (ii) with hydrolysis by 1,2 M HCl in 80 % ethanol (v/v) (acidic ethanol extract (AEE) (Coseteng and Lee, 1987). Spectrophotometric determination of total phenols (TP) was conducted by spectrofotometer (UV/VIS UNICAM HELIOS β, England) using Folin-Ciocalteu reagent (Ough and Amerine, 1988) and the results are presented as equivalent of galic acid (GAE). The antioxidant capacity (AC) was determined only in ethanol extract (EE) by ferric reducing/antioxidant power (FRAP) method (Pulido et al., 2000) and the results are expressed as μmol Trolox equvalent (TE)/L. Flavonoid content (FC) were determined in AEE by diethylene glycol according to Abeysinghe et al. (2007) with modification in results expression. Absorbance were spectrophotometrically measured at 420 nm, results were calculated on hesperidin instead on rutin as a standard, and results were expressed as equivalent of hesperidin (Hesp)/100 mL. Carotenoids were extracted according to Levaj et al (2012) by petroleum ether (Park, 2007), absorbance (λ=450 nm) was spectrophotometrically measured and concentration was calculated by using extinction coefficient (2500) (Vahlquist, 1982) (1):

$$\text{Total carotenoids (as β-carotene) (mg}\times 100\text{ g}^{-1}) = \frac{A_{\lambda max} \cdot 1000 \cdot V_u}{m(g) \cdot E^{\%}_{1\,cm}} \quad (1)$$

$A_{\lambda max}$ = absorbance at 450 nm, V_u = total volume of extract (mL), $E^{\%}_{1\,cm}$ = extinction coefficient in petroleum ether for β-karoten = 2500.

CIELab color parameters was measured by Spectrophotometer CM-3500d, Konika Minolta (Japan). Hue value (H) , chroma (C) and color difference (ΔE) from start sample (juice after pasteurization) and samples after storage were calculated. All analysis was done in duplicate and the results are represented as mean ± standard deviation of duplicates.

Sensory evaluation was achieved by Quantitative descriptive analysis (QDA). A trained panel of twelve people from the faculty staff and students were selected and trained to sensory evaluate mandarin juices using a 10-point scale (1= unexpressed attribute, 10= maximal express attribute). List of attributes was defined and presented at figure 3. The procedure was performed according to ISO 6564, ISO 8587 and ISO 11036 (in a sensory laboratory equipped according to ISO 8589) and in consistence with method from literature (Bursać Kovačević et al., 2008).

Obtained juices were conducted to microbial testing on presence of *Salmonella* strains (HRN EN ISO 6579:2003), *Enterobacteriaceae* (HRN ISO 21528-2:2008), aerobic mesophilic bacteria (HRN EN ISO 4833:2008), yeasts (ISO 21527:2008), molds (ISO 21527:2008), sulfur-reducing clostridium (HRN EN ISO 15213:20049 and *Listeria monocytogenes* (HRN EN ISO 11290-2:1999). Analysis were done at Food Control Centre at Faculty of Food Technology and Biotechnology, University of Zagreb, that is accredited laboratory for official controls, approved for testing food and feed by Croatian Ministry of Agriculture (compliant with HRN ISO/IEC 17025).

RESULTS & DISCUSSION

During mandarin juice processing, the yield was calculated as follows: peel comprise about 20,55% of the total whole fruit weight, while after pressing, juice comprised about 34,71% of the total whole fruit weight and about 43,69% calculated on the weight of peeled fruit. Paolu et al. (2008) reported the yield of 48,30% for Clementine mandarin juice obtained on rotary citrus squeezer. Citrus fruit are abundant in pectin that makes pressing more difficult and less efficient. Therefore, in order to obtain higher yield during processing, an enzymatic pre-treatment before pressing is recommended (Levaj et al. 2012). In comparison to literature data (Levaj et al, 2005, Paolu et al 2008), our results revealed slightly higher values of SS and TA (Table 1). During storage, minor decrease of SS and TA, and increase of pH was recorded. All juices during storage were microbiologically stable (results are not shown).

Concerning phenolic compounds in mandarin juice, two different extraction procedures were used with

the aim to evaluate their efficacy in the extraction of more complex substances. Determination of TP was done in both extracts (EA and AEE), while determination of AC was done only in EA, hence remarkable different results were noticed (Figure 1A and 2A).

Sample		Soluble solids, °Bx	pH	Total acidity, %
Fruit		11,8±0,14	3,56±0,00	1,26±0,03
Juice after press		11,2±0,14	3,56±0,00	1,29±0,04
Juice after past.		11,2±0,00	3,56±0,00	1,12±0,03
Juice after 60	1°C	11,1±0,14	3,68±0,12	0,98±0,00
days	20°C	10,6±0,00	3,75±0,07	1,06±0,00
storage at	37°C	11,2±0,14	3,60±0,00	1,06±0,01

Table 1. Effect of juice processing and storage on physicochemical parameters

Average TP in EE were almost tenfold lower than in AEE, though are in accordance with previous results obtained in EE (Levaj et al 2005, Levaj et al, 2012, Zhang et al 2018). On the other hand, TP in AEE are approximately similar to results (sum of TP in juice sacs, segment membrane, and segment) obtained in acidic methanol extracts Abeysinghe et al. (2007). Different trend of TP changes by those two extractions procedure were observed, too. In EE during juice processing TP decreased in contrast to TP in AEE. Probably, pressing does not allow complete extraction of phenolics from fruit to juice, and thermal treatment cause degradation of phenolics. Further, AEE could improve the extractability of phenolics from the tissue from whole fruit as well as from small particles of tissue, which are extracted in juice. In addition, the extraction efficiency is in opposite relation to particle size and probably short thermal treatment could improve it as well as by AEE mixture of different phenolics structure and thermal stability. That could be the reason why pasteurized juice contained higher values of TP in AEE.

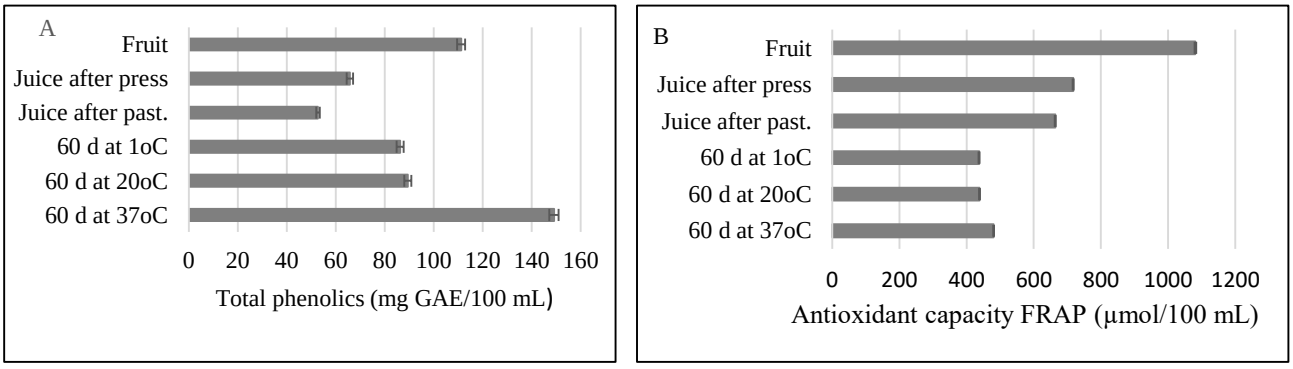


Figure 1. Total phenolics (A) and antioxidant capacity (B) in samples before and after storage at different temperature during 60 days (d)

