ISSN (1230-0322)

2020, Vol. 70, No. 2

Food

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica

Published

by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Published since 1957 as
Roczniki Chemii i Technologii Żywności and Acta Alimentaria Polonica (1975–1991)

SCOPE: The Journal covers fundamental and applied research in food area and nutrition sciences with a stress on interdisciplinary studies in the areas of food, nutrition and related subjects.

POLICY: Editors select submitted manuscripts in relation to their relevance to the scope. Referees are selected from the Advisory Board and from Polish and international scientific centres. Identity of referees is kept confidential.

AUTHORSHIP FORMS referring to Authorship Responsibility, Conflict of Interest and Financial Disclosure, Copyright Transfer and Acknowledgement, and Ethical Approval of Studies are required for all authors.

FREQUENCY: Quarterly – one volume in four issues (March, June, September, December).

COVERED by Web of Science, Current Contents/Agriculture, Biology & Environmental Sciences, Journal Citation Reports and Science Citation Index Expanded, BIOSIS (Biological Abstracts), SCOPUS, FSTA (formerly: Food Science and Technology Abstracts), CAS (Chemical Abstracts), AGRICOLA, AGRO-LIBREX data base, EBSCO, FOODLINE, Leatherhead FOOD RA data base FROSTI, AGRIS and Index Copernicus data bases, Polish Scientific Journals Contents (PSJC) Life Science data base available at http://psjc.icm.edu.pl and any www browser; ProQuest: The Summon, Bacteriology Abstracts, Immunology Abstracts.

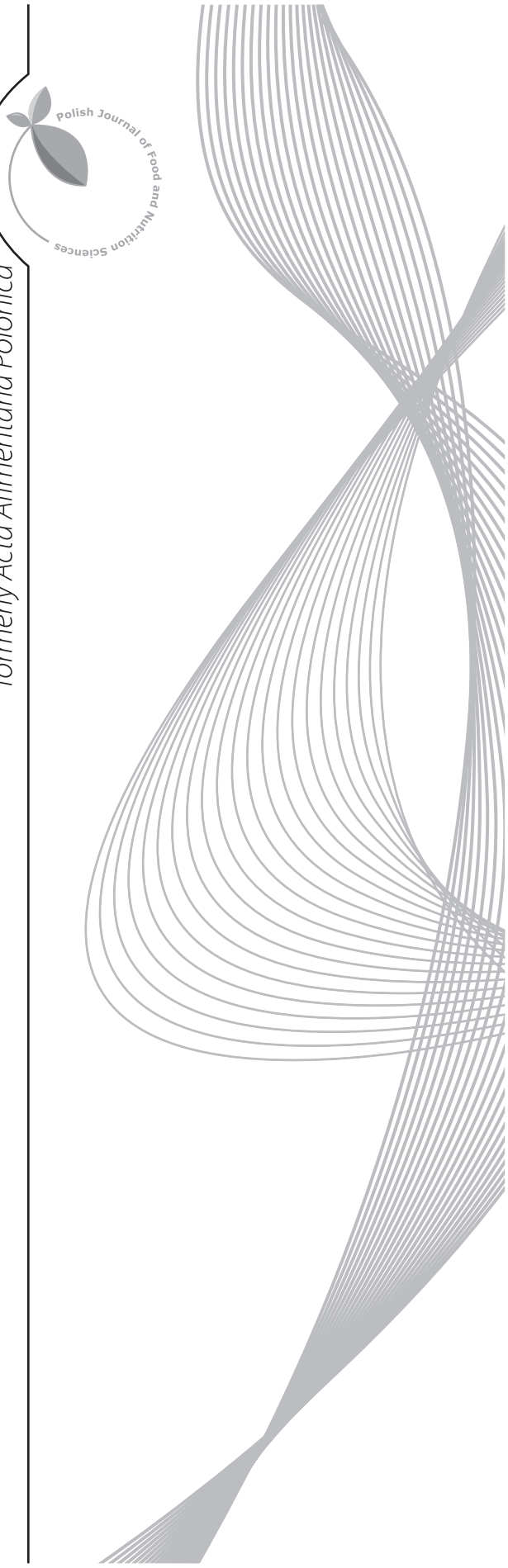
EDITORIAL AND BUSINESS CORRESPONDENCE: Submit contributions (see Instructions to Authors) and address all communications regarding subscriptions, changes of address, etc. to:

CORRESPONDENCE TO: Ms. Joanna Molga
Polish Journal of Food and Nutrition Sciences
Institute of Animal Reproduction and Food Research
of Polish Academy of Sciences
ul. Tuwima 10, 10-747 Olsztyn, Poland
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

ISSN (1230–0322)
2 0 2 0 , Vol. 70, No. 2

Polish Journal of Food and Nutrition Sciences

formerly Acta Alimentaria Polonica



Published

by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn

Pol. J. Food Nutr. Sci. 2020, Vol. 70, No. 2

Advisory Board of PJFNS 2019–2022

Pol. J. Food Nutr. Sci. 2020, Vol. 70, No. 2

POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
covered by CURRENT CONTENTS/AGRICULTURE, BIOLOGY & ENVIRONMENTAL SCIENCES, JOURNAL CITATION REPORTS AND SCIENCE CITATION INDEX EXPANDED, BIOSIS, SCOPUS, IFIS Publishing and CAS abstracts, and AGRO-Librex, FROSTI, PSJC, AGRIS and Index Copernicus data bases
2020, VOL. 70, NO. 2

REVIEW

ORIGINAL PAPERS

Ministerstwo Nauki
i Szkolnictwa Wyższego

KOMUNIKAT

W latach 2019–2020 kwartalnik naukowy ***Polish Journal of Food and Nutrition Sciences*** realizuje zadania z zakresu digitalizacji i upowszechniania publikacji naukowych, które ukazują się na jego łamach oraz utrzymania procedur zapewniających ich oryginalność, w ramach zadania nr 1 pt. ***Digitalizacja artykułów publikowanych w kwartalniku PJFNS w celu zapewnienia otwartego dostępu do nich przez sieć Internet*** oraz zadania nr 2 pt. ***Utrzymanie procedur zabezpieczających oryginalność artykułów naukowych publikowanych w kwartalniku PJFNS***. Oba zadania finansowane są na podstawie umowy nr 607/P-DUN/2019 ze środków Ministra Nauki i Szkolnictwa Wyższego przeznaczonych na działalność upowszechniającą naukę.

ANNOUNCEMENT

In the years 2019–2020, the scientific quarterly *Polish Journal of Food and Nutrition Sciences* accomplishes tasks in the scope of digitalization and dissemination of published articles and in the scope of ensuring originality of published works in the framework of task no. 1: ***Digitalization of scientific manuscripts published in PJFNS quarterly to enable open access to them in the Internet*** and task no. 2: ***Maintenance of procedures assuring originality of scientific manuscripts published in PJFNS quarterly***. Both tasks are financed under the agreement no. 607/P-DUN/2019 from funds of the Minister of Science and Higher Education intended for science dissemination activities.

Subscription

2020 – One volume, four issues per volume. Annual subscription rates are: Poland 150 PLN, all other countries 80 EUR. Prices are subject to exchange rate fluctuation. Subscription payments should be made by direct bank transfer to Bank Gospodarki Żywnościowej, Olsztyn, Poland, account No 17203000451110000000452110 SWIFT code: GOPZPLWOLA with corresponding banks preferably. Subscription and advertising offices at the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, ul. J. Tuwima 10, 10-747 Olsztyn, Poland, tel./fax (48 89) 5234670, fax (48 89) 5240124, e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Zamówienia prenumeraty: Joanna Molga (e-mail: pjfns@pan.olsztyn.pl)

Wersja pierwotna (referencyjna) kwartalnika PJFNS: wersja papierowa (ISSN 1230–0322)
Nakład: 100 egz.; Ark. wyd. 17; Ark. druk. 17
Skład i druk: Mercurius, www.mercurius.com.pl

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 85–99
DOI: 10.31883/pjfns/113272
http://journal.pan.olsztyn.pl

Review article
Section: Food Quality and Functionality

Pigmented Maize (*Zea mays* L.) Contains Anthocyanins with Potential Therapeutic Action Against Oxidative Stress – A Review

*Jesús Miguel Magaña-Cerino[1], Héctor Arturo Peniche-Pavía[2], Axel Tiessen[2,3], Carmen Magdalena Gurrola-Díaz[1]**

[1]Instituto de Investigación en Enfermedades Crónico Degenerativas, Instituto Transdisciplinar de Investigación e Innovación en Salud, Departamento de Biología Molecular y Genómica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, C. Sierra Mojada 950, Col. Independencia, C. P. 44350, Guadalajara, Jalisco, México
[2]Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional Unidad Irapuato, Libramiento Norte Km. 9.6, C. P. 36824, Irapuato Guanajuato, México
[3]Laboratorio Nacional PlanTECC, Libramiento Norte Km 9.6, C. P. 36824 Irapuato, Guanajuato, México

Key words: *Zea mays* L., anthocyanins, oxidative stress, antioxidant, *in vivo* and *in vitro* effect

Different maize (*Zea mays* L.) varieties have been used for thousands of years as a healthy food source in Mesoamerica including pigmented maize. Maize ingestion could contribute to the reduction in the rate of non-communicable diseases and, in turn, to its function as an adjuvant in their management. These diseases are mainly associated with oxidative stress, which is characterized by a redox cell imbalance produced due to pro-oxidant molecules accumulation, inducing irreversible damages. Although the endogenous antioxidant defense system is efficient, exogenous antioxidants are necessary to help to prevent this damage. Bioactive compounds, like anthocyanins, contained in dietary plants exert a major activity against oxidative stress. Could the maize anthocyanins play a curative, preventive or complementary role in the treatment of chronic diseases? Here, we describe the occurrence of anthocyanins from pigmented maize and their chemical structures. Furthermore, the biosynthesis, bioavailability, and stability are also summarized. Finally, many *in vitro* and *in vivo* studies of maize anthocyanins are discussed that demonstrated their nutraceutical potential, antioxidant capacity, and other biological effects. Given the importance of the biological properties of maize anthocyanins, it is necessary to understand the current knowledge and propose further research or clinical studies which allows us to better elucidate the biological mechanism of maize anthocyanins derivatives of several varieties and processes of cooking and combination with other ingredients to enhance their nutritional and health benefits.

INTRODUCTION

Oxidative stress is a condition that begins when the redox balance of living organisms is altered by an excessive formation of radical molecules, which exceeds the endogenous antioxidant capacity [Lobo *et al*., 2010]. Usually, the electron transport chains in the mitochondria utilize oxygen to convert nutrients into adenosine triphosphate (ATP) and through this process generate radicals, which are used to carry out cellular signaling processes. However, various environmental, physical, and chemical factors can cause the excessive generation of these radicals, resulting in the chemical imbalance of biomolecules and cellular stress [McCord, 2000].

The main feature of this condition is an increase in free radicals and reactive species such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (HO^-), singlet oxygen ($^1O_2$), alkoxy radical (RO), and peroxyl radical ($ROO^\bullet$). These compounds cause necrosis, apoptosis, damage, and cell death due to modification in the structure and function of macromolecules such as lipids, proteins (in biological membranes and tissues), and deoxyribonucleic acid (DNA) [Birben *et al*., 2012].

Although the endogenous antioxidant defense system is efficient, sometimes it is overwhelmed and needs exogenous antioxidants. Plant and animal reducing compounds such as vitamin C, vitamin E, carotenoids (xanthophylls and carotenes), anthocyanins, chalcones, isoflavones, tannins, flavandiols, and flavonols can restore the oxidative balance [Kasote *et al*., 2015]. Several epidemiological studies have demonstrated that individuals who consumed fruits, vegetables, and whole grains with anthocyanins, were at a lower risk to develop chronic diseases such as cancer, diabetes, and cardiovascular disease (CVD) [Pandey & Rizvi, 2009]. Most of these beneficial health effects are attributed to exogenous antioxidant compounds ingested from plant sources at different concentrations. Anthocyanins are among the bioactive compounds that stand out for their beneficial properties [He & Giusti, 2010].

Maize is widely distributed and consumed around the world, especially in developing countries. Because of this, some researchers have focused their efforts on the generation of new varieties of pigmented maize through cross-hybridization to obtain new varieties of grains with a greater

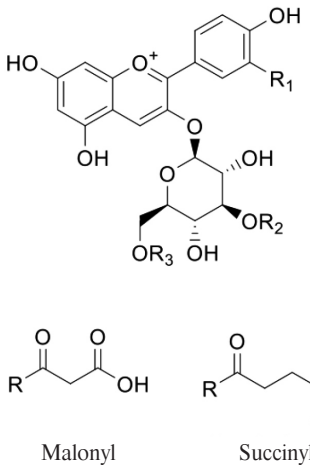
* Corresponding Author: Tel. +52 (33) 10585200 Ext. 33930
E-mail: carmenhpv@yahoo.de (C.M. Gurrola-Díaz)

antioxidant capacity [Tiessen *et al.*, 2017]. Therefore, such improved maize varieties could function as bioactive agents and potential candidates to be included in the prevention and integral management of chronic degenerative diseases, and in reducing their incidence [Chander *et al.*, 2008]. This review aimed to collect data on the effect of anthocyanins present in pigmented maize. They were considered regarding their metabolism, as well as their intervention in the antioxidant and modulating capacity of several cellular processes involved in oxidative stress such as cell growth, cell proliferation and cell death.

OCCURRENCE OF ANTHOCYANINS AND THEIR CHEMICAL STRUCTURES

Anthocyanins are responsible for many colors (blue, blue-black, red, and purple) of leaves, flowers, fruits, seeds, roots, and stems. In cereal grain (a kind of single-seed fruit), the anthocyanins are localized mainly in the cells of pericarp, testa, and aleurone layer, which depends on many structural and regulatory genes [Fan *et al*., 2016; Li *et al*., 2018]. Several plant foods contain different concentrations of anthocyanins including: berries (*Morus* and *Rubus* gender), grapes (*Vitis*), plums and cherries (*Prunus*), red pear (*Pyrus*), red apple (*Malus*), pomegranate (*Punica*), blackcurrants (*Ribes*), raspberry (*Rubus*), strawberries (*Fragaria*), chili (*Capsicum*) [Aza-Gonzalez & Ochoa-Alejo, 2012], camu-camu (*Myrciaria dubia*) [Langley *et al.*, 2015], açaí (*Euterpe oleracea*) [Peixoto *et al.*, 2016], cacao (*Theobroma)* [Chavez-Rivera & Ordoñez-Gomez, 2013], and calyces of *Hibiscus sabdariffa* L. (Jamaica) [Gurrola-Diaz *et al.*, 2010], as well as such red radishes, red peppers, tomatoes, red onions, purple and red-fleshed potatoes (*Solanum Tuberosum* L.) [Jansen & Flamme, 2006], purple cabbage (*Brassica oleracea* var. *capitate*), and eggplant (*Solanum melongena* L.) [Sadilova *et al.*, 2006]. Anthocyanins are also present in grains such as in the color-varieties of corn (*Zea*), wheat (*Triticum*), rice (*Oryza*) [Goufo & Trindale, 2014], and bean (*Phaseolus)* [Takeoka *et al.*, 1997]. These foods (seeds, fruits, and vegetables) are the main source of natural pigments, which providing them unique colors and flavors and make them attractive for consumption, and eventually may serve in a complementary natural treatment of chronic diseases. In maize, they accumulate in structures such as stem, pod, leaves and inflorescences; in the cob, they are found in bracts, rachis, and kernels [Cui *et al.*, 2012]. Chemically, the anthocyanins are polyhydroxy/polymethoxy glycosides derived from the anthocyanidins. They are formed by an anthocyanidin molecule, that is the aglycone, to which a sugar moiety is bound by a β-glycosidic bond or, in some cases, by α-glycosidic bond. There are approximately 20 types of anthocyanidins in nature, but only six: pelargonidin (Pl), cyanidin (Cy), peonidin (Peo), delphinidin (Dp), petunidin (Pt) and malvidin (Mv), are widely distributed [Tsuda, 2012]. The name of these compounds is derived from the plant source from which they were first isolated. The basic chemical structure of aglycones is the flavylium ion, also called 2-phenyl-1-benzopyrylium (2-phenylchromenyl; IUPAC). It consists of a 15-carbon skeleton (C6-C3-C6) organized into two aromatic groups: benzopyrylium/chromenium, and phenolic (also known as hydroxycinnamoyl) (see Figure 1). Because of the trivalent character of oxygen, flavylium typically functions as a cation (oxonium ion) or flavylium salt [De Rosso *et al*., 2008].

The monosaccharides commonly bound by *O*-glycosylation to the anthocyanidins, are glucose and rhamnose followed by galactose, xylose, arabinose, and occasionally, gentiobiose and raffinose [Horbowicz *et al*., 2008; Yonekura-Sakakibara *et al*., 2012; Fang, 2014]. All of them bind to anthocyanidin primarily by the hydroxyl group at the 3-position and secondarily at the 5 or 7-position but can also be found at positions 3', 5', or 6', but rarely at the 4' position. When two sugars are present in the same molecule, they are located in the 3 and 5 hydroxyl groups, producing a more stable structure than when a molecule contains only a single monosac-



R_1	Anthocyanidin
-H	Pelargonidin
-OH	Cyanidin
-OMe	Peonidin

R_2	R_3	Name
H	H	Anthocyanidin-3-O-β-glucopyranoside
H	Malonyl	Anthocyanidin-3-O-(6''-malonyl-glucoside)
Malonyl	Malonyl	Anthocyanidin-3-O-(3'', 6''-dimalonyl-glucoside)
H	Malonyl	Anthocyanidin-3-O-(6''-succinyl-glucoside)
Succinyl	Succinyl	Anthocyanidin-3-O-(3'', 6''-disuccinyl-glucoside)
Malonyl	Succinyl	Anthocyanidin-3-O-(malonyl, succinylglucoside)

FIGURE 1. The structure of anthocyanin compounds isolated from maize. The enzymatic system of maize permits to yield three different anthocyanidins: pelargonidin, cyanidin, and peonidin, as well as their acylated derivatives: malonylated and succinylated ones [Abdel-Aal *et al*., 2006; Fossen *et al*., 2001].

charide. The combination of the anthocyanidins with the different sugars generates more than 700 different anthocyanins [Wallace & Giusti, 2015]. The degree of glycosylation has a significant impact on the biological activity of the anthocyanin, depending on the chemical nature (type of carbohydrate) as well as the number and position of the glycosylation sites [Zhao *et al*., 2014]. Cereals like maize produce anthocyanins monoglycosylated with glucose, and later do acylation with one or two malonyl-CoA or succinyl-CoA [Fossen *et al*., 2001; Abdel-Aal *et al*., 2006] (see Figure 1).

Cyanidin is the major constituent of the pigmented maize grain and exhibits a significant antioxidant activity in comparison with that of the non-pigmented maize. In fact, its phytochemical composition and concentration of bioactive compounds vary depending on grain color, with a wide variety of tones ranging from black, blue, purple, red, yellow to white. Because it is a staple food providing a high anthocyanin content, there has been scientific interest in the study of pigmented maize and its health benefits [Lopez-Martinez *et al*., 2009]. Particularly, a similar profile of anthocyanin has been identified with different extraction methods (microwave, ultrasound, and maceration) like in the case of: cyanidin-3-(6''malonyl)--glucoside, cyanidin-3-*O*-glucoside and mainly peonidin--3-*O*-glucoside, pelargonidin-3-*O*-glucoside, pelargonidin-3--(6''malonyl)-glucoside, and peonidin-3-(6''malonyl)-glucoside [Salinas-Moreno *et al*., 2005a; Yang & Zhai, 2010; Camelo-Méndez *et al*., 2016; Li *et al*., 2017; Chen *et al*., 2018; Fernandez-Aulis *et al*., 2019], however some differences in the anthocyanin profile are due to the environmental factors, maturity stages, and parts of the plant (husk, cob, and leaf) [Fossen *et al*., 2001; Li *et al*., 2008].