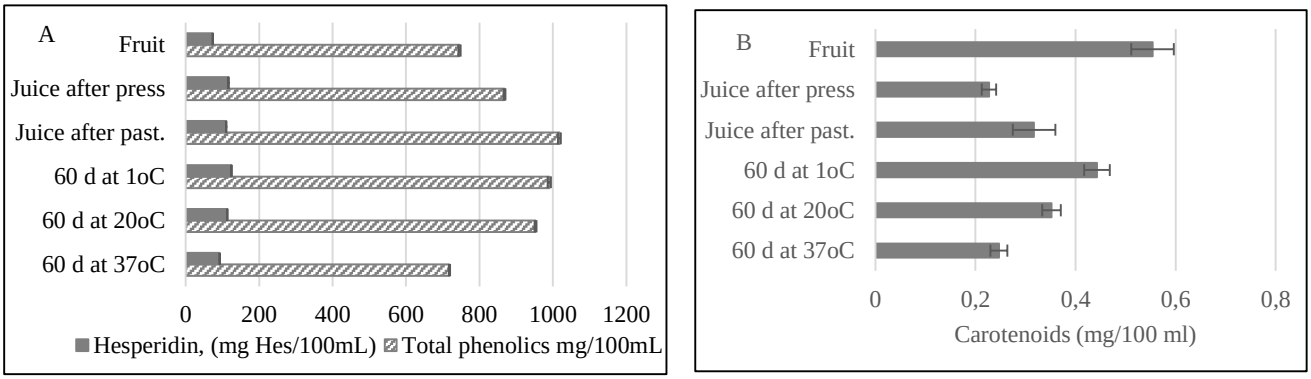


Figure 2. Total phenolics and hesperidin (A) and carotenoids (B) in samples before and after storage at different temperature during 60 days (d)

Moreover, storage time and increased storage temperature affected increase of TP. It could be associated to well-known citrus juice susceptibility to non-enzymatic browning (Handwerk, Coleman, 1988) and less selectivity of FC reagent (Klimzak et. al., 2010). The opposite trend was recorded with AEE, probably because browning pigments in acidic media reacts differently.
AA changes was similar to those observed for TP changes in EE. Correlation coefficient for juice processing samples as well as for juices during storage were higher than 0,9. Changes of FG followed pattern of TP changes in AEE and were in accordance to literature data (Abeysinghe et al., 2007). Hydraulic press definitely is not efficient for carotenoids extraction (Fig 2B) though obtained results are in agreement to the results of Lotha i Khurdiya (1994). In addition, remarkable amount of coloured fruit tissue left in press and was not extracted into juice at all. Pasteurization (thermal treatment) caused increase of TC what is consistent to findings of Plaza et al (2011). Protein denaturation is associated to carotenoid realising from protein or membrane lipids complex and improving its extractability (Plaza et al. 2011). Storage at 37ºC showed negative influence on TC, while other temperatures increase TC. Similar findings were observed in previously published data (Plaza et al. 2011).
With respect to colour parameters, lightness was varying in consistence to TP changes in EE and sensory evaluated color (Table 3) in both, during processing and storage. Juice became darker (lower L* and higher color difference (ΔE)) with storage time and storage temperature.

Table 2 Color parameters in samples before and after storage at different temperature

Sample		L*	a*	b*	C*	h*	ΔE
Fruit		32,72±1,13	27,60±0,99	56,27±2,23	62,67±0,42	0,46±0,57	-
Juice after press		53,41±0,85	20,58±0,67	57,73±0,42	61,29±0,42	1,23±0,57	-
Juice after past		61,26±0,28	15,84±0,28	58,98±0,28	61,07±0,28	1,31±0,48	-
Juice after 60 days storage at	1ºC	51,41±0,57	17,25±0,42	54,08±0,57	56,77±0,42	1,26±0,57	10,60±0.42
	20ºC	46,44±0,71	21,19±0,57	63,78±0,42	67,20±0,42	1,25±0,57	16,88±0,57
	37oC	44,32±0,42	18,79±0,28	59,49±0,57	62,39±0,57	1,27±0,42	17,29±0,57

Though all juices during storage were microbiologically stable, juice stored at 37ºC was not acceptable for sensory evaluation, especially taste (missed on Fig 3), in spite of its microbial stability. Probably, products of non-enzymatic browning (Handwerk, Coleman, 1988) or some enzyme, as PME, was responsible for that consider to thermal resistance of PME during pasteurization compared to spoilage microorganisms (Cinquanta, 2010). As it can be seen at Fig 3 juices before storage and stored at 1ºC were the best-evaluated juices. Generally, sensory scores are not high e.g. mandarin taste was lower scored for the start sample, what is most likely the consequence of pressing instead squeezing mandarin fruit to obtain juice. Press do not allow transfer of whole juice sacs into juices what is probably responsible for lack of mandarin specific taste.

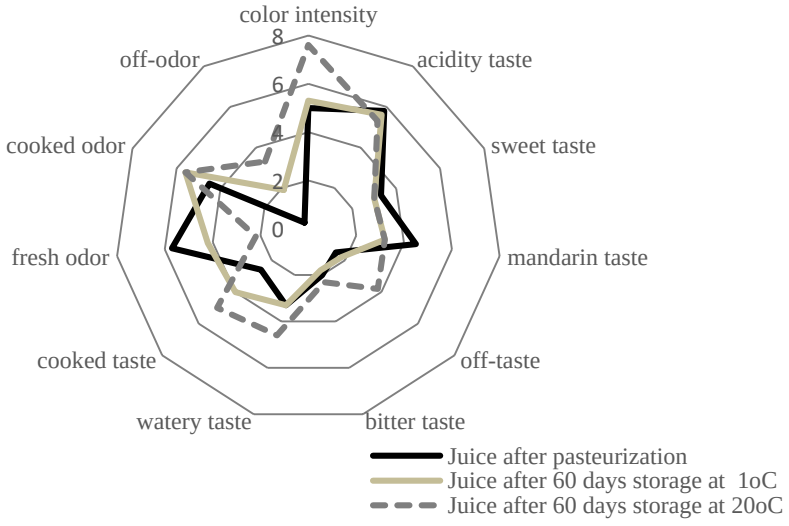


Figure 3. Sensory evaluation of pasteurized and stored mandarin juices

CONCLUSION

Pressing as way to produce mandarin juice is not strongly recommended considering lack of intense mandarin taste of obtained juice. Temperature and storage time affected all investigated parameters as well as sensory properties of juices. Sensorial changes were not very pronounced in the juices stored at 1℃. Similar, stability of biological active compounds was perceived in juices stored at 1^{o}C and 20^{o}C/60 days, while 37^{o}C had detrimental influence on biological and sensory properties of mandarin juice.

REFERENCES

Abeysinghe,D.C., Li, X., Sun , C., Zhang, W., Zhou, C., Chen, K., (2007) Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. Food Chemistry 104, 1338-1344.

Ashurst .R. (ed.) (2005) Chemistry and Technology of Soft Drinks and Fruit Juices, 2nd edn, Blackwell Publishing Ltg., Hereford, UK.