STABILITY OF ANTHOCYANINS

Anthocyanins are unstable and susceptible to degradation upon the effects of temperature, light, enzymes, pH, and oxygen radicals. All these factors can affect their stability as well as their coloration intensity [Cevallos-Casals *et al*., 2004]. In aqueous solutions, anthocyanins suffer structure intramolecular adjustment mainly caused by the electronic deficiency (positive charge) of the flavylium nuclei. In vacuolar pH (4–6), the principal red flavylium (stable at <3) undergoes a proton loss, a water molecule addition, and an intramolecular rearrangement, generating the quinoid form with blue/violet coloration (at pH = 4), the colorless carbinol (at pH = 5), and chalcones with yellow coloration (at pH = 6), respectively [Kallam *et al*., 2017]. If pH keeps rising, the anionic forms start to accumulate, shifting the color of medium to green, when the ionized chalcone and ionized quinoid are in the equilibrium state at pH 8–10 [Levi *et al*., 2004]. At pH values greater than 12, dianion chalcone is the major compound, producing a yellow color in the solution [Petrov *et al*., 2013] (see Figure 2).

Two processes which can affect the stability and color of anthocyanins from maize are: the co-pigmentation, and the acylation [Cooper-Driver, 2001]. The co-pigmentation effect is a non-covalent intermolecular interaction with other phenolic compounds through a type π-stacking interaction (a property of aromatic rings), which enables the hy-

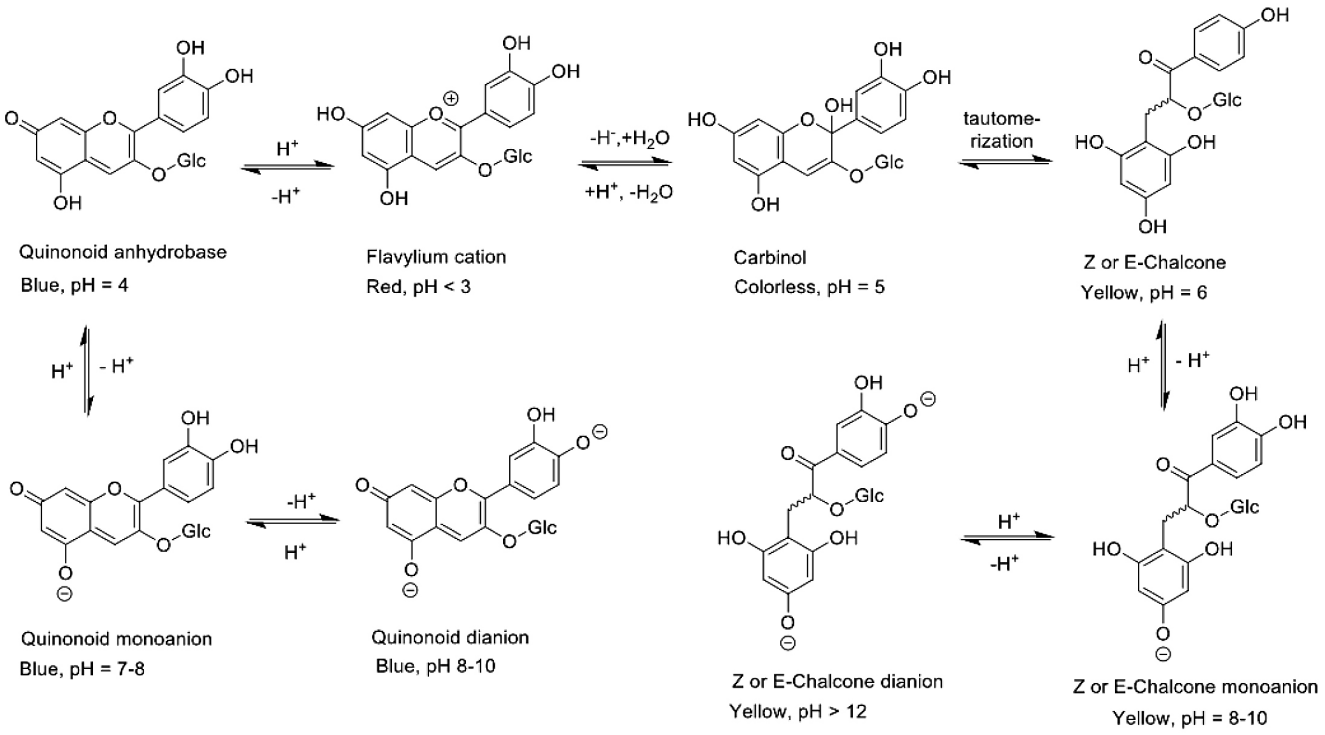


FIGURE 2. Anthocyanin structural changes in aqueous medium at different pH. Cyanidin-3-*O*-glucoside is the structure shown; contains the most abundant aglycone in purple maize. In low pH (<3), the flavylium cation is the major chemical species, quinoid form with blue/violet coloration (pH = 4), colorless carbinol (pH = 5), and chalcones with yellow coloration (pH = 6). The ionized chalcone and quinoid forms are in the equilibrium state at pH 8–10. At pH values greater than 12, the yellow dianion chalcone is the major compound.

perchromic shift in mildly acidic conditions through stabilizing blue quinoid species instead of carbinol compound in an aqueous medium [Mazza & Brouillard, 1990]. The acylation with hydroxyl cinnamic acids (HCAs) can increase the number of interactions due to an extra phenyl ring [Gläßgen & Seitz, 1992]. In the case of maize, the acylation occurs with malonyl and succinyl moieties, which cannot stabilize further the co-pigmentation, as happens for the acylation with HCAs [Harakotr *et al*., 2014]. Most of the aliphatic acylations are lost under an alkaline pH, such as the nixtamalization process [Mora-Rochín *et al*., 2016].

ANTHOCYANINS IN PIGMENTED MAIZE (*ZEA MAYS* L.)

The prehispanic cultures named the maize cob "*centli*" and the kernel "*tlaolli*" in their Nahuatl language. The earlier evidence for maize (*Zea mays* L.) domestication was estimated 8,700 calendrical years ago, found in Xihuatoxtla Shelter in Guerrero, Mexico [Piperno *et al*., 2009]. This grain constitutes one of the main sources of calories, proteins, and vitamins for the rural inhabitants of many regions including Mexico, central and north America. People in these regions have incorporated pigmented maize into their regular diets [Betrán *et al.*, 2000]. There are many varieties of maize with different nutritional values, of which four varieties stand out: blue, red, yellow, and white [de la Parra *et al*., 2007]. Maize is commonly consumed in the form of dry mass flour to make "tortillas", chips, tamales, "gorditas", and many other basic foodstuffs of the Mexican and Latin American (*e.g.* different types of beverages).

Pigmented maize is a major source of starch, proteins, fats, sugars, anthocyanins, salicylic acid, resins, saponins, potassium, sodium salts, sulfur, phosphorus, and other phenolic compounds [Pedreschi & Cisneros-Zevallos, 2007]. High concentrations of anthocyanins and phenolic compounds are distributed in the aleurone and pericarp monolayer of the grain, which provides the characteristic blue, red, purple, and black color to the maize varieties [Espinosa Trujillo *et al.*, 2009]. Red and blue/purple beads are the most common [Salinas *et al.*, 1999] and due to genetic variations, at least 59 races have been described in Mexico, many of them corresponding to variants of pigmented grains [Sanchez *et al.*, 2000; Salinas-Moreno *et al.*, 2013]. Žilić *et al*. [2012] reported differences in the anthocyanins content in several varieties of pigmented maize ranging from 2.50 to 696.07 mg CGE/kg d.m. (cyanidin-3-*O*-glucoside equivalent per kilogram of dry mass). In this work, cyanidin-3-*O*-glucoside was the most abundant anthocyanin contained in the light blue maize variety. In another study conducted on blue maize planted in some regions of Mexico (Querétaro and Chihuahua States), Urias-Lugo *et al.* [2015a] confirmed the highest concentration at 1052 mg Cy3glu/kg (cyanidin-3-*O*-glucoside for each kg of sample) of anthocyanins in the grain.

Anthocyanins in pigmented grains hold the promise of being functional food compounds due to their antioxidant and anti-inflammatory activity, phase II enzyme activators, anti-cell proliferation, and hypoglycemic effect [Tsuda *et al.*, 2003; Urias-Peraldi *et al*., 2013]. Generally, the seeds have a high proportion of pigments in the aleurone layer and, to a lesser extent, in the starchy endosperm [Betrán *et al.*, 2000]. The diversity of the varieties of pigmented maize is due to the physical characteristics such as the variability in the size, density, hardness, and chemical composition of the grain as well as environmental factors such as the climatic conditions where it grows, soil type, cultivation practices and finally, to specific genetic factors of each variety. The interaction of these factors provides to each variety of pigmented maize a unique observable characteristic such as coloration; but also, a different biological activity based on the quantity and profile of secondary metabolites, which enhances its functional food potential. For example, in light red-colored grains, the most abundant anthocyanidin is the pelargonidin [Salinas *et al.*, 1999; Abdel-Aal *et al.*, 2006], while in the blue grains (blue, purple, and black) the cyanidin derivatives prevail [Pedreschi & Cisneros-Zeballos, 2007; Zhao *et al.*, 2009]. In magenta red kernels, cyanidin derivatives predominate but they also contain derivatives of pelargonidin and peonidin [Salinas-Moreno *et al*., 2005a]. Specifically, the main anthocyanins reported in maize are cyanidin 3-*O*-glucoside and pelargonidin 3-*O*--glucoside that have been described in a relatively high proportion (about 40%) as malonylated *i.e.*, cyanidin 3-*O*-(6"-malonyl-glucoside) and cyanidin 3-*O*-(3",6"-dimalonyl-glucoside). The cobs of some landraces may contain kernels with up to three colors [Barrientos-Ramírez *et al*., 2018]. In the blue-grain varieties, the pigment is found in the aleurone layer, in those having light red grain – in the pericarp, and in those with magenta/red grain – it is located in both, the pericarp and aleurone layer [Salinas-Moreno *et al.*, 2012]. There are both acylated and non-acylated anthocyanins in pigmented maize grains. Compared to other cereals, pigmented maize from Mexico exhibits a high concentration of anthocyanins of acylated type, which increases its chemical stability at extreme conditions of pH and temperature [de Pascual-Teresa *et al.*, 2002; Salinas-Moreno *et al.*, 2005a]. The acylated anthocyanins identified in the maize grain were cyanidin-3-*O*--glucoside, pelargonidin-3-*O*-glucoside, and peonidin 3-*O*--glucoside [Styles & Ceska, 1972]. The sugar moiety might bind to one or more acyls (aliphatic acids such as malic, malonic, or succinic acid). In some cases, the acyl radicals can also come from cinnamic acids (*p*-coumaric, caffeic, ferulic or synaptic) acting on glucose and rhamnose [Wang *et al*., 1997].

Several studies support the role of anthocyanins in suppressing free radicals and thereby in helping prevent and/or treat certain diseases such as atherosclerosis, diabetes, hypertension, inflammation, cancer, and also aging [Liu *et al*., 2012]. In addition, there is evidence that hybrid maize may contain high levels of nutraceutical compounds and could be considered an advantage for commercial production of tortillas and related food products with added value [Urias-Peraldi *et al.*, 2013]. Currently in the food industry, maize anthocyanins have been used as dyes of many food products [Salinas-Moreno *et al*., 2005b]. However, the stability and function of maize anthocyanins depends on the type of cooking method, pretreatment, and combinations with other ingredients [Bello-Pérez *et al*., 2016].

"Nixtamalization" is the main process of transformation of maize for consumption that was developed by the Mexicas

before the Precolumbian era. The word derives from the Nahuatl – *nextli*, ashes, and *tamalli* – cooked maize dough. This technology is still widely used in the American continent to obtain a wide variety of food products. The "nixtamalization" process involves chemical, structural, and nutritional changes in the various constituents of the grain [Rojas-Molina *et al.*, 2007]. Because the nixtamalization process is a thermal-alkaline treatment that produces a decrease in anthocyanin content in pigmented maize, techniques such as fractional nixtamalization have been developed, which allows recovering approximately 58% of anthocyanins of pigmented maize probably because the endosperm and embryo layers are removed and nixtamalized separately. However, the nixtamalization by extrusion allows obtaining a greater quantity of anthocyanins in the blue maize (cyanidin 3-*O*-glucoside, equivalent to 620.9 mg/kg) than in those of red, yellow, and white colors. Additionally, different anthocyanin derivatives are formed or even increased during the process, which modifies the anthocyanin profile [Escalante-Aburto *et al*., 2013a,b]. Besides, the nixtamalization processing of Mexican blue maize landraces has been found to increase the relative percentage of non-acylated anthocyanins (cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside) and to decrease the relative percentage of acylated anthocyanins, such as cyanidin 3-*O*-(succinyl-glucoside) and cyanidine-3-*O*-(disuccinyl-glucoside), when compared to the raw kernels [Mora-Rochin *et al.*, 2016]. All this can be useful in the selection of varieties of pigmented maize and their use as food products with a nutraceutical potential; which makes it necessary to search for methods that would improve grain processing and enable retaining a higher concentration of phenolic compounds, and subsequently evaluating the potential benefits to health through daily consumption under a controlled trial.

BIOSYNTHESIS OF ANTHOCYANINS IN PIGMENTED MAIZE

The characteristic coloration of each variety of pigmented maize grain is contained into genes related to the biosynthetic pathway of anthocyanins. This also includes maize varieties with white and yellow grains. The regulation of this pathway is directed by three families of transcriptional factors: *r1*(*red1*)/*b1*(*booster1*) family that belongs to the class of transcription factors type bHLH (basic helix-loop-helix), *c1*(*colorless1*)/*pl1* (*purple plant1*)/*p1*(*pericarp color1*) family corresponding to the MYB-like transcription factors, and WD40 factor PAC1 (*pale aleurone color 1*) [Sharma *et al*., 2011; Hu *et al*., 2016]. The expression of each member of this family occurs in a tissue-specific manner or during plant development. The pigmentation patterns of maize depend to a large extent on the allelic combination of the MYB and bHLH loci [Shen & Petonilo, 2006]. For example, the dark blue color, typical of some grains of maize that develop in tropical climates, is generated by the allelic combination of the regulatory *pl1*/*B1* genes [Lago *et al*., 2013, 2014]. This allelic combination can activate mainly the synthesis of cyanidin-3-*O*-glucoside in the pericarp and some tissues of the maize plant. Whereas *r1*/*c1* combination is required for the accumulation of pelargonidin-3-*O*-glucoside in the aleurone layer [Cone, 2007; Sharma *et al*., 2011; Li *et al*., 2019].

BIOAVAILABILITY OF ANTHOCYANINS

In animal and human studies, it has been reported that anthocyanins are rapidly absorbed as glycosides in the stomach and small intestine, mainly in the jejunum section so that they can reach many peripheric tissues and modulate metabolic changes in the organism [Talavéra *et al.*, 2004; Kay *et al*., 2005]. On the other hand, despite the intake of anthocyanin-rich foods, the plasma concentration of these compounds remains very low [Manach *et al.*, 2005]. It is suggested that they have a low rate of bioavailability [Bitsch *et al.*, 2004; Felgines *et al.*, 2005; Tian *et al.*, 2006]. Some causes of low bioavailability are due to their chemical structure, excretion with feces, instability at neutral pH [Mullen *et al*., 2006; Bub *et al*., 2001], metabolism of the intestinal microbiota [Aura *et al.*, 2005], effect of the intestinal mucosa, and hepatic metabolism. Despite the low bioavailability, some beneficial effects may be caused by the metabolites such as protocatechuic acid (a major metabolite of anthocyanins in humans with antimicrobial and antioxidant effects) [Wang *et al.*, 2010].

The relative composition of the different molecular structures of the anthocyanins coexisting in an aqueous solution at any given time depends on the pH, temperature, concentration, and time. This is particularly important since anthocyanins are exposed to different pH conditions throughout the gastrointestinal tract, which affects their bioavailability and therefore their bioactivity and/or pharmacodynamics [McGhie & Walton, 2007]. Intake of foods rich in anthocyanins for a prolonged time favored the accumulation of these compounds in several tissues but not in the plasma and urine [Kalt *et al.*, 2008].

In addition, the type of food matrix significantly affected the absorption of anthocyanins [Yang *et al.*, 2011]. *In vitro* and *in vivo* studies have shown that the properties of aglycone, sugar, and the presence of acyl radicals can influence the absorption and metabolism of anthocyanins, being biotransformed mainly by conjugation with glucoronic acid and also by methylation reactions [de Ferrars *et al*., 2014].

On other hand, the microbiota plays a special role in the metabolism of anthocyanins, which reach the colon in two ways. In the first one, they cannot be absorbed by the stomach and small intestine whereas in the second, the anthocyanins are excreted in the bile after absorption in the stomach and duodenum. Subsequently, after reaching the colon, the anthocyanins are hydrolyzed to liberate the glycosylated fraction of the aglycone and consequently degrade into phenolic acids, which are more susceptible to reabsorption [Kay, 2006].

In regard to their elimination, the number of anthocyanins excreted by urine is low and the mechanism of excretion depends on how and where anthocyanins are biotransformed. Studies with oral administration of purple maize in broilers showed that the anthocyanins did not affect the biochemical parameters and histopathology characteristics although they were presented in the skin, urine, and feces [Nabae *et al*., 2008; Amnueysit *et al*., 2010]. In most reports,

the mechanism describing the elimination of anthocyanins through feces is limited because anthocyanins are converted in other compounds by microbiota action [Couteau *et al*., 2001]. For example, glucose-bound aglycones are less stable to the action of the β-glucosidase enzyme of the small intestine compared to galactose-binding aglycones. This instability upon intestinal enzymes action can probably be attributed to the action of the microbiota. It should be also noted that acylated anthocyanins with *p*-coumaric acid or a second sugar in their structure are resistant to degradation and render them susceptible to reabsorption in the small intestine [He *et al*., 2005]. Although the bioavailability studies of anthocyanins from other plants could be applied to pigmented maize, not enough *in vitro* and *in vivo* studies have been reported to provide pharmacokinetic data about their bioavailability.

EFFECT OF THE NUTRACEUTICAL POTENTIAL OF ANTHOCYANINS FROM PIGMENTED MAIZE ON OXIDATIVE STRESS

The antioxidant capacity of the extracts of pigmented maize is mainly due to the phenolic compounds that they contain. Of these, the aglycones have a superior activity to that of anthocyanins because of a greater number of sugars in the molecule [Serna-Saldivar *et al*., 2013]. The *in vitro* and *in vivo* studies of anthocyanins have shown a wide spectrum of their antioxidant effects. Among those, stands out the reduction of free radical absorption capacity, the stimulation of phase II enzymes for the detoxification, as well as the reduction of oxidative products formation in the DNA and lipids peroxidation. For example, sheep fed with diets based on anthocyanin-rich maize silage showed no significant change in the plasma total antioxidant status (TAS) in comparison to these administered the control diet [Hosoda *et al*., 2012]. However, it has been reported that the intake of anthocyanins *in vivo* increases plasma/serum antioxidant capacity (PAC) in golden Syrian hamsters and human subjects, respectively [Mazza *et al*., 2002; Auger *et al*., 2004].

Besides, anthocyanins have been found to offer the protection against mutagenesis caused by environmental and carcinogenic toxins and the modulation of specific signaling pathways for cell proliferation [Wang & Stoner, 2008]. Some of these effects are summarized in Table 1, highlighting results from extraction protocols (aqueous or hydroalcoholic). The general biological responses involve the upregulation of enzymes and the activation of mechanisms that protect cells and organisms from oxidant agents (radicals) and from oxidative stress damage (due to the redox imbalance), which is a common feature in the development of non-communicable diseases.

ANTIOXIDANT CAPACITY OF ANTHOCYANINS

The scientific interest in food anthocyanins has increased because they have been shown capable of preventing cellular oxidative stress [Prior, 2003]. The antioxidant activity of anthocyanins depends mainly on the aglycone molecule but may also be affected by covalently bound sugars altering solubility and membrane permeability. Anthocyanins have an adequate chemical structure to act as antioxidants, which allows them to donate hydrogens [Rice-Evans *et al*., 1997; Wang *et al.*, 1999] or electrons to free radicals or to trap them and move them in their aromatic structure [Ramírez--Tortosa *et al.*, 2001]. Optimal antioxidant activity is related to the presence of hydroxyl groups in the 3' and 4' positions of the B ring, which confer molecular stability to the formed radical [Kongpichitchoke *et al*., 2015]. While the free hydroxyl group in the 3 position of the C ring and the 5 position of the ring A, are electron donors [Rice-Evans *et al.*, 1996]. In the same sense, the antioxidant capacity of anthocyanins is due to their flat structure, which allows a greater delocalization of electrons through the rings [Dangles & Elhajji, 1994]. In the maize, it has been reported that maize cyanidin derivatives are more potent antioxidant agents than those of delphinidin. In addition, cyanidin has a greater antioxidant power if the glycosylated sugar is a rhamnose rather than glucose [Abdel-Aal *et al*., 2008].

Furthermore, glucosides identified in the rose variety of maize, like cyanidin, pelargonidin and peonidin, being cyanidin-3-(6-malonyl-glucoside) a malonic derivative cyanidin, are probably responsible for its grain color as well as its nutraceutical value [Barrientos-Ramírez *et al*., 2018]. Accordingly, in one study with waxy corn a significant ($r = 0.70$) correlation was reported between TAC (total anthocyanin content) and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay and a moderate correlation between TPC (total phenolic content), DPPH, and TEAC (Trolox equivalent antioxidant capacity) ($r = 0.60$ and $r = 0.76$, respectively). These results confirm that the antioxidant capacity of anthocyanins depends largely on the type and amount of cyanidin glucosides and not only on the quantity of total polyphenols of the maize kernel [Duangpapeng *et al*., 2019]. On the other hand, in blue maize, the anthocyanin profile showed primarily acylated cyanidins and pro-anthocyanidins in raw and nixtamalized extracts. Results from the DPPH assay showed that the antioxidant capacity in the blue maize tortilla extract had slightly lower values (45.1 μM ET/g fresh weight) than raw blue maize extract (49.2 μM ET/g fresh weight). It can be explained because of the nixtamalization process in the tortilla's manufacturing which modifies the type and content of anthocyanins in the grain. Nevertheless, both extracts reduced the TBARS production in homogenized rat brains (IC_{50} = 792 mg/mL and IC_{50} = 750 mg/mL, respectively). These findings suggest that the nixtamalization process improves the biological anthocyanin's properties [Herrera-Sotero *et al*., 2017]. Finally, these results show also that the antioxidant activity of anthocyanins depends on the type, number, and glycosylation site in the molecule of anthocyanidins contained in the maize kernel as well as methodology used to evaluate the antioxidant capacity of these compounds [Zhao *et al.*, 2014].

OTHER BIOLOGICAL EFFECTS

Long-term ingestion of pigmented maize anthocyanins protected rat hearts against ischemia-reperfusion injury [Toufektsian *et al.*, 2008]. Besides, a study with mice reported that the anthocyanins from black rice, black soybean,

and purple maize increased the fecal butyric acid content, as well as ameliorated diet-induced obesity by reducing both oxidative stress and inflammation [Wu *et al*., 2017]. Other properties of anthocyanins have been described as antimicrobial and antiproliferative activities [Cisowska *et al*., 2011]. These abilities been evaluated in extracts of anthocyanins from some types of native pigmented maize varieties in which the authors identified five anthocyanin compounds: cyanidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, pelargonidin 3-*O*-glucoside, cyanidin 3-*O*-(6''-malonyl--glucoside), and cyanidin 3-*O*-(3'', 6''-dimalonyl-glucoside), [Salinas-Moreno *et al.*, 2005a]. IC_{50} of Chinese purple maize extract was reported against pathological strains of *S. enteritidis* (5 mg/mL), *S. aureus* (10 mg/mL) and *C. albicans* (25 mg/mL) in a dose-dependent manner [Zhao *et al.*, 2009]. Moreover, another study determined the antimicrobial ability of anthocyanins isolated from the cob of purple maize. A significant antifungal activity of anthocyanins was observed in fluconazole-resistant strains of *C. glabrata*, *C. krusei*, and *C. parapsilosis* with MIC (Minimum Inhibitory Concentration) ranging from 0.625 to 2.5 mg/mL [Suket *et al*., 2014]. Furthermore, the antimicrobial activities of free and bound phenolic fractions from Peruvian purple maize were compatible with the survival of *L. helveticus* and *B. longum* (probiotic bacteria). Nonetheless, none of these fractions could inhibit the growth of pathogenic *H. pylori* [Galvez-Ranilla *et al*., 2017]. On the other hand, it has been reported that maize phenolic compounds could decrease starch digestibility by a specific anthocyanin-starch interaction [Hernández-Uribe *et al*., 2007; Camelo-Méndez *et al*., 2016], and also inhibit digestive enzymes involved in the absorption of carbohydrates. Moreover, it has been suggested that the use of pigmented maize flours might help in the formulation of gluten-free products with slowly digestible starches [Rocchetti *et al*., 2018].

Extracts of maize anthocyanins counteracted the proliferation of cancer cell lines. The antiproliferative capacity of anthocyanins was dose-dependent (IC_{50}=0.525 μg/mL) in HT-29 cells [Zhao *et al.*, 2009]. Also, some authors [Urias-Lugo *et al*., 2015b] demonstrated that breast carcinoma (MCF7), hepatic (HepG2), colon carcinoma (Caco2 and HT29), and prostate carcinoma (PC3) cell lines treated with acidified extracts of anthocyanins of hybrid blue maize presented greater antiproliferative effect. The isomer cyanidin malonylglucoside I reduced the cell viability in Caco2, HepG2, MCF7, and PC3 cells, suggesting that acylated compounds inhibit the growth of cancer cells. Cyanidin 3-*O*-glucoside from purple maize suppressed Ras protein levels and inhibited 7,12-dimethylbenzo-anthracene [Guillen-Sanchez *et al.*, 2014], which induce mammary carcinogenesis in rats, suggesting that it may act as a potential chemotherapeutic agent [Fukamachi *et al*., 2008]. In addition, Navolokin *et al*. [2012] studied the effect of anthocyanin extracts from maize and observed reduced endogenous intoxication in rats with transplanted tumor suspension of liver cancer PC-1 cells.

Anthocyanins derived from the variety *Zea mays* var. *Kculli* (purple maize) improved insulin secretion of pancreatic beta cells in db/db mice compared to glimepiride (a sulfonylurea) but also protected pancreatic beta cells from apoptosis in cultures of the HIT-T15 cell line after treatment with the sulfonylurea, which increases the concentration of H_2O_2 [Hong *et al.*, 2013]. Interestingly, the mechanism of action of the anthocyanin extract of purple maize on insulin secretion differed from that of the GLP/GLP receptor/cAMP/PKA signal for insulin secretion. Extracts of purple maize (50 mg/kg purple maize extract) induced phosphorylation of AMP-activating protein kinase (AMPK), decreased expression of phosphoenolpyruvate carboxy kinase (PEPCK) and glucose-6-phosphatase (G6pase) in liver, and also increased the expression of the glucose transporter 4 (GLUT4) of skeletal muscle in C57BL/KsJ db/db mice. The authors suggested that the extracts exhibited antidiabetic activity through the protection of the pancreatic cells against ROS, increased the production and secretion of insulin, and activated the AMPK in the liver [Huang *et al.*, 2015]. In addition, other results indicate that the anthocyanin extract from the purple maize can be used in combination with the conventional treatment of type 2 diabetes and thus may improve the function of the pancreatic beta cells [Kang *et al.*, 2013]. Another study reported that purple maize anthocyanins, in particular cyanidin 3-*O*-glucoside and cyanidin--3-*O*-(6''-malonyl-glucoside), attenuated the proliferation of mesangial cells and protein accumulation of extracellular matrix "mesangium and glomerular basal membrane" (a diabetic glomerulosclerosis characteristic) in human mesangial cells stimulated by a high concentration of glucose through the inactivation of NF-κB translocation. This effect enhanced the signaling of transforming growth factor beta (TGF-β), as well as promoted the degradation of the extracellular matrix [Li *et al.*, 2012a]. Similarly, Roh *et al.* [2016] investigated the anti-inflammatory effects of *Zea mays* L. husk extracts and found a downregulation of the inducible nitric oxide synthase (iNOS) gene expression mediated by NF-κB and AP-1 signaling in RAW256.7 cells.

In a model of *in vitro* inflammation of mono- or co-culture of macrophage or/and adipocyte cells treated with extracts rich in anthocyanins from purple and red maize, Zhang *et al.* [2019] showed an inhibition of NF-κB and JNK pathways *via* regulation of IκBα and JNK phosphorylation. Besides, these extracts decreased pro-inflammatory cytokine production and lipolysis, and enhanced the glucose transporter 4 membrane (GLUT4) translocation [Zhang *et al*., 2019]. Additionally, the anthocyanin-rich purple maize extract increased free fatty acid receptor-1 (FFAR1) and glucokinase (GK) activity in Caco-2, INS-1E, and HepG2 cells culture [Luna-Vital & de Mejía, 2018].

On the other hand, when an anthocyanin complex (prepared with dried petals of blue butterfly pea (*C. ternatea* L.), dried rhizomes of turmeric (*C. longa*) and dried purple waxy corn cobs (*Z. mays* L. ceritina Kulesh) was given to hamsters infected with *Opisthorchis viverrini* (an experimental model of inflammation and periductal fibrosis in liver), Intuyod *et al*. [2014] found reduced number of inflammatory cells and periductal fibrosis in this model. Besides, in the treated animals there were reduced the formation of 8-oxodG (an oxidative DNA damage marker), NF-κB expression, iNOS synthesis, and oxidative stress. Contrarily, the gene expression of CAT, SOD, and GPx was increased in the treated experimental group [Intuyod *et al.*, 2014].

TABLE 1. Antioxidant effect of pigmented maize anthocyanins on *in vivo* and *in vitro* models.

Author, year	Type of trial	Anthocyanin, origin	Extract/Dose/Period of the treatment	Results (main biological effects)
[Tsuda *et al*., 2000]	Rats with hepatic ischemia-reperfusion injury	Cyanidin 3-*O*-β-d-glucoside (C3G), *Zea mays* L.	0.2 g/kg body weight for 14 days	↓oxidative stress
[Ramos-Escudero *et al*., 2012]	*In vitro* cellular antioxidant response in isolated mouse organs (kidney, liver, and brain)	Total polyphenols, flavonoids, flavonols, flavanols, and anthocyanins of purple corn (*Zea Mays* L.)	Purple corn extract 100 mg/mL with or without H_2O_2 (100 μM)	↓lipid peroxidation, ↑endogenous antioxidant enzyme activities (CAT, TPX – total peroxidases, and SOD)
[Tsuda *et al*., 2003]	Male C57BL/6J mice with obesity, hyperglycemia and hyperinsulinemia	Cyanidin 3-glucoside-rich purple corn color (PCC)	2 g/kg diet for 12 weeks	↓insulin resistance, suppressed mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowered the sterol regulatory element binding protein-1 mRNA level in white adipose tissue
[Thiraphatthanavong *et al*., 2014]	Male Wistar rats, experimental diabetic cataract model	Hydroalcoholic extract of purple waxy corn	2, 10, and 50 mg/mL at room temperature for 72 h in enucleated rat lenses	↓lens opacity with MDA level and aldose reductase (AR) activity, ↑GPX activity
[Kang *et al*., 2013]	Adult male db/db mice (C57BLKS/+Lepr^{db} Iar) and their age-matched non-diabetic db/m littermates (C57BLKS/J)	Anthocyanins-rich purple corn extract (*Zea mays* L.)	10 µg/kg of BW PCE (purple corn extract) daily for 8 weeks	Retarded the endothelial proliferation in db/db mouse kidneys by induction of vascular endothelium-cadherin, PECAM-1 and Ki-67 and diminished the mesangial and endothelial induction of angiopoietin (Angpt) proteins
	Human renal mesangial cells (HRMC)		1–20 µg/mL in HRMC conditioned media	↓endothelial expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF)-1α induced by HG-HRMC-CM (high glucose-exposed mesangial conditioned media)
[Mamani-Choquepata *et al*., 2013]	Aortic rings of hypertensive rats stimulated with norepinephrine	Total anthocyanins of corncob without seeds of purple corn (*Zea mays* L.) in three extracts: aqueous, hydro alcoholic, and hydro alcoholic acidified	Increasing doses (0.05, 0.1, 0.5 and 1 mg/L of total anthocyanins) of the different extracts	Vasodilator effect of the purple corn is dependent on the concentration of anthocyanins. Greater vasodilation was the hydro alcoholic extract prepared in ethanol at 70%
[Villasante *et al*., 2015]	Rainbow trout, *Oncorhynchus mykiss*	Purple corn extract; PCE (containing cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside)	5% of PCE in diet for 8 weeks	↓adiposity and ↑percentage of total n-3 and total n-6 polyunsaturated fatty acids (PUFA) in the fish body, ↑expression of GPx1 in erythrocytes and ↓TAC in plasma
[Wattanathorn *et al*., 2015]	Streptozotocin diabetic Male Wistar rats with induction of diabetic neuropathy by chronic constriction	Extract of purple waxy corn (*Zea mays* L.) and ginger *Zingiber officinale* Roscoe (PWCG)	Doses of 100, 200, and 300 mg/kg of BW	↑SOD and GPx activity which in turn decreased oxidative stress status in the lesion nerve
[Hosoda *et al*., 2012]	Suffolk wethers fed a diet supplemented with purple corn pigment	Purple anthocyanin-rich corns (*Zea mays* L.) pigment in the diet	0.5% diet on dry matter basis for 14 days	Enhancement in plasma SOD activity, ↓oxidative stress
[Zhang *et al*., 2014]	Wistar rat fluorosis model	Maize purple plant pigment (MPPP) contains 45.96% cyaniding-3-glucoside and 12.99% 3',4'-dihydroxy anthocyanin-3-glucoside	Water containing 5 g/kg MPPP in the diet with 100 ppm fluoride	↓elevation of MDA levels in blood and liver, Bcl-2 protein expression, ↑Bax protein expression, SOD and GSH-Px activities in kidney and GSH level in liver and kidney
[Arroyo *et al*., 2008]	Male Holtzmann rats with hypertension by (Nitro-L-arginine methyl ester) L-NAME	Atomized hydro alcoholic extract of *Zea mays* L. (Andean purple corn)	Orally once a day for 25 days 500 and 1000 mg/kg of BW	↓in blood pressure and MDA

TABLE 1. Continued...

Author, year	Type of trial	Anthocyanin, origin	Extract/Dose/Period of the treatment	Results (main biological effects)
[Moreno-Loaiza & Paz-Aliaga, 2010]	Male Swiss rat thoracic aortic rings	Extract by maceration of Andean purple corn cobs in 70% ethanol	Doses of the extract: 0.1, 0.5, and 1.0 mg/mL with L-NG-Nitroarginine methyl ester (L-NAME) to verify that the vasodilation depends on nitric oxide synthetase (NOs)	↓in maximum contraction. The vasodilation was inhibited by prior incubation with L-NAME
[Zhang *et al*., 2010]	Male Kunming mice with a cholesterol-rich diet comprising 1.0% cholesterol, 10.0% lard, 0.5% bile salt, and 88.5% commercial chow	Pigments extracted from black glutinous corncob (BGC)	Three cholesterol-rich diet groups were given 200, 500, and 800 mg/kg PBGC for 4 weeks	↓plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and triglyceride (TG). ↓lipid peroxidation and atherogenic index
[Guzman-Geronimo *et al*., 2017]	Male Wistar rat with high-sucrose (30%) diet-induced metabolic syndrome	Blue maize extract (*Zea mays* L.)	Total polyphenols and anthocyanins of 9.97 and 2.92 mg/kg of body weight for 4 weeks	Enhanced HDL-c (High-density lipoprotein cholesterol) and ↓systolic blood pressure, serum triglycerides, total cholesterol, and epididymal adipose tissue weight
[Mex-Alvarez *et al*., 2016]	Male albino mouse (*Mus musculus*) with high fat diet	Purple and red corn extracts (*Zea mays* L.)	Subcutaneous injection of maize extract at dose of 250 mg/kg for 60 days	↓total cholesterol, triglycerides, atherogenic index, and ↑HDL-c
[Li *et al*., 2012b]	Adult male db/db mice (C57BLKS/+Lepr^{db} Iar) and their age-matched non-diabetic db/m littermates (C57BLKS/J)	Anthocyanins-rich polyphenolic extracts of purple corn (PCE) (*Zea mays* L.)	10 mg/kg of BW PCE daily for 8 weeks	↓plasma glucose level, albuminuria, protein expression of nephrin and podocin were repressed, ↓collagen fiber accumulation in kidney glomeruli and CTGF expression *via* retarding TGF-β signaling
	Human renal mesangial cells (HRMC)	Anthocyanins-rich purple corn butanol fraction (PCB) (*Zea mays* L.)	PCB supplemented at concentration of 1–20 μg/mL for 3 days under the condition of high glucose and mannitol	↓production of fibrosis biomarkers of collagen IV and CTGF, promotes the activation via eliciting Tyk2-STAT signaling pathway, ↓expression of PDGF
[Foy Valencia, 2010]	Albino rats with high fat diet-induced hyperlipidemia	Anthocyanin extracts from the crown of *Zea mays L*. Starchy (purple maize)	5% powder and extract aqueous (*Chicha morada*) to 20% for 15 days	↓triglycerides, total cholesterol and LDL-c
[Toufektsian *et al*., 2008]	Male Wistar rats with *ex vivo* and *in vivo* models of ischemia-reperfusion injury	Maize lines nongenetically modified: ACR, ∆-D902, and F1	Maize seed powder replacing 20% from standard pellet for 8 weeks	↑ glutathione levels and infarct size were reduced
[Limsitthichaikoon *et al*., 2018]	Mucosal epithelium of porcine esophagus, male Wistar rats, and patients who were 18–25 years old	Purple waxy corn cobs (*Zea mays* L. var. *ceratina Kulesh*) and blue petals (*Clitoria ternatea* Linn)	Anthocyanin complex (AC), combined *Zea mays* and *Clitoria ternatea* extracts in a mucoadhesive gel for 7 days	Enhanced anthocyanin permeation *in vitro*. In humans and rats, mucoadhesive gel reduced erythema and sizes of oral wounds
[Flores Cortez *et al*., 2018]	Holtzman rats with experimental arthritis with pristane (PIA)	*Zea mays* L., purple variety	100 mg/kg and 1000 mg/kg extracts for 21 days	↓the inflammatory process and radiological modifications of PIA-induced arthritis
[Tian *et al*., 2019]	Lactating Saanen dairy goats	Anthocyanin-rich purple corn (*Zea mays* L.)	Dietary anthocyanin-rich 50% (DM) of purple corn silage for 8 weeks	↑ content of milk lactose, SOD level and TAC in plasma and milk
[Revilla *et al*., 2018]	Corn borer *S. nonagrioides* larvae with oxidative stress induced (H_2O_2)	Black maize kernels	Artificial diet with white and black maize flour for 28 weeks	Antioxidant activity has insecticide effect on young larvae and a positive effect on grown larvae

↓, Reduce;↑, Increase; SOD, superoxide dismutase; GPx, Glutathione peroxidase; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; TC, total cholesterol; PBGC, pigment from black glutinous corncob; CTGF, connective tissue growth factor; PDGF, platelet-derived growth factor; BW, body weight; Bax, bcl-2-like protein 4; MDA, malondialdehyde; TAC, total antioxidant capacity; DM, dry mass.