Bursać Kovačević, D., Vahčić, N., Levaj, B., Dragović- Uzelac, V. (2008). The effect of cultivar and cultivation on sensory profiles of fresh strawberries and their purees. Flavour and Fragrance Journal, 23, 323-332.

CIE, Commission Internationale de l'Eclairage (1976) Official recommendations on uniform colour spaces, colour differences equations and metric colour terms. Paris: France.

Cinquanta L., Albanese D., Cuccurullo G., Di Matteo M. 2010. Effect on Orange Juice of Batch Pasteurization in an Improved Pilot-Scale Microwave Oven Journal of Food Science 75 (1) E46-E50.

Coseteng M.Y., Lee C.Y. 1987. Changes in Apple Polyphenoloxidase and Polyphenol Concentrations in Relation to Degree of Browning. Journal of Food Science, 52, 985-986.

Directive 2012/12/EU of the European Parliament and of the Council of 19 April 2012 amending Council Directive 2001/112/EC relating to fruit juices and certain similar products intended for human consumption

Handwerk R.L., Coleman R.L. 1988. Approaches to citrus browning problem. A review. Journal of Agriculture Food Chemistry. 36 (1) 231-236.

Klimczak I., Małecka M.., Szlachta M., Gliszczyńska-Świgło A., 2007. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. Journal of Food Composition and Analysis 20, 313–322.

Levaj, B., Dragović-Uzelac, V., Bursać, D., Lukešić, Ž., Banović, M., Kovačević Ganić, K., Repajić, M., Sklevicky T., Smernjak, B. 2005. The possibilities of mandarins processing Proceedings of the 2nd Central European Meeting and 5th Croatian Congress of Food Technologists, Biotechnologists and Nutritionists, Opatija 2004., 149-156.

Levaj B., Putnik P., Linardić I., Herceg Z., Režek Jambrak A., Bursać Kovačević D. 2012. Influence of pre-treatment on yield and quality of mandarin juices. Proceedings of 6th Central European Congress / Lević, Jovanka (ur.). Novi Sad: University of Novi Sad, Institute of Food Technology, 874-880

Legua P., Forner J.B., Hernández F., Forner-Giner MA. 2014. Total phenolics, organic acids, sugars and antioxidant activity of mandarin (*Citrus clementina* Hort. ex Tan.): Variation from rootstock. Scientia Horticulturae, 174:60-64.

Lotha, R.E., Khurdiya, D.S. 1994. Effect of methods of juice extraction from Kinnow mandarin on the composition and quality of juice, pomace and peel. Journal of Food Science Technology, 31, 380-384.

Ough, C.S., Amerine, M.A. 1988. Methods for analysis of musts and wine, J.Wiley&Sons, New York pp 205-206.

Park, P.K., E.Y. Kim and K.H. Chu, 2007. Chemical disruption of yeast cells for the isolation of carotenoid pigment Separation and Purification Technology 53 (2) 148-152.

Palou L, Jacas J-A, Marcilla A, Alonso M, del Río MÁ. 2008. Physico-chemical and sensory quality of 'Clemenules' mandarins and survival of the Mediterranean fruit fly as affected by complementary cold and carbon dioxide quarantine treatments. Postharvest Biology and Technology. 48 (3) 443–50.

Pulido, R., Bravo L., Saura-Calixto F. 2000. Antioxidant activity of dietary Polyphenols as determined by modified ferric reducing/antioxidant power assay. Journal of Agriculture and Food Chemistry, 48, 3396-3402.

Vahlquist A., 1982. Vitamin A in human skin. I. Detection and identification of retinoids in normal epidermis. Journal Invest Dermatology. 79, 94-97.

Plazaa L.,Sánchez-Moreno C., De Ancos B., Elez-Martínez P., Martín-Belloso O., Pilar Cano M. 2011. Carotenoid and flavanone content during refrigerated storage of orange juice processed by high-pressure, pulsed electric fields and low pasteurization. LWT - Food Science and Technology 44 (4) 834-839

Zhang H, Yang Y.F., Zhou Z.Q. 2018. Phenolic and flavonoid contents of mandarin (Citrus reticulata Blanco) fruit tissues and their antioxidant capacity as evaluated by DPPH and ABTS methods. Journal of Integrative Agriculture, 17(1) 256–263.

Preparation of wine for the analysis by analytical techniques: NMR, IRMS and ICP-OES – method validation

Miloš, M.[a], Petric, I.V.[b], Jusup, J.[b], Šimon. S.[b], Leder, R.[b], **Banović, M**.[a]

[a]*Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia, mbanovic@pbf.hr*
[b]*Institute of Viticulture and Enology, Croatian Center for Agriculture, Food and Rural Affairs, Jandrićeva 42, 10000 Zagreb, Croatia, renata.leder@hcphs.hr*

ABSTRACT
Stable isotopes and mineral composition of wines have been recognized as indicators of wine's authenticity, and methods for their determination have been developed. Stable isotope ratios of $(D/H)_I$ and $(D/H)_{II}$ of wine is determined by the SNIF-NMR® (*Site Specific Natural Isotope Fractionation - Nuclear Magnetic Resonance*) for the methyl- and the methylene-position of the ethanol molecule. The determination of $^{13}C/^{12}C$ is performed in the ethanol molecule by the IRMS - *Isotope Ratio Mass Spectrometry*. For both methods, ethanol from the wine is obtained using ADCS - *Automated Distillation Control System*. The residue after distillation is ethanol free and therefore suitable for determining the macro- and microelements by the ICP-OES (*Inductively Coupled Plasma – Optical Emission Spectrometry*) method. The aim of this paper was to validate the wine sample preparation by ADCS in order to obtain a distillate with an alcohol grade of 92-93% w/w. Repeatability of the method was determined by distillation of the same sample on four distillation columns in six replicates. Obtained mean value was 92.60 ± 0.23% w/w. The relative standard deviations (RSD) were calculated for each column and they were: 0.15, 0.13, 0.31, and 0.12%, respectively. Determination of the distillate water content was performed by Karl-Fischer titration method and the repeatability of water determination was calculated (RSD = 0.25%). It was concluded that the method is fit for the intended purpose, which is preparation of the wine sample for analysis by analytical techniques: NMR, IRMS and ICP-OES.

Keywords: authenticity; validation method of distillation; wine

INTRODUCTION

Increased consumers awareness of the importance of food safety has been steadily increasing in recent years. There is also an increase in activities that include the adulteration of food products, including wine, for financial gain. This has led to the development of sophisticated analytical procedures for determination of traceability according to geographic and botanical origin and influence of technological processing (Chua et al., 2012, Bandoniene et al., 2013). Although quality awareness of wine producers is on a rather high level, there are still cases of wine production by illegal practices. Stable isotopes and mineral composition of wines have been recognized as genuine indicators of wine's authenticity, and methods for their determination have also been developed (Christoph et al., 2007). Stable isotope ratio of $(D/H)_I$ i $(D/H)_{II}$ of wine is determined by the SNIF-NMR® method for the methyl- and the methylene-position of the ethanol molecule (Martin et al., 1981, Martin et al., 1982). The determination of carbon stable isotope ratio ($^{13}C/^{12}C$) is also performed in the ethanol molecule but by the IRMS (Christoph et al., 2015). For both methods, ethanol from the wine is obtained in the same way, by distillation. The main prerequisite of distillation system according to the OIV method (OIV-MA-AS311-05:R2011) is to enable the extraction of ethanol from the wine without additional isotope fractionation. Between 96 - 98.5% of wine ethanol should be extracted and the alcoholic strength of the obtained distillate should be 92-92% w/w (about 95% vol.). All preparation procedures must be carried out without significant loss of ethanol by evaporation, which would alter the isotope composition of the sample. For the ICP-OES technique it is essential to extract the ethanol from wine because it prevents the plasma formation (Larcher and Nicolini, 2001). Therefore, the residue after sample distillation is ethanol free and suitable for determining the macro- and microelements. By using the residue of the distillation for ICP-OES analysis the consumption of very expensive high purity chemicals is considerably reduced and the usage of the microwave digestion or other means of sample preparation are completely avoided. The aim of this paper was to validate the procedure for preparing the wine sample by ADCS in order to be analysed by aforementioned analytical methods. The ADCS automates the entire process, completely controls the distillation and allows the software to store and process results.