CONCLUSION

Anthocyanins represent a promising class of antioxidants found naturally in plants that enhance their stress resistance and nutritional value through diverse biological mechanisms. Several studies *in vitro* and *in vivo* corroborate the antioxidant and biological effect of maize anthocyanins. The aging of the population and the exponential increase of governmental health expenses make it necessary to implement natural and cost-effective alternatives for the prevention and management of diseases caused by redox disequilibrium and cell aging. Maize is a staple crop widely accepted as food ingredient; therefore, it could be useful as a functional additive to prevent aging, obesity, and metabolic syndrome. Its permanent consumption could also reduce the increase of cases with chronic degenerative diseases such as diabetes, cancer, and cardiovascular diseases. Antioxidant efficacy of pigmented maize anthocyanins has been less documented in both *in vivo* and *in vitro* studies possibly due to the scarce knowledge of their bioavailability and pharmacokinetics. Besides, there is a wide diversity of anthocyanins that can be found in plants. This represents a methodological problem for reductionistic experimental approaches, since it requires the previous analysis of extracts for the exact chemical identification and characterization of the anthocyanins/polyphenols of each sample batch. There are equipment/infrastructure limitations for the determination of molecular structures and the antioxidant *in vitro* activity as well. Uniformity in the elaboration of the extracts, methodologies of quantification, and report of new results is also needed. Another important circumstance to be considered is the food manufacture process that simultaneously alters a large number of molecules. The process and secondary ingredients that best enhance the nutraceutical potential of colored maize are yet unknown, but we might learn the empirical lessons of centuries of the Mesoamerican culinary culture mixing for example, roasted maize, chili, chia seeds, and cacao beans. In conclusion, pigmented maize has properties that need to be exploited and thoroughly investigated. We expect that this review highlights the importance of anthocyanins from pigmented maize as well as its biological effects in order to promote further research in this field and increase the knowledge of the beneficial properties it offers to the health through its consumption.

RESEARCH FUNDING

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT Mexico) to AT. We acknowledge support from the National Laboratory PlanTECC, *Problemas Nacionales e Infraestructura*. We further acknowledge initial funding grants by SAGARPA through CIMMYT and the MasAgro initiative. CONACYT. PN2015––613, LN2018–293362.

ACKNOWLEDGEMENTS

We thank Pedro M. García-López (CUCBA, University of Guadalajara), Andres Estrada Luna and Norma Cecilia Morales Elias for technical support. We also thank Francisco Orozco Montes, Adrian Garcia Casarrubias, Edgar Cubedo Lopez, Sheila Juarez Colunga, Jesus Ruben Torres Garcia, Alberto Camas Reyes and Julio Armando Massange-Sanchez for their help.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

REFERENCES

1. Abdel-Aal, E.S., Abou-Arab, A., Gamel, T., Hucl, P., Young, J., Rabalski, I. (2008). Fractionation of blue wheat anthocyanin compounds and their contribution to antioxidant properties. *Journal of Agricultural and Food Chemistry, 56*(23), 11171–11177.
2. Abdel-Aal, E.S.M., Young, J.C., Rabalski, I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry, 54*(13), 4696–4704.
3. Amnueysit, P., Tatakul, T., Chalermsan, N., Amnueysit, K. (2010). Effects of purple field corn anthocyanins on broiler heart weight. *Asian Journal of Food and Agro-Industry, 3*(3), 319–327.
4. Arroyo, J., Raez, E., Rodríguez, M., Chumpitaz, V., Burga, J., De la Cruz, W., Valencia, J. (2008). Antihypertensive and antioxidant activity of atomized Andean purple corn (*Zea mayz* L.) hydroalcoholic extract in rats. *Revista Peruana de Medicina Experimental y Salud Publica*, *25*(2), 195–199 (in Spanish; English abstract).
5. Auger, C., Laurent, N., Laurent, C., Besançon, P., Caporiccio, B., Teissédre, P.L., Rouanet, J.M. (2004). Hydroxycinnamic acids do not prevent aortic atherosclerosis in hypercholesterolemic golden Syrian hamsters. *Life Sciences*, *74*(19), 2365–2377.
6. Aura, A.M., Martin-Lopez, P., O'Leary, K.A., Williamson, G., Oksman-Caldentey, K.M., Poutanen, K., Santos-Buelga, C. (2005). *In vitro* metabolism of anthocyanins by human gut microbiota. *European Journal of Nutrition*, *44*(3), 133–142.
7. Aza-Gonzalez, C., Ochoa-Alejo, N. (2012). Characterization of anthocyanins from fruits of two Mexican chili peppers (*Capsicum annuum* L.). *Journal of the Mexican Chemical Society, 56*(2), 149–151.
8. Barrientos-Ramírez, L., Ramírez-Salcedo, H.E., Fernández-Aulis, M.F., Ruíz-López, M.A., Navarro-Ocaña, A., Vargas-Radillo, J.J. (2018). Anthocyanins from rose maize (*Zea mays* L.) grains. *Interciencia*, *43*(3), 188–192.
9. Bello-Pérez, L.A., Camelo-Mendez, G.A., Agama-Acevedo, E., Utrilla-Coello, R.G. (2016). Nutraceutic aspects of pigmented maize: digestibility of carbohydrates and anthocyanins. *Agrociencia*, *50*(8), 1041–1063.
10. Betrán, F.J., Bockholt, A.J., Rooney, L.W. (2000). Blue corn. In A.R. Hallauer (Ed.), *Specialty Corns*, Second Edition, CRC Press, Boca Raton, Florida, USA, pp. 305–314.
11. Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, *5*(1), 9–19.
12. Bitsch, R., Netzel, M., Frank, T., Strass, G., Bistch, I. (2004). Bioavailibility and biokinetics of anthocyanins from red grape juice and red wine. *Journal of Biomedicine & Biotechnology, 2004*(5), 293–298.
13. Bub, A., Watzl, B., Heeb, D., Rechkemmer, G., Briviba, K. (2001). Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice. *European Journal of Nutrition*, *40*(3), 113–120.

14. Camelo-Méndez, G.A., Agama-Acevedo, E., Sanchez-Rivera, M.M., Bello-Pérez, L.A. (2016). Effect on *in vitro* starch digestibility of Mexican blue maize anthocyanins. *Food Chemistry*, *211*, 281–284.
15. Cevallos-Casals, B.A., Cisneros-Zevallos, L. (2004). Stability of anthocyanin-based aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic and natural colorants. *Food Chemistry*, *86*(1), 69–77.
16. Chander, S., Meng, Y.J., Zhang, Y.R., Yan, J.B., Li, J.S. (2008). Comparison of nutritional traits variability in selected eighty-seven inbreds from Chinese maize (*Zea mays* L.) germplasm. *Journal of Agricultural and Food Chemistry, 56*(15), 6506–6511.
17. Chavez-Rivera, R., Ordoñez-Gomez, E. (2013). Total polyphenols, anthocyanins and antioxidant capacity (DPPH and ABTS) during processing of cocoa liquor and cocoa powder. *Revista ECIPerú, 10*(1), 42–50 (in Spanish; English abstract).
18. Chen, L., Yang, M., Mou, H., Kong, Q. (2018). Ultrasound-assisted extraction and characterization of anthocyanins from purple corn bran. *Journal of Food Processing and Preservation*, *42*(1), art. no. e13377.
19. Cisowska, A., Wojnicz, D., Hendrich, A.B. (2011). Anthocyanins as antimicrobial agents of natural plant origin. *Natural Product Communications*, *6*(1), 149–156.
20. Cone, K.C. (2007). Anthocyanin synthesis in maize aleurone tissue. In O.-A. Olsen (Ed.) *Endosperm. Developmental and Molecular Biology,* Springer, Berlin, Heidelberg, Germany, pp. 121–139.
21. Cooper-Driver, G.A. (2001). Contributions of Jeffrey Harborne and co-workers to the study of anthocyanins. *Phytochemistry*, *56*(3), 229–236.
22. Couteau, D., McCartney, A.L., Gibson, G.R., Williamson, G., Faulds, C.B. (2001). Isolation and characterization of human colonic bacteria able to hydrolyse chlorogenic acid. *Journal of Applied Microbiology*, *90*(6), 873–881.
23. Cui, L., Gao, R., Dong, S., Zhang, J., Liu, P., Zhang, H., Meng, J., Shi, D. (2012). Effects of ear shading on the anthocyanin contents and quality of kernels in various genotypes of maize. *Australian Journal of Crop Science, 6*(4), 704–710.
24. Dangles, O., Elhajji, H. (1994). Synthesis of 3-methoxy- and 3-(β-D-glucopyranosyloxy) flavylium ions. Influence of the flavylium substitution pattern on the reactivity of anthocyanins in aqueous solution. *Helvetica Chimica Acta*, *77*(6), 1595–1610.
25. de Ferrars, R.M., Czank, C., Zhang, Q., Botting, N.P., Kroon, P.A., Cassidy, A., Kay, C.D. (2014). The pharmacokinetics of anthocyanins and their metabolites in humans. *British Journal of Pharmacology*, *171*(13), 3268–3282.
26. de la Parra, C., Serna Saldivar, S.O., Hai, L.R. (2008). Effect of processing on the phytochemical profiles and antioxidant activity of corn production of masa, tortillas and tortilla chips. *Journal of Agricultural and Food Chemistry, 55*(10), 4177–4183.
27. de Pascual-Teresa, S., Santos-Buelga, C., Rivas-Gonzalo, J. (2002). LC-MS analysis of anthocyanins extracts from purple corn cob. *Journal of the Science of Food and Agriculture, 82*(9), 1003–1006.
28. De Rosso, V.V., Moran Vieyra, F.E., Mercadante, A.Z., Borsarelli, C.D. (2008). Singlet oxygen quenching by anthocyanin's flavylium cations. *Free Radical Research*, *42*(10), 885–891.
29. Duangpapeng, P., Lertrat, K., Lomthaisong, K., Paul Scott, M., Suriharn, B. (2019). Variability in anthocyanins, phenolic compounds and antioxidant capacity in the tassels of collected waxy corn germplasm. *Agronomy – Basel*, *9*(3), art. no. 158.
30. Escalante-Aburto, A., Ramirez-Wong, B., Torres-Chavez, P.I., Barron-Hoyos, J.M., Figueroa-Cardenas, J.D., Lopez-Cervantes, J. (2013a). The nixtamalization process and its effect on anthocyanin content of pigmented maize, a review. *Revista Fitotecnia Mexicana, 36*(4), 429–437 (in Spanish; English abstract).
31. Escalante-Aburto, A., Ramirez-Wong, B., Torres-Chavez, P.I., Figueroa-Cardenas, J.D., Lopez-Cervantes, J., Barron-Hoyos, J.M., Morales Rosas, I. (2013b). Effect of extrusion processing parameters on anthocyanin content, physicochemical properties of nixtamalized blue corn expanded extrudates. *CyTA – Journal of Food, 11*, SI, 29–37.
32. Espinosa Trujillo, E., Mendoza Castillo, M., Castillo Gonzalez, F., Ortiz Cereceres, J., Delgado Alvarado, A., Carrillo Salazar, A. (2009). Anthocyanin accumulation in pericarp and aleurone layer of maize kernel and their genetic effects on native pigmented varieties. *Revista Fitotecnia Mexicana, 32*(4), 303–309 (in Spanish; English abstract).
33. Fan, X., Fan, B., Wang, Y., Yang, W. (2016). Anthocyanin accumulation enhanced in Lc-transgenic cotton under light and increased resistance to bollworm. *Plant Biotechnology Reports*, *10*(1), 1–11.
34. Fang, J. (2014). Bioavailability of anthocyanins. *Drug Metabolism Reviews, 46*(4), 508–520.
35. Felgines, C., Talavera, S., Texier, O., Gil-Izquierdo, A., Lamaison, J.L., Remesy, C. (2005). Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *Journal of Agricultural and Food Chemistry, 53*(20), 7721–7727.
36. Fernandez-Aulis, F., Hernandez-Vazquez, L., Aguilar-Osorio, G., Arrieta-Baez, D., Navarro-Ocana, A. (2019). Extraction and identification of anthocyanins in corn cob and corn husk from Cacahuacintle maize. *Journal of Food Science*, *84*(5), 954–962.
37. Flores-Cortez, D., Villalobos-Pacheco, E., Rojo-Mejia, A., Palomino-Yamamoto, P., Martin-Ramírez, Y. (2018). Osteoarticular protection of *Zea may*s L. purple variety (purple corn) in experimental arthritis in rats. *Revista Peruana de Medicina Experimental y Salud Pública*, *35*(3), 441–448 (in Spanish; English abstract).
38. Fossen, T., Slimestad, R., Andersen, Ø.M. (2001). Anthocyanins from maize (*Zea mays*) and reed canarygrass (*Phalaris arundinacea*). *Journal of Agricultural and Food Chemistry, 49*(5), 2318–2321.
39. Foy Valencia, E. (2010). Los efectos de las antocianinas extraídas de *Zea mays* L. (maíz morado) sobre las hiperlipidemias en ratas albinas. *Biotempo,* 10, 32–38 (in Spanish; English abstract).
40. Fukamachi, K., Imada, T., Ohshima, Y., Xu, J., Tsuda, H. (2008). Purple corn color suppresses Ras protein level and inhibits 7,12-dimethylbenz[a]antracene-induced mamary carcinogenesis in the rat. *Cancer Science, 99*(9), 1841–1846.
41. Galvez Ranilla, L., Christopher, A., Sarkar, D., Shetty, K., Chirinos, R., Campos, D. (2017). Phenolic composition and evaluation of the antimicrobial activity of free and bound phenolic fractions from a Peruvian purple corn (*Zea mays* L.) accession. *Journal of Food Science*, *82*(12), 2968–2976.
42. Gläßgen, W.E., Seitz, H.U. (1992). Acylation of anthocyanins with hydroxycinnamic acids *via* 1-O-acylglucosides by protein preparations from cell cultures of *Daucus carota* L. *Planta*, *186*(4), 582–585.

43. Goufo, P., Trindale, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. *Food Science and Nutrition, 2*(2), 75–104.
44. Guillen-Sanchez, J., Mori-Arismendi, S., Paucar-Menacho, L. (2014). Characteristics and functional properties of purple corn (*Zea mays* L.) var. subnigroviolaceo. *Scientia Agropecuaria, 5,* 211–217 (in Spanish; English abstract).
45. Gurrola-Diaz, C., Garcia-Lopez, P., Sanchez-Enriquez, S., Troyo-Sanroman, R., Andrade-Gonzalez, I., Gomez-Leyva, J. (2010). Effects of *Hibiscus sabdariffa* extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). *Phytomedicine, 17*(7), 500–505.
46. Guzman-Geronimo, R.J., Alarcon-Zavaleta, T.M., Oliart-Ros, R.M., Meza-Alvarado, J.E., Herrera-Meza, S., Chavez-Servia, J.L. (2017). Blue maize extract improves blood presure, lipid profiles, and adipose tissue in high-sucrose diet-induced metabolic syndrome in rats. *Journal of Medicinal Food, 20*(2), 110–115.
47. Harakotr, B., Suriharn, B., Tangwongchai, R., Scott, M. P., Lertrat, K. (2014). Anthocyanins and antioxidant activity in coloured waxy corn at different maturation stages. *Journal of Functional Foods*, *9*, 109–118.
48. He, J., Giusti, M. (2010). Anthocyanins: Natural colorants with health-promoting properties. *Annual Review of Food Science and Technology, 1*, 163–187.
49. He, J., Magnuson, B.A., Giusti, M.M. (2005). Analysis of anthocyanins in rat intestinal contents-impact of anthocyanin chemical structure on fecal excretion. *Journal of Agricultural and Food Chemistry, 53*(8), 2859–2866.
50. Hernández-Uribe, J.P., Agama-Acevedo, E., Islas-Hernández, J.J., Tovar, J., Bello-Pérez, L.A. (2007). Chemical composition and *in vitro* starch digestibility of pigmented corn tortilla. *Journal of the Science of Food and Agriculture*, *87*(13), 2482–2487.
51. Herrera-Sotero, M.Y., Cruz-Hernández, C.D., Trujillo-Carretero, C., Rodríguez-Dorantes, M., García-Galindo, H.S., Chávez--Servia, J.L., Oliart-Ros, R.M., Guzmán-Gerónimo, R.I. (2017). Antioxidant and antiproliferative activity of blue corn and tortilla from native maize. *Chemistry Central Journal*, *11*(1), art. no. 110.
52. Hong, S.H., Heo, J.I., Kim, J.H., Kwon, S.O., Yeo, K.M., Bakowska-Barczak, A.M., Kolodziejczyk, P., Ryu, O.H., Choi, M.K., Kang, Y.H., Lim, S.S., Suh, H.W., Huh, S.O., Lee, J.Y. (2013). Antidiabetic and beta cell-protection activities of purple corn anthocyanins. *Biomolecules & Therapeutics, 21*(4), 284–289.
53. Horbowicz, M., Kosson, R., Grzesiuk, A., Dębski, H. (2008). Anthocyanins of fruits and vegetables – their occurrence, analysis and role in human nutrition. *Vegetable Crops Research Bulletin, 68*, 5–22.
54. Hosoda, K., Miyaji, M., Matsuyama, H., Haga, S., Ishizaki, H., Nonaka, K. (2012). Effect of supplementation of purple pigment from anthocyanin-rich corn (*Zea mays* L.) on blood antioxidant activity and oxidation resistance in sheep. *Livestock Science, 145*(1–3), 266–270.
55. Hu, C.Y., Li, Q.L., Shen, X.F., Quan, S., Lin, H., Duan, L., Wang, Y.F., Luo, Q., Qu, G.R., Han, Q., Lu, Y., Zhang, D.B., Yuan, Z., Shi, J.X. (2016). Characterization of factors underlying the metabolic shifts in developing kernels of colored maize. *Scientific Reports*, *6*, art. no. 35479.
56. Huang, B., Wang, Z., Park, J., Ryu, O., Choi, M., Lee, J.-Y., Kang, Y., Lim, S. (2015). Anti-diabetic effect of purple corn extract on C57BL/KsJ db/db mice. *Nutrition Research and Practice,* 9(1), 22–29.
57. Intuyod, K., Priprem, A., Limphirat, W., Charoensuk, L., Pinlaor, P., Pairojkul, C., Lertrat, K., Pinlaor, S. (2014). Anti-inflammatory and anti-periductal fibrosis effects of an anthocyanin complex in *Opisthorchis viverrini*-infected hamsters. *Food and Chemical Toxicology, 74*, 206–215.
58. Jansen, G., Flamme, W. (2006). Coloured potatoes (*Solanum Tuberosum* L.) – Anthocyanin content and tuber quality. *Genetic Resources and Crop Evolution, 53*(7), 1321–1331.
59. Kallam, K., Appelhagen, I., Luo, J., Albert, N., Zhang, H.B., Deroles, S., Hill, L., Findlay, K., Andersen, O.M., Davies, K., Martin, C. (2017). Aromatic decoration determines the formation of anthocyanic vacuolar inclusions. *Current Biology*, *27*(7), 945–957.
60. Kalt, W., Blumberg, J., McDonald, J., Vinqvist-Tymchuk, M., Fillmore, S., Graf, B., O'Leary, J., Milbury, P. (2008). Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *Journal of Agricultural and Food Chemistry, 56*(3), 705–712.
61. Kang, M.-K., Lim, S.S., Lee, J.-Y., Yeo, K.M., Kang, Y.-H. (2013). Anthocyanin-rich purple corn extract inhibit diabetes-associated glomerular angiogenesis. *PloS One, 8*(11), art. no. e79823.
62. Kasote, D.M., Katyare, S.S., Hegde, M.V., Bae, H. (2015). Significance of antioxidant potential of plants and its relevance to therapeutic applications. *International Journal of Biological Sciences*, *11*(8), 982–991.
63. Kay, C. (2006). Aspects of anthocyanin absorption, metabolism and pharmacokinetics in humans. *Nutrition Research Reviews, 19*(1), 137–146.
64. Kay, C.D., Mazza, G., Holub, B.J. (2005). Anthocyanins exist in the circulation primarily as metabolites in adult men. *Journal of Nutrition, 135*(11), 2582–2588.
65. Kongpichitchoke, T., Hsu, J.L., Huang, T.C. (2015). Number of hydroxyl groups on the B-ring of flavonoids affects their antioxidant activity and interaction with phorbol ester binding site of PKCδ C1B domain: *in vitro* and *in silico* studies. *Journal of Agricultural and Food Chemistry*, *63*(18), 4580–4586.
66. Lago, C., Cassani, E., Zanzi, C., Landoni, M., Trovato, R., Pilu, R. (2014). Development and study of a maize cultivar rich in anthocyanins: coloured polenta, a new functional food. *Plant Breeding*, *133*(2), 210–217.
67. Lago, C., Landoni, M., Cassani, E., Doria, E., Nielsen, E., Pilu, R. (2013). Study and characterization of a novel functional food: purple popcorn. *Molecular Breeding*, *31*(3), 575–585.
68. Langley, P.C., Pergolizzi, J.V., Taylor, R., Ridgway, C. (2015). Antioxidant and associated capacities of camu camu (*Myrciaria dubia*): a sistematic review. *Journal of Alternative and Complementary Medicine, 21*(1), 8–14.
69. Levi, M.A.B., Scarminio, L.S., Poppi, R.J., Trevisan, M.G. (2004). Three-way chemometric method study and UV-Vis absorbance for the study of simultaneous degradation of anthocyanins in flowers of the *Hibiscus rosa-sinensys* species. *Talanta*, *62*(2), 299–305.
70. Li, C.Y., Kim, H.W., Won, S.R., Min, H.K., Park, K.J., Park, J.Y., Ahn, M.S., Rhee, H.L. (2008). Corn husk as a potential source of anthocyanins. *Journal of Agricultural and Food Chemistry*, *56*(23), 11413–11416.

71. Li, J., Kang, M.-K., Kim, J.-K., Kim, J.-L., Kang, S.-W., Lim, S.S., Kang, Y.-H. (2012b). Purple corn anthocyanins retard diabetes-associated glomerulosclerosis in mesangial cells and db/db mice. *European Journal of Nutrition, 51*(8), 961–973.
72. Li, J., Lim, S., Lee, J.-Y., Kim, J.-K., Kang, S.-W., Kim, J.-L., Kang, Y-H. (2012a). Purple corn anthocyanins dampened high-glucose-induced mesangial fibrosis and inflammation: possible renoprotective role in diabetic nephropathy. *The Journal of Nutritional Biochemistry, 23*(4), 320–331.
73. Li, Q., Somavat, P., Singh, V., Chatham, L., de Mejia, E.G. (2017). A comparative study of anthocyanin distribution in purple and blue corn coproducts from three conventional fractionation processes. *Food Chemistry*, *231*, 332–339.
74. Li, T.C., Zhang, W., Yang, H.Y., Dong, Q., Ren, J., Fan, H.H., Zhang, X., Zhou, Y.B. (2019). Comparative transcriptome analysis reveals differentially expressed genes related to the tissue-specific accumulation of anthocyanins in pericarp and aleurone layer for maize. *Scientific Reports*, *9*, art. no. 2485.
75. Li, X., Qian, X., Lǔ, X., Wang, X.H., Jia, N., Zhang, M.S., Ren, M.J. (2018). Upregulated structural and regulatory genes involved in anthocyanin biosynthesis for coloration of purple grains during the middle and late grain-filling stages. *Plant Physiology and Biochemistry*, *130*, 235–247.
76. Limsitthichaikoon, S., Khampaenjiraroch, B., Damrongrungruang, T., Limphirat, W., Thapphasaraphong, S. Priprem, A. (2018). Topical oral wound healing potential of anthocyanin complex: animal and clinical studies. *Therapeutic Delivery*, *9*(5), 359–374.
77. Liu, Y.X., Song, X., Zhang, D., Zhou, F., Wang, D., Wei, Y., Gao, F.Y., Xie, L.Y., Jia, G., Wu, W., Ji, B. (2012). Blueberry anthocyanins: protection against ageing and light-induced damage in retinal pigment. *British Journal of Nutrition, 108*(1), 16–27.
78. Lobo, V., Patil, A., Phatak, A., Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy Review*, *4*(8), 118–126.
79. Lopez-Martinez, L.Y., Oliart-Ros, R.M., Valerio-Alfaro, G., Lee, C.-H., Parkin, K.L., Garcia, H.S. (2009). Antioxidant activity, phenolic compounds and anthocyanins content of eighteen strains of Mexican maize. *LWT – Food Science and Technology, 42*(6), 1187–1192.
80. Luna-Vital, D.A., de Mejia, E.G. (2018). Anthocyanins from purple corn activate free fatty acid-receptor 1 and glucokinase enhancing *in vitro* insulin secretion and hepatic glucose uptake. *PLoS One*, *13*(7), art. no. e0200449.
81. Mamani-Choquepata, R., Mamani-Quispe, P., Manchego-Rosado, L., Moreno-Loaiza, O., Paz-Aliaga, A. (2013). Dose effect of anthocyanins of three extracts of *Zea Mays* L. (purple corn) in the vasodilation of rat aortic rings. *Revista Peruana de Medicina Experimental y Salud Publica, 30*(4), 714–728 (in Spanish).
82. Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition, 81*(1), 230S-242S.
83. Mazza, G., Brouillard, R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry*, *29*(4), 1097–1102.
84. Mazza, G., Kay, C.D., Cottrell, T., Holub, B.J. (2002). Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *Journal of Agricultural and Food Chemistry*, *50*(26), 7731–7737.
85. McCord, J.M. (2000). The evolution of free radicals and oxidative stress. *American Journal of Medicine*, *108*(8), 652–659.
86. McGhie, T., Walton, M. (2007). The bioavailability and absorption of anthocyanins: towards a better understanding. *Molecular Nutrition & Food Research, 51*(6), 702–713.
87. Mex-Alvarez, R., Garma-Quen, P., Bolivar-Fernandez, N., Guillen-Morales, M., Tut-Heredia, J. (2016). Influence of the maize varieties *Zea mays* L. of Campeche on the weight gain in mice. *Revista Iberoamericana de Ciencias, 3*(5), 106–113 (in Spanish; English abstract).
88. Mora-Rochin, S., Gaxiola-Cuevas, N., Gutierrez-Uribe, J.A., Milan-Carrillo, J., Milan-Noris, E.M., Reyes-Moreno, C., Serna-Saldivar, S.O., Cuevas-Rodriguez, E.O. (2016). Effect of traditional nixtamalization on anthocyanin content and profile in Mexican blue maize (*Zea mays* L.) landraces. *LWT – Food Science and Technology*, *68,* 563–569.
89. Moreno-Loaiza, O., Paz-Aliaga, A. (2010). Vasodilator effect mediated by nitric oxide of the *Zea mays* L. (andean purple corn) hydroalcoholic extract in aortic rings of rat. *Revista Peruana de Medicina Experimental y Salud Publica,* 27(4), 527–531 (in Spanish; English abstract).
90. Mullen, W., Edwards, C.A., Crozier, A. (2006). Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl-and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. *British Journal of Nutrition*, *96*(1), 107–116.
91. Nabae, K., Hayashi, S.M., Kawabe, M., Ichihara, T., Hagiwara, A., Tamano, S., Tsushima Y., Uchida, K., Koda, T., Nakamura, M., Ogawa, K., Shirai, T. (2008). A 90-day oral toxicity study of purple corn color, a natural food colorant, in F344 rats. *Food and Chemical Toxicology*, *46*(2), 774–780.
92. Navolokin, N.A., Polukonova, N.V., Maslyakova, G.N., Bucharskaya, A.B., Durnova, N.A. (2012). Effect of extracts of *Gratiolla Officinalis* and *Zea Mays* on the tumor and the morphology of the internal organs of rats with transplanted liver cancer. *Russian Open Medical Journal, 1*(2), 1–4.
93. Pandey, K.B., Rizvi, S.I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 270–278.
94. Pedreschi, R., Cisneros-Zevallos, L. (2007). Phenolic profiles of Andean purple corn (*Zea mays* L.). *Food Chemistry*, *100*(3), 956–963.
95. Peixoto, H., Roxo, M., Krstin, S., Rohrig, T., Richling, E., Wink, M. (2016). An anthocyanin-rich extract of Açai (*Euterpe precatoria* Mart.) increases stress resistance and retards aging-related markers in *Caenorhabditis elegans*. *Journal of Agriculture and Food Chemistry, 64*(6), 1283–1290.
96. Petrov, V., Diniz, A.M., Cunha-Silva, L., Parola, A.J., Pina, F. (2013). Kinetic and thermodynamic study of 2'-hydroxy-8-methoxyflavylium. Reaction network interconverting flavylium cation and flavanone. *RSC Advances*, *3*(27), 10786–10794.
97. Piperno, D.R., Ranere, A.J., Holst, I., Iriarte, J., Dickau, R. (2009). Starch grain and phytolith evidence for early ninth millennium BP maize from the Central Balsas River Valley, Mexico. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(13), 5019–5024.

98. Prior, R.L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *The American Journal of Clinical Nutrition, 78*(3), 570S-578S.
99. Ramirez-Tortosa, C., Andersen, Ø.M., Gardner, P.T., Morrice, P.C., Wood, S.G., Duthie, S.J., Collins, A.R, Duthie, G.G. (2001). Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biology and Medicine*, *31*(9), 1033–1037.
100. Ramos-Escudero, F., Muñoz, A.M., Alvarado-Ortíz, C., Alvarado, A., Yáñez, J.A. (2012). Purple corn (*Zea mays* L.) phenolic compounds profile and its assessment as an agent against oxidative stress in isolated mouse organs. *Journal of Medicinal Food*, *15*(2), 206–215.
101. Revilla, P., Soengas, P., Malvar, R.A. (2018). Effects of antioxidant activity of black maize in corn borer larval survival and growth. *Spanish Journal of Agricultural Research*, *16*(1), art. no. e1004.
102. Rice-Evans, C.A., Miller, N.J., Paganga, G. (1996). Structure--antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*(7), 933–956.
103. Rice-Evans, C.A., Miller, N.J., Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152–159.
104. Rocchetti, G., Giuberti, G., Gallo, A., Bernardi, J., Marocco, A., Lucini, L. (2018). Effect of dietary polyphenols on the *in vitro* starch digestibility of pigmented maize varieties under cooking conditions. *Food Research International*, *108*, 183–191.
105. Roh, K.-B., Kim, H., Shin, S., Kim, Y.-S., Lee, J.-A., Kim, M.O., Jung, E., Lee, J., Park, D. (2016). Anti-inflammatory effects of *Zea mays* L. husk extracts. *BMC Complementary and Alternative Medicine, 16*(1), art. no. 298.
106. Rojas-Molina, I., Gutierrez-Cortez, E., Palacios-Fonseca, A., Baños, L., Pons-Hernandez, J.L., Guzman-Maldonado, S.H., Pineda-Gomez, P., Rodriguez, M.E. (2007). Study of structural and thermal changes in endosperm of quality protein maize during nixtamalization process. *Cereal Chemistry, 84*(4), 304–312.
107. Sadilova, E., Stintzing, F.O., Carle, R. (2006). Anthocyanins, colour and antioxidant properties of eggplant (*Solanum molongena* L.) and violet pepper (*Capsicum annum* L.) peel extracts. *Zeitschrift for Naturforschung C – A Journal of Biosciences, 61*(7–8), 527–535.
108. Salinas, M., Soto, H., Martinez, B., Gonzalez, H., Ortega, P. (1999). Análisis de antocianinas en maíces de grano azul y rojo provenientes de cuatro razas. *Revista de Fitotecnia Mexicana, 22*(2), 161–174 (in Spanish; English abstract).
109. Salinas-Moreno, Y., Cruz-Chavez, F., Diaz-Ortiz, S., Castillo--Gonzalez, F. (2012). Pigmented maize grains from Chiapas, physical characteristics, anthocyanin content and nutraceutical value. *Revista de Fitotecnia Mexicana, 35*(1), 33–41 (in Spanish; English abstract).
110. Salinas-Moreno, Y., Garcia-Salinas, C., Coutiño-Estrada, B., Vidal-Martinez, V. (2013). Content and type variability of anthocyanins in blue/purple colored grains of Mexican maize populations. *Revista de Fitotecnia Mexicana, 36*(3), 285–294 (in Spanish; English abstract).
111. Salinas-Moreno, Y., Rubio Hernández, D., Díaz Velázquez, A. (2005b). Extraction and use of pigments from maize grains (*Zea mays* L.) as colorants in yogurt. *Archivos Latinoamericanos de Nutrición*, *55*(3), 293–298 (in Spanish; English abstract).
112. Salinas-Moreno, Y., Salas-Sanchez, G., Rubio-Hernandez, D., Ramos-Lobato, N. (2005a). Characterization of anthocyanins extracts from maize kernels. *Journal of Chromatographic Science, 43*(9), 483–487.
113. Sanchez J.J., Goodman, M.M., Stuber, C.W. (2000). Isozymatic and morphological diversity in the races of maize of Mexico. *Economic Botany*, 54(1), 43–59.
114. Serna-Saldivar, S., Gutierrez-Uribe, J., Mora-Rochin, S., Garcia-Lara, S. (2013). Nutraceutical potential of native maize and changes during traditional and extrusion processing. *Revista de Fitotecnia Mexicana, 36*(3), 295–304 (in Spanish; English abstract).
115. Sharma, M., Cortes-Cruz, M., Ahern, K.R., McMullen, M., Brutnell, T.P., Chopra, S. (2011). Identification of the *Pr1* gene product completes the anthocyanin biosynthesis pathway of maize. *Genetics*, *188*(1), 69–79.
116. Shen, L.Y., Petolino, J.F. (2006). Pigmented maize seed via tissue-specific expression of anthocyanin pathway gene transcription factors. *Molecular Breeding*, *18*(1), 57–67.
117. Styles, E., Ceska, O. (1972). Flavonoid pigments in genetic strains of maize. *Phytochemistry, 11*(10), 3019–3021.
118. Suket, N., Srisook, E., Hrimpeng, K. (2014). Antimicrobial activity of the anthocyanins isolated from purple field corn (*Zea mays* L.) cob against Candida spp. *Journal of Pharmacy and Biological Sciences*, *9*(4), 40–44.
119. Takeoka, G.R., Dao, L.T., Full, G.A., Wong, R.Y., Harden, L.A., Edwards, R.A., Berrios, J.D. (1997). Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins. *Journal of Agricultural and Food Chemistry, 45*(9), 3395–3400.
120. Talavéra, S., Felgines, C., Texier, O., Besson, C., Manach, C., Lamaison, J., Rémésy, C. (2004). Anthocyanins are efficiently absorbed from the small intestine in rats. *Journal of Nutrition, 134*(9), 2275–2279.
121. Thiraphatthanavong, P., Wattanathorn, J., Muchimapura, S., Thukham-Mee, W., Wannanon, P., Tong-un, T., Suriharn, B., Lertrat, K. (2014). Preventive effect of *Zea mays* L. (purple waxy corn) on experimental diabetic cataract. *BioMed Research International*, 2014, art. no. 507435.
122. Tian, Q.G., Giusti, M.M., Stoner, G.D., Schwartz, S.J. (2006). Urinary excretion of black raspberry (*Rubus occidentalis*) anthocyanins and their metabolites. *Journal of Agricultural and Food Chemistry, 54*(4), 1467–1472.
123. Tian, X.Z., Paengkoum, P., Paengkoum, S., Chumpawadee, S., Ban, C., Thongpea, S. (2019). Short communication: Purple corn (*Zea mays* L.) stover silage with abundant anthocyanins transferring anthocyanin composition to the milk and increasing antioxidant status of lactating dairy goats. *Journal of Dairy Science, 102*(1), 413–416.
124. Tiessen, A., Cubedo-Ruiz, E.A., Winkler, R. (2017). Improved representation of biological information by using correlation as distance function for heatmap cluster analysis. *American Journal of Plant Sciences, 8*(3), 502–516.
125. Toufektsian, M.C., de Longeril, M., Nagy, N., Salen, P., Donati, M.B., Giordano, L., Mock, H.P., Peterek, S., Matros, A., Petroni, K., Pilu, R., Rotilio, D., Tonelli, C., de Leiris, J., Bouchet, F., Martins, C. (2008). Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. *Journal of Nutrition, 138*(4), 747–752.