MATERIALS & METHODS

Determination of alcoholic strength of the wine samples was performed by Alcolyzer (Patent Anton Paar®; Anton Paar, Austria), a near infrared spectrometer. One wine sample of appropriate quantity was distillated in six replicates at four distillation columns of ADCS – *Automated Distillation Control System* (Eurofins, Nantes, France), operated by the ADSC V1.1.9.0 software. Karl Fischer DL31 volumetric titrator (Mettler Toledo, Greifensee, Swizterland) operated by LabX light software was used for the determination of the distillate water content (% w/w) in all obtained distillates in order to calculate the alcoholic strength (%w/w) and yield of each performed distillation. Eurokarl Windows v.1.0.0.0 software was used for transfer of the obtained alcoholic strength data to the ADSC V1.1.9.0 software. Reagents used for the Karl-Fischer titration were Titrant 5, Solvent and 1% water standard for the standardization procedure of the solvent were obtained from Merck (Darmstadt, Germany). The requirements for the distillation procedure are described in the OIV method (OIV-MA-AS311-05:R2011). Two interlaboratory comparison samples from two different providers (*Bureau Interprofessionnel d'Etudes Analytiques*, Paris, France – BIPEA: "17Wines" proficiency testing scheme and Eurofins, Nantes, France: "Food analysis using Isotopic Techniques – Proficiency Testing Scheme" – FIT-PTS 2016 Round 3) were destillated by ADCS followed by determination of stable isotope ratio of $^{13}C/^{12}C$ (OIV-MA-AS312-06:R2001) in obtained ethanol by IRMS Delta V Plus coupled with elemental analyzer FlashEA 1112 Series (Thermo Fischer Scientific, Bremen, Germany). The sample was measured against the certified reference material BCR-656 obtained at Institute for Reference Materials and Measurements (Geel, Belgium) calibrated by the primary international reference (Vienna-Pee-Dee Belemnite V-PDB). Chemicals used for filling the combustion reactor for conversion the sample ethanol in carbon dioxide were copper (II) oxide, silver cobaltous/cobaltic oxide and chromic (III) oxide obtained from thermo Fischer Scientific, Bremen, Germany. The determination of Ca, Cu, Fe and Pb was conducted in the wine residue after the distillation by 2000 Optima ICP-OES, Perkin Elmer, Shelton, Connecticut, USA. Standard solutions of four elements were obtained by Merck (Darmstadt, Germany) as well as the 60 % ultrapure HNO_3 which was used diluted to 2% by ultrapure water at 18 MΩ/cm resistivity. 2% HNO_3 was used for dilution of the residue after distillation by 1:1. Five wine samples (three white and two red) were distillated and obtained ethanol was sent to the Service Commun des Laboratoires, Bordeaux, France, for the stable isotope analyses of $(D/H)_I$ and $(D/H)_{II}$ by NMR (OIV-MA-AS311-05:R2011; 400 MHz instrument of Bruker, Darmstadt, Germany).

RESULTS & DISCUSSION

Determination of repeatability of the method was carried out by distillation of the same sample at four distillation columns of the ADCS (Figure 1) in six replicates. The RSDs were calculated for each column and for all distillations in total. The results of 24 distillations of the same wine sample with the alcoholic strength of 11.73% vol. are shown in the Table 1.

To confirm the validity of each distillation, determination of the distillate water by Karl-Fischer titration method was performed and the obtained yield of all distillations were between 97.4% and 100.0% which means that almost all ethanol from wine is transferred to the distillate. The total mean value of the alcoholic strength of distillates was 92.60 ± 0.23% w/w. The RSDs were calculated for each column and they were: 0.15, 0.13, 0.31, and 0.12%, respectively. The data were tested by the Grubbs test for the outliers (Grubbs, 1950). There was one value of alcoholic strength (93.21% w/w, the first replicate at column No. 3) designated as furthest from the rest but it was still not an outlier.

Although the column No. 3 have shown the highest RSD, the obtained values for alcoholic strength are still between 92 and 93% w/w as required by the OIV method (OIV, 2018).

Validation of the Karl-Fischer volumetric titration method of water content determination and subsequent alcoholic strength of the distillate and yield of the distillation was conducted thought the repeatability calculation (Table 2).

The standardization procedure of the Karl-Fisher solvent was performed by the certified reference material with the known water content, followed by the titration of the 10 replicates of the same wine distillate. The obtained mean water content value was 7.13% w/w with the standard deviation of 0.02 % w/w and the repeatability was expressed as RSD = 0.25%.

Figure 1. ADCS – *Automated Distillation Control System*

Table 1. Repeatability of distillations performed by ADCS

Column No.	Replicates	Yield (%)	Water (% w/w)	Alcoholic strenght (% w/w)	Average (% w/w)	Standard deviatio (% w/w)	RSD (%)
1	1	97.4	7.48	92.52	92.54	0.14	0.15
	2	98.1	7.23	92.77			
	3	100.0	7.38	92.62			
	4	97.7	7.58	92.42			
	5	98.0	7.57	92.43			
	6	98.0	7.54	92.46			
2	1	97.9	7.26	92.74	92.66	0.12	0.13
	2	98.0	7.50	92.50			
	3	97.9	7.28	92.72			
	4	97.4	7.33	92.67			
	5	97.7	7.45	92.55			
	6	97.8	7.20	92.80			
3	1	97.5	6.79	*93.21*	92.81	0.29	0.31
	2	97.9	7.36	92.64			
	3	97.8	6.95	93.05			
	4	97.9	7.41	92.59			
	5	97.5	7.12	92.88			
	6	97.6	7.54	92.46			
4	1	98.0	7.56	92.44	92.38	0.11	0.12
	2	98.3	7.79	92.21			
	3	98.3	7.73	92.27			
	4	98.2	7.56	92.44			
	5	98.6	7.57	92.43			
	6	98.4	7.53	92.47			
Minimum		97.4	6.79	92.21			
Maximum		100.0	7.79	93.21			
Average		98.0	7.40	92.60			
Standard deviation		0.53	0.23	0.23			

Table 2. Repeatability of distillate water content determination by Karl Fischer volumetric titration

Replicate	1	2	3	4	5	6	7	8	9	10
Water in the distillate (% w/w)	7.10	7.15	7.14	7.13	7.15	7.12	7.12	7.15	7.15	7.12
Average (% w/w)					7.13					
Standard deviation (% w/w)					0.02					
RSD (%)					0.25					

In order to confirm the fitness of the described method for the wine sample preparation, the laboratory have also participated in the interlaboratory comparison organised by Eurofins, Nantes, France, for IRMS stable isotope $^{13}C/^{12}C$ determination and in the interlaboratory comparison organised by BIPEA, Paris, France for ICP-OES analysis of four metals (Ca, Cu, Fe and Pb).
Results showed (Table 3) that laboratory has good measurement capabilities and that ADCS preparation method is suitable for these analytical techniques. The following values are presented: number of participating laboratories, reference value, minimum and maximum permissible deviation interval, result obtained by the laboratory and Z-score, from which the success of laboratories participation in interlaboratory comparisons is being estimated. For all reported results the Z-scores were between -2.00 and 2.00 demonstrating high quality of performed sample preparation followed by compliant results obtained by the appropriate analytical technique (Hovind et al., 2011).