126. Tsuda, T. (2012). Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. *Molecular Nutrition & Food Research, 56*(1), 159–170.
127. Tsuda, T., Horio, F., Osawa, T. (2000). The role of anthocyanins as an antioxidant under oxidative stress in rats. *Biofactors, 13*(1–4), 133–139.
128. Tsuda, T., Horio, F., Uchida, K., Aoki, H., Osawa, T. (2003). Dietary cyanidin 3-*O*-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *Journal of Nutrition, 133*(7), 2125–2130.
129. Urias-Lugo, D.A., Heredia, J.B., Muy-Rangel, M.D., Valdez-Torres, J.B., Serna-Saldivar, S.O., Gutierrez-Uribe, J.A. (2015b). Anthocyanins and phenolic acids of hybrid and native blue maize (*Zea mays* L.) extracts and their antiproliferative activity in mammary (MCF7), liver (HepG2), colon (Caco2 and HT29) and prostate (PC3) cancer cells. *Plant Foods for Human Nutrition, 70*(2), 193–199.
130. Urias-Lugo, D.A., Heredia, J.B., Serna-Saldivar, S.O., Muy-Rangel, M.D., Valdez-Torres, J.B. (2015a). Total phenolics, total anthocyanins and antioxidant capacity of native and elite blue maize hybrids (*Zea mays* L.). *CyTA – Journal of Food*, *13*(3), 336–339.
131. Urias-Peraldi, M., Gutierrez-Uribe, J.A., Preciado-Ortiz, R.E., Cruz-Morales, A.S., Serna-Saldivar, S.O., Garcia-Lara, S. (2013). Nutraceutical profiles of improved blue maize (*Zea mays*) hybrids for subtropical regions. *Field Crops Research*, 141, 69–76.
132. Villasante, A., Patro, B., Chew, B., Becerra, M., Wacyk, J., Overturf, K., Powell, M.S, Hardy, R.W. (2015). Dietary intake of purple corn extract reduces fat body content and improves antioxidant capacity and *n*-3 polyunsaturated fatty acid profile in plasma of rainbow trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society, 46*(4), 381–394.
133. Wallace, T.C., Giusti, M.M. (2015). Anthocyanins. *Advances in Nutrition*, *6*(5), 620–622.
134. Wang, D., Wei, X., Yan, X., Jin, T., Ling, A. (2010). Protocatechuic acid, a metabolite of anthocyanins, inhibits monocyte adhesion and reduces atherosclerosis in apolipoprotein E-deficient mice. *Journal of Agricultural and Food Chemistry, 58*(24), 12722–12728.
135. Wang, H., Cao, G.H., Prior, R.L. (1997). Oxygen radical absorbing capacity of anthocyanins. *Journal of Agricultural and Food Chemistry*, *45*(2), 304–309.
136. Wang, H., Nair, M.G., Strasburg, G.M., Chang, Y.C., Booren, A.M., Gray, J.I., DeWitt, D.L. (1999). Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *Journal of Natural Products*, *62*(2), 294–296.
137. Wang, L.S., Stoner, G.D. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters*, *269*(2), SI, 281–290.
138. Wattanathorn, J., Thiraphatthanavong, P., Muchimapura, S., Thukhammee, W., Lertrat, K., Suriharn, B. (2015). The combined extract of *Zingiber officinale* and *Zea mays* (purple color) improves neuropathy, oxidative stress, and axon density in streptozotocin induced diabetic rats. *Evidence-Based Complementary and Alternative Medicine,* 2015, art. no. 301029.
139. Wu, T., Guo, X.Q., Zhang, M., Yang, L., Liu, R., Yin, J.J. (2017). Anthocyanins in black rice, soybean and purple corn increase fecal butyric acid and prevent liver inflammation in high fat diet-induced obese mice. *Food & Function*, *8*(9), 3178–3186.
140. Yang, M., Kooh, S.I., Song, W.O., Chun, O.K. (2011). Food matrix affecting anthocyanin bioavailability: Review. *Current Medicinal Chemistry*, 18, 291–300.
141. Yang, Z., Zhai, W. (2010). Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (*Zea mays* L.). *Innovative Food Science Emerging Technologies*, *11*(1), 169–176.
142. Yonekura-Sakakibara, K., Fukushima, A., Nakabayashi, R., Hanada, K., Matsuda, F., Sugawara, S., Inoue, E., Kuromori, T., Ito, T., Shinozaki, K., Wangwattana, B., Yamazaki, M., Saito, K. (2012). Two glycosyltransferases involved in anthocyanin modification delineated by transcriptome independent component analysis in *Arabidopsis thaliana*. *Plant Journal*, *69*(1), 154–167.
143. Zhang, Q., Luna-Vital, D., de Mejia, E.G. (2019). Anthocyanins from colored maize ameliorated the inflammatory paracrine interplay between macrophages and adipocytes through regulation of NF-κB and JNK-dependent MAPK pathways. *Journal of Functional Foods*, *54*, 175–186.
144. Zhang, Z., Yang, L., Ye, H., Du, X.F., Gao, Z.M., Zhang, Z.-L. (2010). Effects of pigment extract from black glutinous corncob in a high-fat-fed mouse model of hyperlipidemia. *European Food Research and Technology, 230*(6), 943–946.
145. Zhang, Z., Zhou, B., Wang, H.H., Wang, F., Song, Y.L., Liu, S.N., Xi, S.H. (2014). Maize purple plant pigment protects against fluoride-induced oxidative damage of liver and kidney in rats. *International Journal of Environmental Research and Public Health, 11*(1), 1020–1033.
146. Zhao, C.L., Chen, Z.J., Bai, X.S., Ding, C., Long, T.J., Wei, F.G., Miao, K.R. (2014). Structure-activity relationships of anthocyanidin glycosylation. *Molecular Diversity, 18*(3), 687–700.
147. Zhao, X.Y., Zhang, C., Guigas, C., Ma, Y., Corrales, M., Tauscher, B., Hu, X.S. (2009). Composition, antimicrobial activity, and antiproliferative capacity of anthocyanin extracts of purple corn (*Zea mays* L.) from China. *European Food Research and Technology, 228*(5), 759–765.
148. Žilić, S., Serpen, A., Akıllıoğlu, G., Gökmen, V., Vančetović, J. (2012). Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (*Zea mays* L.) kernels. *Journal of Agricultural and Food Chemistry*, *60*(5), 1224–1231.

Submitted: 23 May 2019. Revised: 19 July and 15 October 2019. Accepted: 22 October 2019. Published on-line: 17 December 2019.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 101–111
DOI: 10.31883/pjfns/117716
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

High Voltage Electrical Discharges and Ultrasound-Assisted Extraction of Phenolics from Indigenous Fungus-Resistant Grape By-Product

Ante Lončarić[1], Antun Jozinović[1], Tihomir Kovač[1], Nebojša Kojić[2], Jurislav Babić[1], Drago Šubarić[1]*

[1]Faculty of Food Technology Osijek, Josip Juraj Strossmayer University of Osijek, Franje Kuhača 20, 31000 Osijek, Croatia
[2]Vupik plus d.o.o., Sajmište 113c, 32000 Vukovar, Croatia

Key words: grape by-product, phenolics, high voltage electrical discharges, ultrasound-assisted extraction

The possible use of high voltage electrical discharges (HVED) at different frequencies (20, 50, and 100 Hz) and ultrasound-assisted extraction (UAE) at different temperatures (20, 40, and 80°C) for the recovery of phenolics from indigenous fungus-resistant grape by-product was evaluated. All extractions were performed over the period of 5, 10, and 15 min and with methanol- and ethanol-based solvents. Grape pomace (the grape by-product) was collected during the preparation of grape jams. The main phenolics identified in grape pomace were anthocyanins, including malvidin, delphinidin, peonidin-3-*O*-glucoside, and cyanidin-3-*O*-glucoside. Beside anthocyanins, phenolic acids, flavan-3-ols, and flavonols were identified. The HVED-assisted extraction showed to be a superior extraction method for obtaining high yields of all analysed compounds. The highest amount of total phenolics (3023.57 mg GAE/100 g DM) was extracted from grape pomace, using 50% (*v/v*) ethanol and 60 kV/cm HVED at 100 Hz for 15 min. The antioxidant activity of the HVED extract was 2.17 mmol Trolox/g DM. The highest yield of the identified phenolics from grape pomace was obtained with electric field intensity of 60 kV/cm and total energy input of 22.27 kJ/kg, during the extraction.

INTRODUCTION

Plant phenolics are organic compounds found abundantly in plants with potential health benefits. According to the Transparency Market Research, global demand for phenolics in 2018 was estimated at USD 873.7 million [Ameer *et al.,* 2017]. One way of meeting the high market requirements is exploitation of fruit by-products to extract and isolate phenolic compounds. During the production of wine, grape jams and juices, huge amounts of the grape pomace (GP) are obtained as the by-product. In Europe, one way of exploiting grape pomace is for obtaining the enocyanins, *i.e.* food colorants E-163, which are anthocyanins isolated from red wine pomace. Current data on phenolics profile of GP vary greatly between different studies [Teixeira *et al.,* 2014]. It is found that GP contains flavan-3-ols, flavonols, anthocyanins, and phenolic acids [Yang *et al.,* 2009]. Grape pomace consists mainly of grape skin which in red grape varieties is rich with anthocyanins, including malvidin, cyanidin, delphinidin, peonidin, petunidin, and their glucosides. So far, most of the researches on the utilization of grape pomace have been made with the pomace produced from processing of conventional grape varieties. However, the data on the phenolics profile of varieties of fungus-resistant grapes (FRG) showed that FRG are rich in phenolic acids, dihydrochalcones, stilbenes, flavonols, flavan-3-ols, and anthocyanins [Kontić *et al.,* 2016; Ehrhardt *et al.,* 2014]. The FGR were bred by interspecific cross-breeding between the Mediterranean variate *Vitis vinifera* and American varieties with the aim to develop varieties resistant to fungal diseases, including powdery and downy mildews, and grey rot. FRG were introduced in Europe in the 19th century to counter the invasion of the vermin *Phylloxera* (*Viteus vitifoliae*) [Noah, 2016]. Although production of wine from some FRG including the *Noah, Othello, Isabelle, Jacquez, Clinton*, and *Herbemont* varieties is forbidden due to Regulation EU No. 1308/2013, FRG are still grown in several European countries. Fungus-resistant grapes are consumed mainly as fresh fruit; however, due to their high resistance and high yields, they represent a good raw material for the production of different products such as juices, jams, jellies, *etc*. The GP obtained after production of such products from FRG has a high content of phenolic compounds due to incomplete extraction during the processing. These phenolic compounds are of interest because they have a positive effect on human health through their antioxidant, antibacterial, and anticarcinogenic potential, and the capability to prevent cardiovascular diseases, *etc*. [Rasouli *et al.,* 2017].

Nowadays, there is a trend in exploring non-conventional, green methods for the extraction of phenolic compounds. High voltage electrical discharges (HVED) is one of them. The HVED enhance the rate of extracted phenolic compounds per initial plant material at low energy input during processing [Li *et al.,* 2019; Boussetta *et al.,* 2012]. Applying high voltage

* Corresponding Author: Tel.: 0038531 244 350;
E-mail: ante.loncaric@ptfos.hr (A. Loncarić)

between two electrodes submersed in water, or water solution, accelerates electrons that electrify water molecules creating the barrage of electrons called a streamer, and if the applied electric field is intense enough, the streamer propagates from the positive to the negative electrode. Consequently, chemical reactions and physical processes such as shock waves, cavitation and production of active species, will occur as well [Touya *et al.,* 2006]. These processes result in a rupture of the cell plant tissue which greatly improves extraction of intracellular compounds from plant material [Liu *et al.,* 2011]. The other method is ultrasound-assisted extraction (UAE) which is one of the most common extraction methods [Trojanowska *et al.,* 2019; Bakht *et al.,* 2018] used for recovering phenolics from plant material. UAE enhances a possibility to improve extraction yields while reducing the use of solvents, providing the opportunity to use greener alternative solvents and enhancing extraction of heat-sensitive components. The mass transfer during UAE is intensified by the implosion that creates high temperature and pressure spots between the solution and the solid matrix [Da Porto *et al.,* 2013]. Ultrasound-assisted extraction proved to be more efficient at different temperatures and with different solvents used for extraction, than shaking water bath extraction method.

Considering solvents used for the extraction of phenolics, the most used ones are water, methanol, and ethanol. In food systems, ethanol and water are preferred extraction solvents because of the hygiene, low cost, and abundance in addition to being compatible with health [Moure *et al.,* 2001].

The objective of this study was to compare the extraction methods for the extraction of phenolics from indigenous fungus-resistant grape by-product and to evaluate the extracts obtained. This by-product has not been studied in depth before (to the best of our knowledge) and could be a good source of bioactive phenolic compounds. The reason for comparison of extraction methods is to enable the recognition of new green methods for the extraction of valuable bioactive compounds from grape by-products and to obtain extracts rich with phenolic compounds.

MATERIAL AND METHODS

Materials

Gallic acid, catechin, epicatechin, *p*-coumaric acid, chlorogenic acid, caffeic acid, quercetin, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin, delphinidin, Folin–Ciocalteu reagent (FC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were purchased from Sigma Aldrich (Darmstad, Germany). Grape pomace of *Isabell* variety, belonging to FRG varieties, was obtained from local producers from Slatina, Croatia. The grape pomace was obtained after pressing the grapes and removing the juice, which was used for jam production. Grape pomace was freeze-dried using a freeze-dryer (Christ Freeze Dryer, Gamma 2–20, Osterode, Germany) at -55°C under 0.220 mbar.

Determination of physicochemical properties of grape by-product

Total acidity were measured by titration with 0.1 M NaOH with phenolphthalein as an indicator. Results were expressed as g of tartaric acid equivalents per 100 g of GP (g/100 g). pH was measured using a table top pH-meter (FiveEasy FE20, Mettler Toledo, Giessen, Germany). GP color was determined with a colorimeter (Minolta CR-300, Kioto, Japan) using the L*a*b* system.

Phenolic compounds extraction

Extractions of phenolics from GP powder were performed using two different solvents (50% (*v/v*) of ethanol and 1% (*v/v*) HCl or 50% (*v/v*) of methanol and 1% (*v/v*) HCl) and two techniques supporting extraction. All extractions were carried out in triplicate with a solvent to solid ratio of 50:1 (*v/w*).

High voltage electrical discharges treatment of GP powder suspensions was performed on an experimental apparatus (Inganiare CPTS1, Croatia) consisting of a high voltage power source which enabled the 30 kV discharges in a 600-mL chamber with electrodes of a needle-plate geometry. A sharp stainless steel needle 2.5 mm in diameter was used. A positive pulse voltage was applied to the stainless steel cylindrical needle (2.5 mm in diameter) and a stainless disc (45 mm in diameter) was used as a ground electrode with the distance between the electrodes of 5 mm. The treatment was conducted at room temperature (25°C) and on the magnetic stirrer at different HVED frequencies (20, 50, 100 Hz) and time (5, 10, 15 min).

Ultrasound-assisted extraction was performed at 35 KHz at different temperatures (20, 40, 80°C) and time (5, 10, 15 min) (Bandelin Sonorex Digitech, Berlin, Germany). Additionally, GP powder extraction without the assisted technique (as control) was carried out on a magnetic stirrer at 40°C for 30 min using 50% (*v/v*) ethanol acidified with 1% (*v/v*) HCl. After a suitable extraction time, for all types of extractions, suspensions were centrifuged (Multifuge 3 L-R Centrifuge, Heareus, Hanau, Germany) at 25°C for 15 min (6596.2 × *g*) and supernatants were filtered through 0.45 μm PTFE syringe-tip filter (Chromafil Xtra, Macherey-Nagel, Germany).

Determination of total phenolics content and antioxidant activity

The total phenolics content (TPC) was determined by employing an FC reagent according to a procedure described by Loncaric *et al.* [2014]. Briefly, 0.2 mL of the extract was mixed with 1.8 mL of deionized water, 10 mL of FC (1:10, *v/v*), and 8 mL of 7.5% sodium carbonate in test tubes. The development of blue color was monitored at 765 nm after 120 min (Jenway 6300 spectrophotometer, Bibby Scientific, Stone, UK). The values obtained were interpolated on a gallic acid calibration curve and expressed as mg of gallic acid equivalents per 100 g of dry matter (DM) of GP (mg GAE/100 g DM).

The antioxidant activity (AOA) was measured using a DPPH radical according to the methodology described by Brand-Williams *et al.* [1995]. The reaction mixture consisted of 0.2 mL of the extract and 3 mL of 0.5 mM DPPH radical solution in ethanol. The changes in the color of radical from deep violet to light yellow were measured at 517 nm using a UV-Vis spectrophotometer (Jenway 6300) and the results were expressed as mmol of Trolox equivalents per g of GP DM (mmol Trolox/g DM).

Determination of phenolics by HPLC

High performance liquid chromatography was performed with the Varian LC system (Palo Alto, CA, USA) equipped with a ProStar 230 solvent delivery module, and ProStar 330 photodiode array detector (PDA). Reverse phase chromatography analyses were carried out with the OmniSpher C18 column (4.6 × 250 mm) packed with 5 μm diameter particles (Varian, USA). Mobile phase consisted of 0.5% (*v/v*) water solution of phosphoric acid (solvent A) and 100% HPLC grade acetonitrile (solvent B); elution was conducted according to Loncaric *et al.* [2014] and injection volumes were 20 μL. The UV-Vis absorption spectra of the standards as well as the compounds present in samples were recorded in the range of 190 to 600 nm. Quantification has been performed by external standard calibration. The content of identified phenolics was expressed as mg per 100 g of GP DM (mg/100 g DM).

Statistical analysis

All measurements were done on three extracts and data were expressed as mean ± standard deviation. Normal distribution and homogeneity of variances was tested using the Shapiro-Wilkov and Levenovu test, respectively, after which the experimental data were analyzed statistically by one-way analysis of variance (ANOVA). Fisher's LSD was calculated to detect significant difference ($p \leq 0.05$) between the mean values. MS Excel (StatPlus, AnalystSoft Inc., Walnut, CA, USA) statistical program was used for statistical analysis.

RESULTS AND DISCUSSION

Effect of control extraction on the physicochemical properties of grape pomace and its phenolic profile and antioxidant activity

The physicochemical properties of grapes and their by-products in general are highly influenced by grape variety, growing areas, cultural practices, and growing year [Stewart, 2013]. A dark red powder was obtained after freeze-drying and grinding grape pomace. The results of analyses of the physicochemical properties of FRG by-product powder are given in Table 1. Color parameters of the GP powder indicate dark red color of the powder (values of L* and a* parameters were positive) and the value of coordinate b* indicates a slightly purple color. The results obtained for total acidity and pH are in the range of those reported by other authors for FRG varieties from different growing areas [Slegers *et al.,* 2015; Vos, 2014; Nisbet *et al.,* 2014]. The low pH value (3.16) of the grape pomace is a consequence of phenolics present in it [Bustamante *et al.,* 2008]. As with the physicochemical properties, the content of individual phenolics can also vary significantly from one FRG variety to another [Pedneault & Provost, 2016; Ehrhardt *et al.,* 2014]. In our study, the GP powder had a high content of total phenolics (260.43 mg GAE/100 g DM) and a high antioxidant activity (6.92 mmol Trolox/g DM) compared to literature data [Pedneault & Provost, 2016; Yang *et al*., 2009]. The most abounded phenolics in the investigated by-product were anthocyanins (163.68 mg/100 g DM); malvidin, delphinidin, peonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, which is in accordance with the findings of Balík *et al*. [2013]. The second most abounded phenolic was quercetin (19.85 mg/100 g DM) from the flavonol family followed by flavan-3-ols (16.62 mg/100 g DM), including catechin and epicatechin. The content of phenolic acids was 15.02 mg/100 g DM with identified caffeic acid, *p*-coumaric acid, gallic acid, and chlorogenic acid.

Effect of high voltage electrical discharge and ultrasound-assisted extractions on total phenolics content and antioxidant activity of grape pomace

Figures 1 and 2 present the total phenolics content and the antioxidant activity of GP extracted with HVED and UAE. The HVED enabled a five-fold intensification of total phenolics extraction compared to UAE. This corresponds to the findings of Boussetta *et al.* [2012] who have noted in their study that HVED extraction intensity of total phenolics from grape pomace, seeds, skins, and stems was increased 7 times compared to the control extraction. The highest TPC (3023.57 mg GAE/100 g DM) and AOA (2.17 mmol Trolox/g DM) were obtained upon the extraction with the ethanol-based solvent, HVED at 100 Hz, treatment time of 15 min,

TABLE 1. Physicochemical properties, phenolic profile and antioxidant activity of grape pomace*.

Parameter	Value
Total acidity (g/100 g)	0.23±0.01
pH	3.16±0.01
Color	
L*	9.26±0.03
a*	11.68±0.12
b*	-0.59±0.09
Individual phenolic content (mg/100 g DM)	
Gallic acid	1.86±0.06
Catechin	8.63±0.03
Epicatechin	7.99±0.05
p-Coumaric acid	3.16±0.02
Chlorogenic acid	1.43±0.11
Caffeic acid	8.57±0.08
Quercetin	19.85±0.03
Cyanidin-3-*O*-glucoside	16.81±1.11
Peonidin-3-*O*-glucoside	28.64±0.98
Malvidin	74.56±1.26
Delphinidin	43.67±0.43
TPC (mg GAE/100 g DM)	260.43±0.14
AOA (mmol Trolox/g DM)	6.92±0.05

*grape pomace extracted using acidified 50% (*v/v*) ethanol without additional assistance (control extraction).
Results are expressed as mean ± SD (n=3). TPC – total phenolic content; AOA – antioxidant activity (DPPH assay), DM – dry matter.

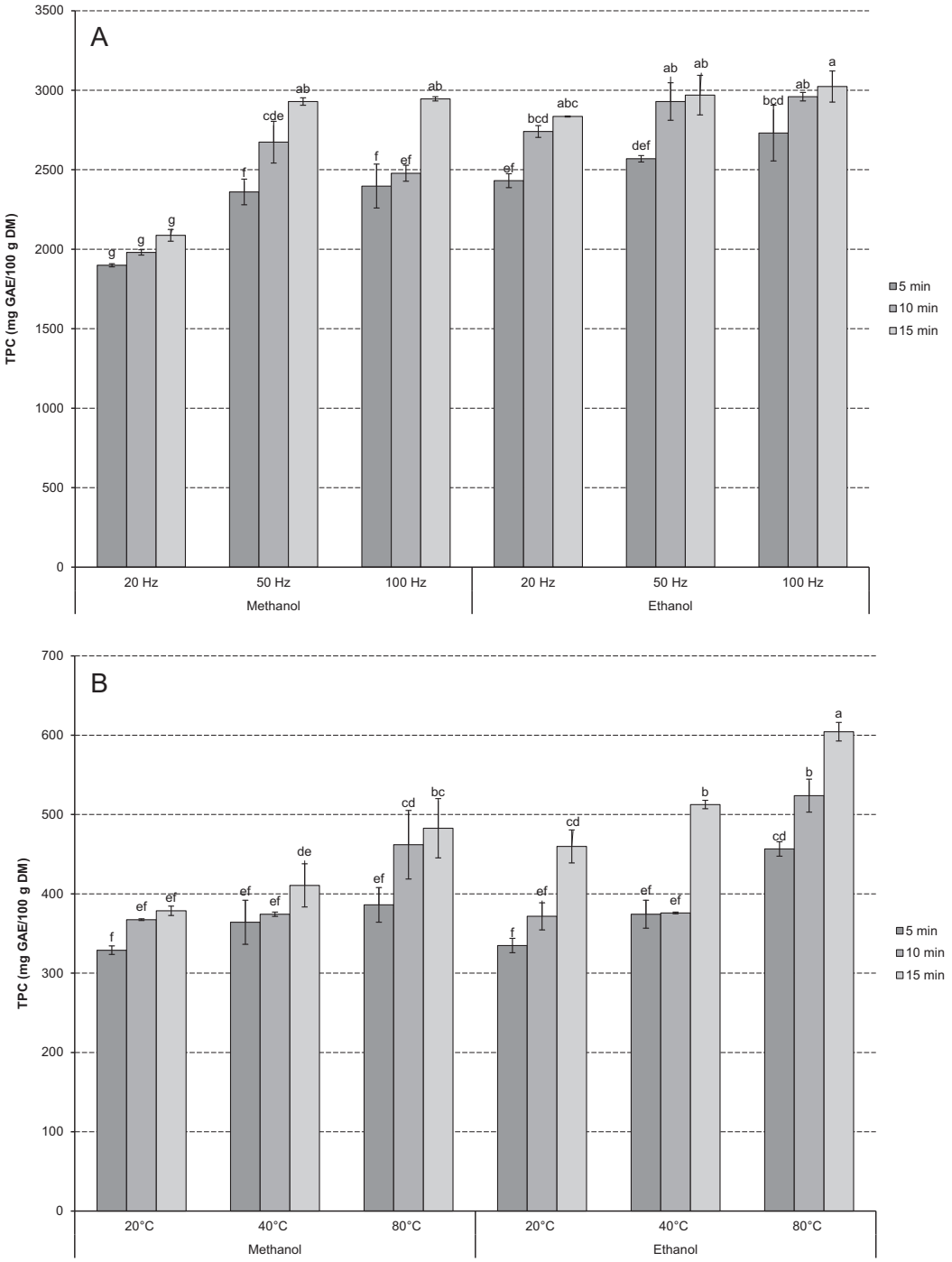


FIGURE 1. Total phenolic content (TPC) of grape pomace extracted with high voltage electrical discharges (HVED) treatment (A) and with ultrasound assistance (UAE) (B) for 5, 10 and 15 min. Results are expressed as means and SD (n=3) and calculated on the dry matter (DM) basis. Different letters above bars indicate significant differences between means at 95% confidence level as obtained by the LSD test.

and electric field intensity of 60 kV/cm. However, these values did not differ significantly ($p>0.05$) from those obtained when shorter extraction times at 100 Hz and also lower HVED frequency (50 Hz) were used. Considering UAE, the highest TPC (604.42 mg GAE/100 g DM) and AOA (1.52 mmol Trolox/g DM) were achieved at 80°C, and treatment time of 15 min. For total phenolics content and antioxidant activity, increasing the time and temperature during UAE produced higher recoveries with significant differences ($p\leq0.05$). The extraction of phenolic compounds from the plant material is influenced by their chemical nature, the sample size, extraction method, and extraction time as well as the structural and compositional features of the plant matrix and the strength with which they are bound to the plant matrix. However, when it comes to the solubility, differences in the structure of phenolic compounds determine their solubility in solvents [Shi *et al*., 2003b]. A mixture of some organic solvent (ethanol or methanol) and water proved to be more efficient in extracting phenolic compounds than the mono-component solvent system, which was reported by other authors for different products

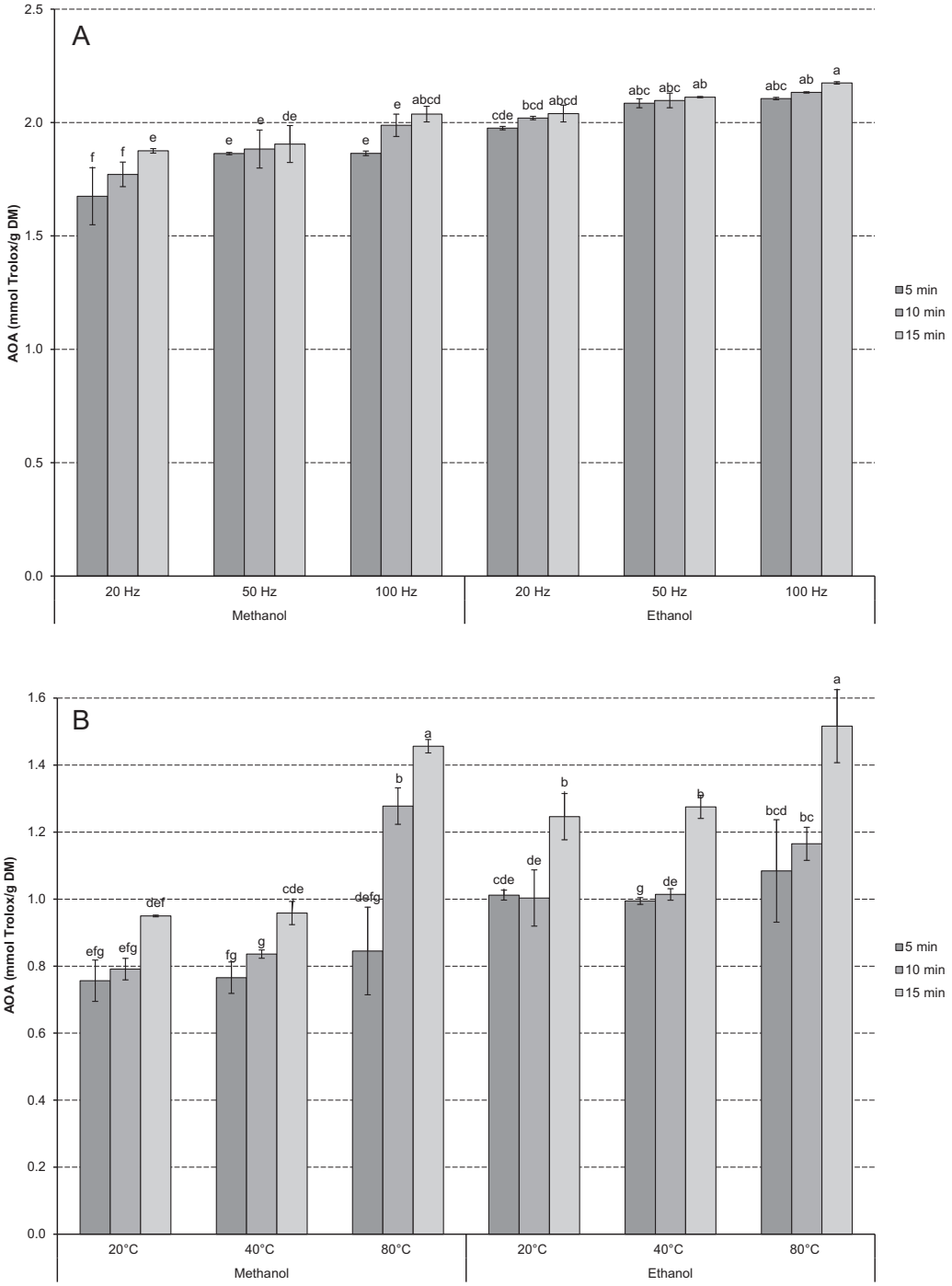


FIGURE 2. Antioxidant activity (AOA) of grape pomace extracted with high voltage electrical discharges (HVED) treatment (A) and with ultrasound assistance (UAE) (B) for 5, 10 and 15 min. Results are expressed as means and SD (n=3) and calculated on the dry matter (DM) basis. Different letters above bars indicate significant differences at 95% confidence level as obtained by the LSD test.

[Yan *et al*., 2018; Spigno *et al*., 2007; Pinelo *et al*., 2005]. When it comes to the extraction of phenolics from grape by-products, some authors [Do *et al*., 2014; Shi *et al*., 2003a] reported aqueous ethanol as a superior solvent while others [Pinelo *et al*., 2005] reported aqueous methanol as a superior solvent for the phenolics extraction. Therefore, the type of extraction solvent as well as the extraction method have a significant impact on the extraction yield of individual polyphenols from each individual plant material. There are some reports concerning optimization of extraction conditions of phenolic compound content and antioxidant activities of some plant foods but as some researches indicated the optimal procedure to be usually different for different plant matrices and different extraction methods [Rababah *et al*., 2010; Pellegrini *et al*., 2007].

Effect of high voltage electrical discharge-assisted extraction on phenolic profile of grape pomace

Table 2 shows contents of individual phenolics in grape pomace determined after HVED-assisted extraction. The effect of HVED extraction on the extraction of individual phe-

TABLE 2. Phenolic profile of grape pomace extracted with high voltage electrical discharges (HVED) treatment (mg/100 g DM).

Extraction conditions		Gallic acid	*p*-Coumaric acid	Caffeic acid	Catechin	Epicatechin	Quercetin	Cyanidin--3-*O*-glucoside	Peonidin--3-*O*-glucoside	Malvidin	Delphinidin
5 min											
MeOH	20 Hz	1.13±0.01^{o*}	3.66±0.08^{j}	28.25±0.02^{m}	8.66±0.04^{o}	7.95±0.09^{q}	16.24±0.02^{q}	17.62±0.03^{n}	28.97±0.01^{j}	79.61±0.08^{n}	42.19±0.09^{o}
	50 Hz	2.18±0.05^{j}	3.78±0.04^{i}	40.02±0.01^{h}	15.24±0.04^{j}	15.36±0.09^{l}	25.37±0.01^{l}	17.93±0.02^{m}	29.44±0.00^{i}	82.62±0.09^{j}	46.32±0.01^{i}
	100 Hz	2.28±0.01^{i}	4.78±0.01^{f}	39.05±0.03^{i}	22.93±0.01^{n}	19.69±0.03^{n}	25.13±0.01^{m}	19.42±0.04^{i}	31.64±0.09^{e}	86.51±0.13^{f}	49.15±0.11^{e}
EtOH	20 Hz	1.10±0.03^{op}	2.80±0.04^{m}	24.88±0.04^{o}	5.47±0.07^{q}	8.82±0.07^{p}	20.09±0.03^{o}	16.44±0.02^{p}	27.45±0.07^{l}	77.71±0.12^{p}	42.11±0.16^{o}
	50 Hz	1.41±0.04^{n}	2.92±0.01^{l}	33.81±0.07^{l}	23.34±0.06^{i}	14.10±0.04^{o}	28.98±0.07^{i}	17.12±0.02^{o}	28.19±0.05^{k}	79.15±0.09^{o}	44.08±0.13^{l}
	100 Hz	1.63±0.01^{l}	3.18±0.03^{k}	42.33±0.05^{f}	25.76±0.09^{h}	25.72±0.14^{h}	31.18±0.03^{g}	18.57±0.05^{k}	28.91±0.02^{j}	80.19±0.10^{m}	46.41±0.09^{i}
10 min											
MeOH	20 Hz	1.48±0.04^{m}	3.69±0.04^{ij}	39.08±0.10^{i}	19.61±0.04^{m}	18.46±0.10^{m}	16.73±0.09^{p}	19.55±0.14^{h}	29.84 ±0.08^{h}	82.41±0.13^{k}	44.82±0.04^{k}
	50 Hz	2.91±0.04^{f}	4.48±0.05^{g}	43.95±0.01^{e}	39.25±0.04^{f}	36.81±0.13^{f}	28.94±0.10^{i}	20.87±0.09^{e}	30.15 ±0.08^{g}	85.74±0.11^{g}	48.12±0.04^{fg}
	100 Hz	3.80±0.02^{c}	5.14±0.04^{e}	41.01±0.07^{g}	34.07±0.04^{g}	31.96±0.24^{g}	25.47±0.06^{k}	22.61±0.08^{b}	33.69 ±0.09^{b}	89.63±0.09^{b}	49.78±0.09^{c}
EtOH	20 Hz	1.06±0.05^{p}	3.67±0.04^{j}	26.83±0.01^{n}	7.27±0.08^{p}	20.07±0.30^{k}	21.47±0.09^{n}	18.11±0.04^{l}	28.88 ±0.13^{j}	79.61±0.16^{n}	43.10±0.07^{n}
	50 Hz	2.10±0.07^{k}	3.73±0.05^{ij}	38.57±0.04^{j}	46.73±0.05^{e}	36.58±0.18^{f}	30.45±0.11^{h}	18.96±0.06^{j}	29.73 ±0.08^{h}	81.19±0.11^{l}	46.09±0.03^{j}
	100 Hz	2.42±0.06^{h}	3.88±0.06^{h}	48.48±0.10^{b}	49.74±0.02^{c}	40.19±0.12^{e}	31.34±0.04^{f}	20.14±0.12^{f}	31.18 ±0.07^{f}	83.11±0.14^{j}	47.98±0.01^{g}
15 min											
MeOH	20 Hz	2.18±0.05^{j}	5.69±0.16^{d}	39.13±0.07^{i}	22.36±0.02^{l}	23.17±0.12^{j}	27.32±0.05^{j}	20.11±0.08^{f}	31.55±0.16^{e}	86.91±0.15^{e}	48.19±0.14^{f}
	50 Hz	2.62±0.03^{g}	5.84±0.06^{c}	44.11±0.07^{d}	48.22±0.05^{d}	41.24±0.21^{d}	33.11±0.02^{e}	22.64±0.01^{b}	32.39±0.01^{c}	89.33±0.13^{c}	50.08±0.09^{b}
	100 Hz	3.47±0.01^{d}	6.80±0.02^{a}	47.54±0.04^{c}	52.17±0.06^{b}	44.77±0.26^{c}	41.02±0.03^{b}	26.84±0.01^{a}	34.57±0.06^{a}	92.27±0.11^{a}	51.83±0.01^{a}
EtOH	20 Hz	3.19±0.05^{e}	5.69±0.03^{d}	35.16±0.02^{k}	22.66±0.03^{k}	24.65±0.49^{i}	33.37±0.02^{d}	19.89±0.01^{g}	30.12±0.11^{g}	81.24±0.09^{l}	43.64±0.08^{m}
	50 Hz	4.71±0.06^{a}	5.63±0.02^{d}	48.53±0.02^{b}	49.74±0.01^{c}	45.76±0.29^{b}	38.28±0.03^{c}	21.64±0.06^{d}	31.67±0.10^{e}	83.67±0.16^{h}	46.87±0.04^{h}
	100 Hz	4.21±0.05^{b}	5.97±0.06^{b}	54.09±0.01^{a}	53.68±0.07^{a}	53.19±0.29^{a}	45.31±0.06^{a}	22.44±0.04^{c}	32.08±0.09^{d}	88.09±0.15^{d}	49.56±0.11^{d}

Results are expressed as mean ± SD (n=3). Extracts were obtained after 5, 10 and 15 min of extraction in methanol (MeOH) and ethanol (EtOH) based solutions. Different letters in superscripts in each column indicate significant differences between means at 95% confidence level as obtained by the LSD test, DM – dry matter.

nolics varied. For all identified phenolic acids (gallic acid, *p*-coumaric and caffeic acids), all investigated parameters had a significant influence ($p \leq 0.05$) on the extraction yields. The highest extraction yield of gallic acid (4.71 mg/100 g DM) was achieved in the ethanol-based solvent after 15 min of extraction and HVED frequency of 50 Hz. The yields of *p*-coumaric and caffeic acids were increased with increasing the HVED frequency and with the duration of the extraction. The highest contents of *p*-coumaric acid (6.80 mg/100 g DM) and caffeic acid (54.09 mg/100 g DM) were achieved in GP after 15 min and HVED frequency extraction of 100 Hz in methanol-based and ethanol-based solvents, respectively. Both flavan-3-ols (catechin and epicatechin) were extracted with similar yields under each individual HVED extraction condition. Both, extraction time and HVED frequency significantly ($p \leq 0.05$) influenced the extraction yield of flavan-3-ols with their highest content in GP obtained after 15 min at 100 Hz in the ethanol-based solution. The highest content of quercetin in GP (45.31 mg/100 g DM) was obtained after extraction at HVED frequency of 100 Hz and treatment time of 15 min in the ethanol-based solvent. Other authors also reported better solubility of quercetin in ethanol-water mixtures compared with methanol-water mixtures [Razmara *et al.,* 2010]. All four identified anthocyanins were better extracted in the methanol-based solvent. The highest yields of those anthocyanins were obtained at HVED frequency of 100 Hz. However, extraction yields of the other two identified anthocyanins: peonidin-3-*O*-glucoside and malvidin, were significantly ($p \leq 0.05$) influenced by all process parameters. The highest contents of cyanidin-3-*O*-glucoside (26.84 mg/100 g DM), peonidin-3-*O*-glucoside (34.57 mg/100 g DM), malvidin (92.27 mg/100 g DM), and delphinidin (51.83 mg/100 g DM) were obtained in GP extracted by the methanol-based solvent after 15 min and frequency of 100 Hz. When choosing a solvent for the HVED extraction, water is a good choice since it is capable of dissolving most of the organic matter. However, some phenolics are poorly soluble in water and to increase their solubility it is useful to add organic solvents such as ethanol or methanol [Yan *et al.,* 2018], but if the organic solvent concentration is too high it will decrease solvent conductivity. Solvents used in this study had similar conductivity, *i.e.* 112.4 mS/cm in the case of the ethanol-based solvent and 111.1 mS/cm in the case of the methanol-based one. It is interesting that so far most of the HVED extractions were conducted in a relatively low liquid to solid ratio. However, this study showed that a higher liquid to solid (50:1, *v/w*) ratio could provide good phenolic yields, which is also supported by the findings of Barba *et al.* [2015]. In their study, they achieved a better yield of phenolic extraction from raw rapeseed (1.00 mg GAE/100 g) and rapeseed press cake (559.17 mg GAE/100 g) with a higher liquid to solid ratio (20:1, *v/w*) compared to the lower ratio (5:1, *v/w*) [Barba *et al.,* 2015].

However, it should be pointed out that for the extraction of phenolics with HVED, energy input (Eq. 1) is a most demanding factor combined with electric field intensity (Eq. 2) and treatment time [Rajha *et al.,* 2015].

$$W_{HVED} = \frac{E_p \times n}{m} \quad \text{Eq. 1}$$

$$E = \frac{V}{d} \quad \text{Eq. 2}$$

where: W_{HVED} is the energy input (kJ/kg), Ep is the energy of one pulse (kJ), m is the mass of suspension (kg); and E is the electric field intensity (kV/cm); V is the peak voltage (kV); d is the distance between electrodes (cm). Clearly, with the longer treatment time the energy input is higher, which leads to more complete destruction of plant cell structure and to extraction enhancement [Parniakov *et al.,* 2016]. Consequently, the yield would increase after a longer treatment time. However, higher energy inputs, over 100 kJ/kg, could lead to the degradation of target compounds or to the generation of H_2O_2 radicals [Rosello-Soto *et al.,* 2015]. In this study, the highest yields were obtained when treating the suspension for 15 min which gives an energy input of 22.27 kJ/kg, the electric field intensity was set on 60 kV/cm and energy of one impulse to 0.15 J. The energy input for treatment times of 5 and 10 min at 100 Hz was 7.43 and 14.85 kJ/kg, respectively.