Table 3. Results of interlaboratory comparisons (ILC) for wine samples prepared by ADCS

Analitical technique	**Parameter**	**No. of laboratories**	**Reference value**	**Min**	**Max**	**Laboratory result**	**Z-score**
ILC organised by Eurofins, Nantes, France / wine sample No. 16/3/F							
NIR	Alcoholic strenght (% vol.)	11	11.17	10.94	11.52	11.12	-0.48
Karl-Fischer tittration	Distillation yield (%)	12	n/a	96.00	99.55	98.70	n/a
IRMS	$^{13}C/^{12}C$ (‰ V-PDB)	11	-26.44	-26.78	-26.15	-26.33	0.75
ILC organised by BIPEA, Paris, France / wine sample No. 37-0317-0029							
ICP-OES	Ca (mg/L)	45	78	62	94	79	0.13
	Cu (mg/L)	51	0.11	0.00	0.23	0.13	0.33
	Fe (mg/L)	69	2.4	1.4	3.4	2.4	0.00
	Pb (mg/L)	16	0.014	0.000	0.034	0.032	1.80

n/a…not applicable

Table 4. Results of NMR analysis of wine samples prepared by ADCS

Analitical technique	**Sample No.**	**Parameter**	**Value (ppm)**	**Average (ppm)**	**Min (ppm)**	**Max (ppm)**	**Stdev (ppm)**
NMR	1	(D/H)I	102,73				
	2	(D/H)I	98,82				
	3	(D/H)I	104,39	100,85	98,82	104,39	2,54
	4	(D/H)I	99,24				
	5	(D/H)I	99,09				
	1	(D/H)II	127,93				
	2	(D/H)II	128,77				
	3	(D/H)II	125,03	127,05	125,03	128,77	1,47
	4	(D/H)II	127,32				
	5	(D/H)II	126,21				

Five samples prepared by ADCS in the laboratory were analyzed by NMR at Service Commun des Laboratoires, Bordeaux, France, showing also good quality of sample preparation. Results of NMR analysis are presented in the Table 4. Ratio of the deuterium and hydrogen at methyl position of the ethanol molecule was between 98.83 ppm and 104.39 ppm and at the methylene position between 125.03 ppm and 128.77 ppm which is in accordance to Christoph et al. (2015).
Presented results showed that the wine distillation by ADCS is appropriate method for extracting the ethanol from wine and subsequent analysis of ethanol by NMR and IRMS, and for the analysis of wine residue by ICP-OES.

CONCLUSION

Based on the presented method validation results we can conclude that laboratory successfully implemented the ADCS sample preparation method. The criteria for yield of distillation, water content determination, ethanol content in the distillate and repeatability of distillations were fulfilled. Laboratory has successfully participated in interlaboratory comparisons which was confirmed by obtained Z-scores that were between -2.00 and 2.00 for all tested parameters. Finally, it can be concluded that the method is fit for the intended purpose which is preparation of the wine samples for analysis by analytical techniques: NMR, IRMS and ICP-OES.

REFERENCES

Bandoniene D., Zettl D., Meisel T., Maneiko M. 2013. Suitability of elemental fingerprinting for assesing the geographic origin of pumpkin (*Cucurbita pepo* var. styriaca) seed oil. Food Chemistry 136 (3-4), 1533-1542.
Christoph N., Rossmann A., Schlicht C., Voerkelius S. 2007. Wine authentication using stable isotope ratio analysis: Significance of geographic origin, climate, and viticultural parameters. ACS Symposium Series 952: 166-179.
Christoph N., Hermann A., Wachter H. 2015. 25 Years authentication of wine with stable isotope analysis in the European Union –Review and outlook. BIO Web of Conferences 5: 02020.
Chua L. S., Abdul-Rahman N. L., Sarmidi M .R., Aziz R. I. 2012. Multi-elemental composition and physical properties of honey samples from Malaysia. Food Chemistry 135(3), 880-887.
Compendium of International Methods of Analysis of Wines and Musts. 2018. International Organization of vine and wine – OIV (OIV-MA-AS311-05:R2011, OIV-MA-AS312-06:R2001) Paris, France.
Grubbs F.E. 1950. Sample criteria for testing outlying observations. Annals of Mathematical Statistics. 21(1), 27-58.
Hovind H., Magnusson B., Krysell M., Lund U., Mäkinen I. 2011. Internal Quality Controll – Handbook for Chemical Laboratories. Nordtest. NT Technical Report 569. Norway.
Larcher R., Nicolini G. 2001. Survey of 22 mineral elements in wines from Trentino (Italy) using ICP-OES. Italian Journal of Food Science 13(2), 233-241.
Patent Anton Paar® (US 6,690,015; AT 406711) Method for the spectroscopic determination of ethanol concentration in an aqueous sample, European Patent Office, EP 1073896 B1.
Martin G.J., Martin M.L., Mabon F., Michon M.J. 1982. Identification of the origin of natural alcohols by natural abundance hydrogen-2 nuclear magnetic resonance. Analytical Chemistry. 54(13), 2380-2382.
Martin G.J., Martin M.L. 1981. Deuterium labelling at the natural abundance level as studied by high field quantitativeH NMR. Tetrahedron Letters 22(36), 3525-3528.

Acknowledgements: authors are sincerely grateful to the Service Commun des Laboratoires, Bordeaux, France, for performing the NMR analyses.

Macroelements and specialized metabolites content under salt stress in hydroponically grown rocket

Nina Toth[a], Luka Jerončić[b], Lepomir Čoga[c], Božidar Benko[a], Sanja Fabek Uher[a], Jana Šic Žlabur[d]

[a] *University of Zagreb Faculty of Agriculture, Department of Vegetable Crops, Zagreb, Croatia (ntoth@agr.hr)*
[b] *University of Zagreb Faculty of Agriculture, Graduate Study Horticulture-Vegetable Growing, Zagreb, Croatia*
[c] *University of Zagreb Faculty of Agriculture, Department of Plant Nutrition, Zagreb, Croatia (lcoga@agr.hr)*
[d] *University of Zagreb Faculty of Agriculture, Department of Agricultural Technology, Storage and Transport, Zagreb, Croatia,(jszlabur@agr.hr)*

ABSTRACT

Salt stress at different plant species may affect the reduction or increase in the content of dry matter, biogenic elements and specialized metabolites. The goal of this research was to determine the effect of salt stress on specialized metabolites accumulation and macroelements' content of hydroponically grown rocket which belongs to the group of functional food. Salt stress was realized by adding NaCl (2.92, 5.27 and 7.62 g L^{-1}) into the standard nutrient solution with EC-value 3 dS m^{-1}, which showed increase in EC-values (5, 7 and 9 dS m^{-1}), respectively. The trial was set up in a floating system according to a randomized block design with three replications. Salt stress had negative effect on dry matter and content of macroelements (N, P, K, Ca and Mg), while the amount of Cl and Na was increased linearly. Salt stress had positive effect on vitamin C and total phenols content. The highest concentration of NaCl resulted in the significantly highest vitamin C content (88.07 mg 100 g^{-1} fresh weight). The highest total phenols content (173.6 and 173.4 mg GAE 100 g^{-1} fresh weight), without significant difference, was achieved at rocket grown in nutrient solutions with EC 7 and 9 dS m^{-1}. The equal content of total chlorophyll (0.9 and 0.8 mg g^{-1} fresh weight) was determined at nutrient solution with standard and the highest EC-values (3 and 9 dS m^{-1}), respectively. Nutrient solutions with higher NaCl concentrations resulted in significantly lower content of total glucosinolates in rocket leaves than the standard nutrient solution, as well as with the lowest NaCl concentration.

Key words: Eruca sativa Mill.*; vitamin C; total glucosinolates; phenols; chlorophyll*

INTRODUCTION

Rocket (*Eruca sativa* Mill.), leafy vegetable suitable for growing in floating hydroponics, according to Cavaiuolo and Ferrante (2014), contains various compounds (vitamins, chlorophylls, carotenoids, glucosinolates, phenols, minerals etc.) with beneficial effect on human health. Hydroponic cultivation of vegetables in greenhouses provides management of all abiotic factors which result in optimal conditions for plant growth and high yield. Various forms of stress can stimulate plants in the synthesis of specialized metabolites (Selmar and Kleinwächter, 2013). Therefore, exposure of vegetables to controlled stress can induce enhanced synthesis of bioactive compounds and increase the vegetables' quality (Barbieri et al. (2010). Salt stress, depending on the intensity, in different plant species causes decrease or increase in the content of specialized metabolites, but also of dry matter and biogenic elements (Bonasia et al., 2017). Zuccarini (2007) stated that in salt stress conditions plants accumulated less potassium and more calcium, while Prasad et al. (2014) determined that increased NaCl concentration affected reduced absorption of potassium, nitrogen, phosphorus and calcium. In order to elucidate previous inconsistent results, the aim of the research was to determine the effect of salt stress on specialized metabolites accumulation and macroelements' content of hydroponically grown rocket.

MATERIALS & METHODS

The trial was carried out in heating greenhouse of Department of Vegetable Crops, University of Zagreb Faculty of Agriculture. The experiment was set up in a floating system as a single-factor trial according to a randomized block design with three replications. It included rocket cultivar 'Coltivata' and 4 salt stress treatments with NaCl concentration (0 – control, 2.92, 5.27 and 7.62 g L^{-1}) i.e. EC value (3 – control, 7, 5 and 9 dSm^{-1}) of nutrient solution. During period of 38 days from sowing (10/4/2017) to harvest (18/5/2017) of rocket rosettes with 6 to 8 developed leaves, abiotic parameters were in optimum range for rocket growth (mean daily air temperature 19 to 27 °C and relative

humidity 38 to 71%; nutrient solution parameters: pH 5.8 to 7.2, dissolved oxygen concentration 3 to 8 mg L^{-1} and EC value as stated).

Chemical analyses of minerals content in rocket leaves were performed in laboratory of Department of Plant Nutrition, University of Zagreb Faculty of Agriculture as follows: total nitrogen, phosphorus, potassium, calcium, and magnesium content (expressed % on dry matter) according to the standard methods (AOAC, 1995) by atomic absorption spectrometry (AAS SOLAR THERMO SCIENTIFIC).

Chemical analyses of specialized metabolites were performed in laboratory of Department of Agricultural Technology, Storage and Transport, University of Zagreb Faculty of Agriculture. For determination of bioactive compounds and pigments, the following methods were carried out: vitamin C content (mg 100 g^{-1}) according to the standard method (AOAC, 2002), total phenol content (mg GAE 100 g^{-1}) according to the Ough and Amerine (1988), total glucosionolates (%) according to the method described by Jezek i sur. (1999) and antioxidant capacity (mmol TE kg^{-1}) by ABTS method (Miller et al., 1993) spectrophotometrically (Shimadzu UV 1650 PC). Pigment compounds, specifically total chlorophyll (chlorophyll a and b) and total carotenoids were determined according to the method described by Holm (1954) and Wettstein (1957). The absorbance of pigment extracts was measured spectrophotometrically (Shimadzu UV 1650 PC) at 662, 644 and 440 nm with acetone (p.a.) as a blank probe. The absorbance values were recalculated according to the Holm-Wettstein equations (mg L^{-1}) while the final result of the pigment content was expressed in mg g^{-1}:

$$chlorophyll\ a = 9.784\ \times A_{662} - 0.990\ \times A_{644}[mg\ L^{-1}],$$
$$chlorophyll\ \ b = 21.426\ \times A_{644} - 4.65\ \times A_{662}[mg\ L^{-1}],$$
$$chlorophyll\ a + b = 5.134\ \ \times A_{662} + 20.436\ \times A_{644}[mg\ L^{-1}\ ],$$
$$carotenoids = 4.695 \times A_{440} - 0.268 \times (chlorophyll\ a + b)[mg\ L^{-1}].$$

The results were statistically analyzed in the SAS program version 9.3 (SAS, 2010). The results were subjected to one-way variance analysis (ANOVA). The mean values were compared with the t-test (LSD) and were considered significantly different at $p \leq 0.01$. Different letters in tables indicate significant differences between mean values and also express standard deviation (±SD).

RESULTS & DISCUSSION

Dry matter and macroelements

The NaCl concentration of nutrient solution (NS) had significant effect on dry matter (DM) content of rocket leaves (data not shown). Statistical differences in DM content (12.66, 10.58, 9.6 and 8.48%) between rocket grown in NS with different NaCl concentration (7.62, 5.27, 2.92 and 0 g L^{-1}, respectively) indicate that salt stress had positive effect on DM accumulation. These results were consistent with statements of Bonasia et al. (2017) and contrary of Barbieri et al. (2010) that DM content increased only to the NaCl concentration of 2.92 g L^{-1}, while further increase of salt stress had decreasing effect. Table 1 shows significant effect of salt stress on content of main macroelements (nitrogen - N, phosphorus - P, potassium - K, calcium - Ca and magnesium - Mg). There is an obvious decreasing trend in the content of all researched macroelements caused by increasing EC value of NS due to addition of NaCl.

Table 1. The content of macroelements (%) on dry matter of rocket leaves

Treatment	N	P	K	Ca	Mg
EC 3	6.57±0.07 A	0.60±0,03 A	8,24±0,3 A	1.33±0,06 A	0.34±0,02 A
EC 5	5.50±0,05 B	0.56±0,01 B	5,75±0,2 B	1.09±0,01 B	0.28±0,01 B
EC 7	5.19±0,01 C	0.44±0,01 C	5,21±0,1 C	0.99±0,01 C	0.22 C
EC 9	5.11±0,03 C	0.47±0,01 C	5,03±0,1 C	0.86±0,02 D	0.21 C

EC 3- standard nutrient solution; EC 5- nutrient solution with 2.92 g L^{-1} NaCl; EC 7- nutrient solution with 5.27 g L^{-1} NaCl; EC 9- nutrient solution with 7.62 g L^{-1} NaCl; Different letters indicate significant differences between mean values within each column at $p \leq 0.01$.

This is in agreement with statements of Prasad et al. (2014) about reduced absorption of N, K, P and Ca in salt stress conditions. The statistically highest content of analyzed macroelements was achieved by rocket grown in control NS without NaCl (EC 3). Significant difference in N, P, K and Mg content

were not determined between treatments EC 7 and EC 9 i.e. NS with NaCl concentration of 5.27 and 7.62 g L^{-1}, respectively, which achieved the lowest content of those macroelements. This result indicates that salt stress with higher NaCl concentration than 5.27 g L^{-1}, did not significantly affected the content of stated macroelements.

Effect of NaCl concentration of NS on chlorine (Cl) and sodium (Na) content in rocket leaves was also statistically significant (Table 2). The highest Cl and Na content was determined in rocket grown in NS with highest NaCl concentration and vice versa, the lowest content of these elements was determined in rocket from control NS without NaCl. EC increase from 3 to 5 dS m^{-1} resulted with the multiple increase (3.7 and 12 times) of Cl and Na content in rocket leaves. By further EC increase from 5 to 7 dS m^{-1} and from 7 to 9 dS m^{-1} the trend of Cl and Na increase was continued, however, it was noticeably smaller than previously stated for control NS (EC 3) and with the smallest salt stress (EC 5). Content of Cl and Na in rocket growing in NS EC 7 were 1.8 and 1.6 times higher than in NS EC 5 while in NS EC 9 were only 1.3 and 1.2 times higher than in NS EC 7. According to Britto and Kronzucker (2006) in conditions of extremely higher ion concentration of NS, ratio of released and absorbed ions by plant root is decreasing due to increased ion release. The markedly highest Cl and Na ratio had rocket from control NS (EC 3), while salt stress resulted in lower ratio which increased slightly with NaCl concentration increase.

Table 2. The content (mg kg^{-1}) and ratio of chlorine and sodium of fresh rocket leaves

Treatment	Cl	Na	Cl : Na
EC 3	870.33±26 D	201.33±7 D	4.32
EC 5	3219±74 C	2491.33±26 C	1.29
EC 7	5775±90 B	4082.67±42 B	1.41
EC 9	7447.67±25 A	4851.67±95 A	1.53

EC 3- standard nutrient solution; EC 5- nutrient solution with 2.92 g L^{-1} NaCl; EC 7- nutrient solution with 5.27 g L^{-1} NaCl; EC 9- nutrient solution with 7.62 g L^{-1} NaCl; Different letters indicate significant differences between mean values within each column at $p \leq 0.01$.

Specialized metabolites

Specialized metabolites exhibit different responses to stress conditions regardless of biotic or abiotic impacts. Salinity stress is one of the main challenges in vegetable production since some vegetable species are extremely intolerant to it, while in opposite most of species from Brassicacae family show good salt tolerance (Wink, 2010). Table 3 shows significant statistical differences between researched treatments for all analyzed specialized metabolites of rocket fresh leaves. The lowest vitamin C content was determined in rocket grown in control NS (EC 3) and NS with 5.27 g L^{-1} NaCl (EC 7), while even 60% higher value was determined in treatment EC 9 with the highest concentration of NaCl in NS. Different authors cited different effects of salinity on vitamin C content. Barbieri et al. (2010) concluded that salt stress has a beneficial effect on the increase of vitamin C content while Bonasia et al. (2017) cites opposite results. Plant species differ in the accumulation of phenolic compounds as a reaction to salt stress, which positively affected the content of total phenols but only to a certain limit value, depending on the plant species (Rezazadeh et al., 2012). In this research significant increase of total phenol content (TPC) was determined by increasing the NaCl concentration in NS (Table 3). The highest TPC was determined in treatment EC 7 and EC 9, respectively, in rocket cultivated in NS with the highest concentration of NaCl (5.27 and 7.62 g L^{-1}). Same trend was also determined for total non-flavonoid content (TNFC), which also increased with the increase of NaCl concentration of NS. Salt stress positively affected the accumulation of analyzed phenolic compounds with an overall increase of TPC for 15% and TFNC for 26% between control treatment (EC 3) and EC 9 treatment. Glucosinolates (GLS) accumulation is stimulated by various stress conditions, especially abiotic (Kim and Ishi, 2007). Significantly higher total GLS content was determined in treatments EC 3 and EC 5, respectively, in rocket cultivated in NS with lower NaCl concentration. Higher NaCl concentration contributed to a reduction of GLS (Table 3). Obtained results are in agreement with similar research on rocket conducted by Bonasia et al. (2017), which is

in disagreement with characteristics of mentioned plant species. Rocket has a high tolerance to higher presence of salt in the soil and tends to grow and propagate on salty and dry soils (Garg and Sharma, 2014). Therefore, it is possible that the intensity of salt stress which would stimulate the GLS synthesis was not achieved by conditions set in this research. Antioxidant capacity of treatments is significantly different (Table 3) with the highest capacity in control treatment (EC 3) and the lowest in EC 7 treatment. Namely, antioxidant capacity is directly related to the content of bioactive compounds which indicates that rocket with highest content of mentioned specialized metabolites exhibits the highest antioxidant capacity. According to the results from this research, treatments with the highest determined content of GLS show a higher antioxidant capacity.

Table 3. The content of specialized metabolites and antioxidant capacity of fresh rocket leaves

Treatment	VIT C (mg 100 g^{-1})	TPC (mg GAE 100 g^{-1})	TNFC (mg GAE 100 g^{-1})	GLS (%)	ANT_CAP (mmol TE kg^{-1})
EC 3	56.43±2.91 C	150.33±1.26 B	65.61±2.47 B	0.45±0.01 A	2.03±1.12 A
EC 5	62.52±3.16 B	135.15±0.90 C	53.51±0.79 C	0.45±0.21 A	2.01±0.94 AB
EC 7	53.68±0.63 C	173.63±2.69 A	84.80±5.00 A	0.39±0.01 B	1.99±3.94 B
EC 9	88.07±0.01 A	173.44±1.33 A	82.75±3.60 A	0.40±0.01 B	2.00±1.82 AB

EC 3- standard nutrient solution; EC 5- nutrient solution with 2.92 g L^{-1} NaCl; EC 7- nutrient solution with 5.27 g L^{-1} NaCl; EC 9- nutrient solution with 7.62 g L^{-1} NaCl; VIT C- vitamin C content; TPC- total phenol content; TNFc- total non-flavonoid content; GLS- total glucosinolates content; ANT_CAP- antioxidant capacity. Different letters indicate significant differences between mean values within each column at $p \leq 0.01$.

Pigment compounds, such as chlorophylls and carotenoids as one of the most widespread, also show a significant role as specialized metabolities. Different researches indicate the negative impact of salt stress on chlorophyll content (Taibi et al., 2016; Bonasia et al., 2017), as confirmed by the results of this study. Statistically significant differences between treatments were determined for chlorophyll a (CHa), total chlorophyll (TCH) and total carotenoid (TC) content (Table 4). Highest chlorophyll content (Cha and TCH) was determined in control treatment (EC 3), while even under mild salt stress conditions (EC 5) a decrease of mentioned pigments was recorded. Except TCH content, a mild decrease of total carotenoid content (TC) was also recorded, but with one exception, the highest TC content was determined in treatment EC 7. It is important to emphasize that mentioned decrease of analyzed pigment content (TCH) in salt stress conditions is not so evident and according to literature data different vegetable species show a large deviation in the concentration of NaCl at which the pigments are reduced (Neocleous et al., 2014). Still, for rocket, a threshold tolerance level to a salt stress is not determined. The fact that rocket is a species well tolerant to salty and dry soil should not exclude the possibility that at higher NaCl concentrations the accumulation of pigments will be greater.

Table 4. The content of pigment compounds of fresh rocket leaves

Treatment	CHa (mg g^{-1})	CHb (mg g^{-1})	TCH (mg g^{-1})	TC (mg g^{-1})
EC 3	0.55±0.04 A	0.34±0.06	0.90±0.09 A	0.15±0.01 AB
EC 5	0.42±0.04 B	0.31±0.04	0.73±0.07 AB	0.13±0.02 B
EC 7	0.46±0.04 B	0.26±0.03	0.72±0.07 B	0.17±0.01 A
EC 9	0.47±0.02 AB	0.34±0.01	0.80±0.01 AB	0.12±0.02 B

EC 3- standard nutrient solution; EC 5- nutrient solution with 2.92 g L^{-1} NaCl; EC 7- nutrient solution with 5.27 g L^{-1} NaCl; EC 9- nutrient solution with 7.62 g L^{-1} NaCl; CHa- chlorophyll a content; CHb- chlorophyll b content; TCH- total chlorophyll content; TC- total carotenoid content. Different letters indicate significant differences between mean values within each column at $p \leq 0.01$. Column without letters shows non-significant differences between mean values at $p \leq 0.01$.

CONCLUSION

Despite the positive effect of salt stress on dry matter content of rocket leaves, it negatively affected the content of macroelements, but increase of NaCl concentration of nutrient solution above 5.27 g L^{-1} did not affect the further significant reduction of the nitrogen, phosphorus, potassium and magnesium content. Salt stress differentially affects the accumulation of different bioactive compounds in the rocket leaves. Salt stress had a positive impact on the accumulation of vitamin C and total phenol, while on the accumulation of total chlorophyll and glucosinolates had adverse effects. More significant accumulation of vitamin C was determined only at the highest concentration of NaCl (EC 9) in nutrient solution, while the more significant accumulation of total phenols was determined at a NaCl concentration of 5.27 g L^{-1} (EC 7).

REFERENCES

AOAC (1995). Official methods of Analysis 16th Ed. Association of Official Analytical Chemists, Washington, USA.

AOAC (2002). Official methods of Analysis 17th Ed. Association of Official Analytical Chemists, Washington, USA.

Barbieri G., Bottino A., Di Stasio E., Vallone S., Maggio A. (2010). Proline and light as quality enhancers of rocker (*Eruca sativa* L.) grown under saline conditions. Scientia Horticulturae, 128: 393-400.

Bonasia A., Lazzizera C., Elia A., Conversa G. (2017). Nutritional, biophysical and physiological characteristics of wild rocket genotypes as affected by soilless cultivation system, salinity level of nutrient solution and growing period. Frontiers in Plant Science, 8(300), doi: 10.3389/fpls.2017.00300. eCollection 2017.

Britto D.T., Kronzucker H.J. (2006). Futile cycling at the plasma membrane: a hallmark of low-affinity nutrient transport. Trends in Plant Science, 11(11): 529-34.

Cavaiuolo M., Ferrante A. (2014). Nitrates and glucosinolates as strong determinants of the nutritional quality in rocket leafy salads. Nutrients, 6(4): 1519-1538.

Garg G., Sharma V. (2014). *Eruca sativa* (L.): Botanical Description, Crop Improvement, and Medicinal Properties. Journal of Herbs, Spices & Medicinal Plants, 20(2): 171-182.

Holm, G. (1954). Chlorophyll mutations in barley, Acta Agriculturae Scandinavica, 4, 457-471.

Jezek J., Haggett B.G.D., Atkinson A., Rawson, D.M., 1999. Determination of glucosinolates using their alkaline degradation and reaction with ferricyanide. Journal of Agricultural and Food Chemistry, 47: 4669-4674.

Kim S.J., Ishii G. (2007). Effect of storage temperature and duration on glucosinolate, total vitamin C and nitrate contents in rocket salad (Eruca sativa Mill.). Journal of the Science of Food and Agriculture, 87:966-973.

Miller, N.J., Diplock, A.T., Rice-Evans, C., Davies, M.J., Gopinathan, V., Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates, Clinical Science, 84, 407-412.

Neocleous D., Koukounaras A., Siomos A. S., Vasilakakis M. (2014). Changes in photosynthesis, yield, and quality of baby lettuce under salinity stress. Journal of Agricultural Science and Technology, 16(6): 1335-1343.

Ough, C.S., Amerine, M.A. (1988). Methods for Analysis of Musts and Wines, John Wiley and Sons, New York.

Prasad S.M., Parihar P., Singh V.P. (2014). Effect of salt stress on nutritional value of vegetables. Biochemistry & Pharmacology 3(2).

Rezazadeh A., Ghasemnezhad A., Barani M., Telmadarrehei T. (2012) Effect of Salinity on Phenolic Composition and Antioxidant Activity of Artichoke (Cynara scolymus L.) Leaves. Research Journal of Medicinal Plants, 6: 245-252.

Selmar D., Kleinwachter M. (2013). Stress enhances the synthesis of secondary plant products: The impact of stress-related over-reduction on the accumulation of natural products. Plant Cell Physiology, 54(6): 817–826.

Taïbi K., Taïbi F., Abderrahim L.A., Ennajah A., Belkhodja M., Mulet J.M. (2016). Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in Phaseolus vulgaris L. South African Journal of Botany, 105, 306-312.

Wettstein, D. (1957). Chlorophyll letale und der submikroskopische Formwechsel der Plastiden, Experimental Cell Research, 12, 427-434.

Wink, M. (2010). Functions and Biotechnology of Plant Secondary Metabolites. Wiley-Blackwell (2nd ed.), Oxford, UK.

Zuccarini P. (2007). Mychorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. Plant and Soil Environment, 53(7): 283-289.

Author index

SPONSORS

Ministry of
Science and Education

Ministry of Agriculture

Ministry of Health

Croatian Chamber
of Economy

Croatian Academy
of Sciences and Arts

Lika-Senj County

Varaždin County

Zadar County

Istrian County

SPONSORS

PODRAVKA

Jamnica ~1828~

IREKS

KemoLab d.o.o.

KEFO SINCE 1949

ALPHA CHROM

Ru-Ve

cromaris

KOBIS

gnosis advanced biotech

HRVATSKO DRUŠTVO ZA BIOTEHNOLOGIJU
CROATIAN SOCIETY OF BIOTECHNOLOGY
KROATISCHE GESELLSCHAFT FÜR BIOTECHNOLOGIE
SOCIÉTÉ CROATE DU GENIE BIOLOGIQUE

SANTA ANA GASTRO SUVENIRI