Effect of ultrasound-assisted extraction on phenolic profile of grape pomace

The ultrasound power (35 kHz) used in this experiment is in the domain (18–40 kHz) for achieving much stronger ultrasound effects such as disruption of biological cell walls and cell membranes, as well as particle size reduction, as reported by Novak *et al.* [2008].

The effect of UAE on the contents of individual phenolics determined in GP is shown in Table 3. The phenolic acid profile was differently affected by the extraction parameters applied. The ethanol-based solvent proved better for the extraction of gallic acid and caffeic acid and the methanol-based one for the extraction of *p*-coumaric acid. Difference in polarity could influence the content of phenolic acids in the extracts, as the methanol polarity is 6.6 compared to 5.2 for ethanol [Betancourt *et al.,* 2008]. However, both extraction time and temperatures significantly ($p \leq 0.05$) influenced the extraction of phenolic acids. The highest extraction yields of gallic acid (2.32 mg/100 g DM) and caffeic acid (36.19 mg/100 g DM) were achieved in the ethanol-based solvent after 15 min at 80°C. The highest extraction yield of *p*-coumaric acid (5.87 mg/100 g DM) was obtained in the methanol-based solvent after 15 min at 80°C. The highest extraction yields of flavan-3-ols, including catechin (40.25 mg/100 g DM) and epicatechin (23.02 mg/100 g DM), were obtained in the ethanol-based solvent after 15 min at 80°C. Both epicatechin and catechin yields were significantly influenced by solvent type ($p \leq 0.05$), which was also noted during the HVED extraction. Other authors also reported that UAE of catechins was more efficient in the ethanol-water solvent and after longer extraction time [Alonso *et al.,* 1991]. The highest extraction yield of quercetin (24.97 mg/100 g DM) was obtained in the ethanol-based solvent after 15 min at 80°C. Anthocyanins were better extracted in the methanol-based solvent as in the HVED case. However, all identified anthocyanins were significantly ($p \leq 0.05$) influenced by all applied parameters equally. The higher extraction yields were obtained by increasing the temperature and extraction time. The high-

TABLE 3. Phenolic profile of grape pomace determined after ultrasound assisted extraction (UAE) (mg/100 g DM).

Extraction conditions		Gallic acid	*p*-Coumaric acid	Caffeic acid	Catechin	Epicatechin	Quercetin	Cyanidin--3-*O*-glucoside	Peonidin--3-*O*-glucoside	Malvidin	Delphinidin
5 min											
MeOH	20°C	0.27±0.01^{m}	0.58±0.01^{k}	19.02±0.05^{m}	10.65±0.02^{n}	5.16±0.03^{m}	3.93±0.03^{lm}	13.15±0.01^{o}	26.51±0.09^{n}	77.36±0.07^{l}	40.33±0.06^{n}
	40°C	0.50±0.03^{l}	0.85±0.00^{j}	19.87±0.05^{l}	11.66±0.08^{m}	5.63±0.00^{l}	7.57±0.01^{g}	16.11±0.00^{l}	27.33±0.07^{k}	77.98±0.06^{k}	41.21±0.04^{l}
	80°C	1.03±0.01^{ijk}	1.09±0.01^{h}	20.52±0.04^{j}	13.81±0.18^{k}	8.37±0.00^{j}	9.03±0.39^{f}	18.46±0.04^{f}	29.61±0.07^{e}	81.61±0.08^{f}	45.63±0.05^{f}
EtOH	20°C	0.89±0.01^{k}	0.26±0.01^{n}	18.08±0.07^{o}	6.66±0.04^{q}	8.46±0.03^{j}	3.69±0.16^{m}	12.53±0.06^{q}	24.19±0.08^{p}	73.31±0.08^{r}	38.62±0.04^{q}
	40°C	0.98±0.08^{jk}	0.34±0.02^{m}	18.56±0.06^{n}	8.66±0.04^{o}	10.99±0.01^{gh}	4.42±0.13^{k}	13.09±0.03^{p}	25.08±0.07^{o}	74.15±0.05^{q}	38.99±0.06^{p}
	80°C	1.46±0.03^{ef}	0.35±0.02^{m}	20.69±0.04^{j}	14.87±0.05^{i}	13.37±0.25^{d}	14.75±0.25^{c}	17.59±0.03^{j}	26.87±0.08^{l}	78.46±0.08^{j}	41.32±0.08^{k}
10 min											
MeOH	20°C	0.98±0.03^{jk}	1.95±0.05^{f}	21.69±0.02^{i}	13.52±0.05^{l}	7.87±0.04^{k}	5.33±0.35^{j}	16.11±0.01^{l}	28.61±0.07^{h}	79.31±0.04^{i}	41.36±0.04^{k}
	40°C	1.03±0.03^{ijk}	2.31±0.04^{e}	22.78±0.04^{h}	14.48±0.04^{j}	7.94±0.04^{k}	8.76±0.07^{f}	18.52±0.00^{e}	28.99±0.08^{g}	80.39±0.05^{h}	44.67±0.02^{g}
	80°C	1.10±0.05^{hij}	2.57±0.02^{d}	25.16±0.26^{f}	26.14±0.01^{c}	11.06±0.03^{g}	12.26±0.18^{e}	20.82±0.02^{b}	32.67±0.07^{b}	86.11±0.05^{b}	48.31±0.02^{b}
EtOH	20°C	1.29±0.08^{efg}	0.38±0.01^{m}	20.26±0.03^{k}	7.18±0.08^{p}	9.33±0.12^{i}	4.04±0.34^{l}	15.55±0.04^{n}	26.67±0.07^{m}	75.87±0.08^{p}	39.26±0.04^{o}
	40°C	1.28±0.07^{fgh}	0.44±0.06^{l}	25.03±0.26^{f}	11.66±0.02^{m}	10.70±0.00^{h}	8.69±0.28^{f}	15.93±0.03^{m}	27.94±0.04^{j}	76.49±0.05^{n}	40.56±0.03^{m}
	80°C	1.47±0.03^{de}	0.85±0.08^{j}	27.57±0.02^{e}	17.69±0.02^{f}	16.70±0.01^{c}	16.81±0.20^{b}	17.94±0.01^{h}	29.37±0.05^{f}	80.56±0.09^{g}	45.73±0.03^{e}
15 min											
MeOH	20°C	1.20±0.20^{ghi}	2.00±0.01^{f}	27.73±0.07^{e}	15.48±0.07^{h}	8.49±0.02^{j}	5.88±0.18^{i}	17.88±0.02^{i}	30.54±0.04^{d}	82.64±0.04^{d}	44.39±0.03^{h}
	40°C	1.65±0.03^{cd}	2.76±0.03^{c}	31.21±0.04^{d}	15.99±0.00^{g}	9.17±0.56^{i}	7.48±0.09^{g}	19.36±0.01^{d}	32.62±0.07b	84.82±0.04^{c}	47.61±0.07^{c}
	80°C	1.69±0.37^{c}	5.87±0.01^{a}	34.53±0.17^{c}	25.46±0.04^{d}	11.99±0.08^{f}	14.90±0.25^{c}	24.43±0.03^{a}	33.97±0.09^{a}	89.91±0.06^{a}	49.82±0.09^{a}
EtOH	20°C	1.81±0.06^{bc}	1.79±0.00^{g}	24.01±0.05^{g}	27.12±0.05^{b}	12.43±0.31^{e}	7.06±0.19^{h}	17.23±0.02^{k}	28.37±0.07^{i}	76.34±0.07^{o}	41.62±0.04^{j}
	40°C	1.95±0.04^{b}	1.03±0.03^{i}	35.32±0.09^{b}	22.82±0.04^{e}	19.44±0.23^{b}	14.05±0.03^{d}	18.06±0.04^{g}	29.61±0.08^{e}	76.99±0.04^{m}	43.55±0.05^{i}
	80°C	2.32±0.08^{a}	3.27±0.05^{b}	36.19±0.03^{a}	40.25±0.03^{a}	23.02±0.14^{a}	24.97±0.05^{a}	20.37±0.07^{c}	31.12±0.05^{c}	82.49±0.07^{n}	47.08±0.04^{d}

Results are expressed as mean ± SD (n=3). Extracts were obtained after 5, 10 and 15 min of extraction in methanol (MeOH) and ethanol (EtOH) based solutions. Different letters in superscripts in each column indicate significant differences between means at 95% confidence level as obtained by the LSD test, DM – dry matter.

est yields of cyanidin-3-*O*-glucoside (24.43 mg/100 g DM), peonidin-3-*O*-glucoside (33.97 mg/100 g DM), malvidin (89.91 mg/100 g DM), and delphinidin (49.82 mg/100 g DM) were obtained in the methanol-based solvent after 15 min of extraction at 80°C. Our findings are consistent with previous reports despite obvious differences in extraction conditions and plant materials. With UAE of dried chokeberries in 50% (*v/v*) ethanol, Ćujić *et al.* [2016] reported that the total anthocyanin contents were significantly greater after longer period of extraction. Wang *et al.* [2018] found the highest yields of TPC and TFC with extraction time of 15 min when extracting blueberry leaves using ultrasound-negative pressure cavitation extraction. The highest yields in all UAE experiments were obtained at 80°C (Table 3), this is because at higher temperatures diffusion coefficient and generally solubility of solute are increased [Palma & Taylor, 1999]. As during the HVED extractions, liquid to solid ratio is another important parameter since it facilitates the mass transfer driving force. The phenolic acid yields obtained in this experiment with the liquid to solid (50:1, *v/w*) ratio confirm this (Table 3). The results obtained are consistent with the findings of Bamba *et al.* [2018] who investigated the influence of extraction conditions on ultrasound-assisted recovery of bioactive phenolics from blueberry pomace and their antioxidant activity. Accordingly, the optimal extraction solvent was 50% (*v/v*) ethanol, and extraction was more efficient with a higher liquid to solid ratio and longer treatment time.

CONCLUSION

The FRG by-product was rich in phenolics, especially anthocyanins. The HVED-assisted extraction of grape pomace showed to be a superior extraction method for obtaining high yields of anthocyanins, phenolic acids, flavan-3-ols, and flavonols. The best parameters for extracting phenolics were: electric field intensity of 60 kV/cm, energy of one impulse of 0.15 J, treatment time of 15 min, and HVED frequency of 100 Hz, giving the total energy input of 22.27 kJ/kg during the extraction. Regarding UAE, it was shown that higher yields were achieved at higher temperature (80°C) and at longer treatment time (15 min), with ultrasound power set on 35 kHz. Since phenolics have different solubility, applying a solvent system with medium polarity seems to be more effective and appropriate to maximize contents of individual phenolics in the extract. Depending on the phenolic compound of interest, ethanol- or methanol-based solvents should be used.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

REFERENCES

1. Alonso, E., Bourzeix, M., Revilla, E. (1991). Suitability of water--ethanol mixtures for the *Vitis vinifera* seeds contained in a winery by-product. *Seed Science and Technology*, *19*(3), 545–552.
2. Ameer, K., Shahbaz, H.M., Kwon, J-H. (2017). Green extraction methods for polyphenols from plant matrices and their byproducts: A review. *Comprehensive Reviews in Food Science and Food Safety*, *16*(2), 295–315.
3. Bakht, A., Geesi, M.H., Riadi, Y., Imran, M., Ali, I., Jawed Ahsan, M., Ajmal, N. (2018). Ultrasound-assisted extraction of some branded tea: Optimization based on polyphenol content, antioxidant potential and thermodynamic study. *Saudi Journal of Biological Sciences*, *26*(5), 1043–1052.
4. Balík, J., Kumsta, M., Rop, O. (2013). Comparison of anthocyanins present in grapes of *Vitis vinifera* L. varieties and interspecific hybrids grown in the Czech Republic. *Chemical Papers*, *67*(10), 1285–1292.
5. Bamba, B., Shi, J., Tranchant, C., Xue, S., Forney, C., Lim, L.T. (2018). Influence of extraction conditions on ultrasound-assisted recovery of bioactive phenolics from blueberry pomace and their antioxidant activity. *Molecules, 23*(7), art. no. 1685.
6. Barba, F.J., Boussetta, N., Vorobiev, E. (2015). Emerging technologies for the recovery of isothiocyanates, protein and phenolic compounds from rapeseed and rapeseed press-cake: Effect of high voltage electrical discharges. *Innovative Food Science & Emerging Technologies*, *31*, 67–72.
7. Betancourt, A.O. (2008). Analysis, extraction and recovery of poly-3-hydroxybutyrate present in biomass *Université du Québec à Montréal Thesis*, pp. 45–55 [https://archipel.uqam.ca/1045/1/M10166.pdf].
8. Boussetta, N., Reess, T., Vorobiev, E., Lanoiselle, J.L. (2012). Pulsed electrical discharges: principles and application to extraction of biocompounds, In: N. Lebovka, E. Vorobiev, F. Chemat (Eds.), *Enhancing Extraction Processes in the Food Industry*, Taylor & Francis – CRC Press, USA, pp. 145–172.
9. Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT – Food Science and Technology*, *28*(1), 25–30.
10. Bustamante, M.A., Moral, R., Paredes, C., Pérez-Espinosa, A., Moreno-Caselles, J., Pérez-Murcia, M.D. (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. *Waste Management*, *28*(2), 372–380.
11. Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., Ibrić, S. (2016). Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. *Food Chemistry, 194*, 135–142.
12. Da Porto, C., Porretto, E., Decorti, D. (2013). Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. *Ultrasonics Sonochemistry*, *20*(4), 1076–1080.
13. Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Ju, Y-H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila* aromatic. *Journal of Food and Drug Analysis*, *22*(3), 296–302.
14. Ehrhardt, C., Arapitsas, P., Stefanini, M., Flick, G., Mattivi, F. (2014). Analysis of the phenolic composition of fungus-resistant grape varieties cultivated in Italy and Germany using UHPLC––MS/MS. *Journal of Mass Spectrometry*, *49*(9), SI, 860–869.
15. Kontić, J.K., Rendulić Jelušić, I., Tomaz, I., Preiner, D., Marković, Z., Stupić, D., Andabaka, Ž., Maletić, E. (2016). Polyphenolic composition of the berry skin of six fungus-resistant red grapevine varieties. *International Journal of Food Properties*, *19*(8), 1809–1824.

16. Li, Z., Fan, Y., Xi, J. (2019). Recent advances in high voltage electric discharge extraction of bioactive ingredients from plant materials. *Food Chemistry*, *277*, 246–260.
17. Liu, D., Vorobiev, E., Savoire, R., Lanoisellé. J-L. (2011). Intensification of polyphenols extraction from grape seeds by high voltage electrical discharges and extract concentration by dead-end ultrafiltration. *Separation and Purification Technology, 81*(2), 134–140.
18. Loncaric, A., Dugalic, K., Mihaljevic, I., Jakobek, L., Pilizota, V. (2014). Effects of sugar addition on total polyphenol content and antioxidant activity of frozen and freeze-dried apple purée. *Journal of Agricultural and Food Chemistry, 62*(7), 1674−1682.
19. Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Siniero, J., Dominguez, H., Nunez, M.J., Parajo, J.C. (2001). Natural antioxidants from residual sources. *Food Chemistry, 72*(2), 145–171.
20. Nisbet, M.A., Martinson, T.E., Mansfield, A.K. (2014). Accumulation and prediction of yeast assimilable nitrogen in New York wine grape cultivars. *American Journal of Enology and Viticulture*, *65*(3), 325–332.
21. Noah, A. (2016). *Forbidden Fruits: The fabulous destiny of Noah, Othello, Isabelle, Jacquez, Clinton and Herbemont*, Brussels and Vienna, April 2016, 1–7 [https://www.arche-noah.at/files/arche_noah_april_2016_direct_producers_forbidden_fruits_executive_summary_1.pdf]
22. Novak, I., Janeiro, P., Seruga, M., Oliveira-Brett, A.M. (2008). Ultrasound extracted flavonoids from four varieties of Portuguese red grape skins determined by reverse-phase high-performance liquid chromatography with electrochemical detection. *Analytica Chimica Acta*, *630*(2), 107–115.
23. Palma, M.L., Taylor, L.T. (1999). Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. *Journal of Chromatography A, 849*(1), 117–124.
24. Parniakov, O., Barba, F.J., Grimi, N., Lebovka, N., Vorobiev, E. (2016). Extraction assisted by pulsed electric energy as a potential tool for green and sustainable recovery of nutritionally valuable compounds from mango peels. *Food Chemistry, 192*, 842–848.
25. Pedneault, K., Provost, C. (2016). Fungus resistant grape varieties as a suitable alternative for organic wine production: Benefits, limits, and challenges. *Scientia Horticulturae, 208,* SI, 57–77.
26. Pellegrini, N., Colombi, B., Salvatore, S., Brenna, O.V., Galaverna, G., Del Rio, D., Bianchi, M., Bennett, R., Brighenti, F. (2007). Evaluation of antioxidant capacity of some fruit and vegetable foods: efficiency of extraction of a sequence of solvents. *Journal of the Science of Food and Agriculture, 87*(1), 103–111.
27. Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., Nunez, 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.
28. Rababah, T.M., Banat, F., Rababah, A., Ereifej, K., Yang, W. (2010). Optimization of extraction conditions of total phenolics, antioxidant activities, and anthocyanin of oregano, thyme, terebinth, and pomegranate. *Journal of Food Science, 75*(7), C626–C632.
29. Rajha, H.N., Boussetta, N., Louka, N., Maroun, R.G., Vorobiev, E. (2015). Electrical, mechanical, and chemical effects of high--voltage electrical discharges on the polyphenol extraction from vine shoots. *Innovative Food Science & Emerging Technologies*, *31*, 60–66.
30. Rasouli, H., Farzaei, M.H., Khodarahmi, R. (2017). Polyphenols and their benefits: A review. *International Journal of Food Properties*, *20*, Suppl. 2, 1700–1741.
31. Razmara, R.S., Daneshfar, A., Sahraei, R. (2010). Solubility of quercetin in water + methanol and water + ethanol from (292.8 to 333.8) K. *Journal of Chemical & Engineering Data, 55*(9), 3934–3936.
32. Regulation (EU) no 1308/2013 of the European Parliament and of the Council of 17 December 2013, establishing a common organisation of the markets in agricultural products and repealing Council Regulations (EEC) No 922/72, (EEC) No 234/79, (EC) No 1037/2001 and (EC) No 1234/2007. [http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1491852068735&uri=CELEX:32013R1308)].
33. Rosello-Soto, E., Barba, F.J., Parniakov, O., Galanakis, C.M., Lebovka, N., Grimi, N., Vorobiev, E. (2015). High voltage electrical discharges, pulsed electric field, and ultrasound assisted extraction of protein and phenolic compounds from olive kernel. *Food and Bioprocess Technology*, *8*(4), 885–894.
34. Shi, J., Yu, J., Pohorly, J., Young, C., Bryan, M. (2003a). Optimization of the extraction of polyphenols from grape seed meal by aqueous ethanol solution. *Journal of Food, Agriculture and Environment, 1*(2), 42–47.
35. Shi, J., Yu, J., Pohorly, J.E., Kakuda, Y. (2003b). Polyphenolics in grape seeds – biochemistry and functionality. *Journal of Medicinal Food, 6*(4), 291–299.
36. Slegers, A., Angers, P., Ouellet, É., Truchon, T., Pedneault, K. (2015). Volatile compounds from grape skin, juice and wine from five interspecific hybrid grape cultivars grown in Québec (Canada) for wine production. *Molecules, 20*(6), 10980–11016.
37. Spigno, G., Tramelli, L., De Faveri, D.M. (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *Journal of Food Engineering, 81*(1), 200–208.
38. Stewart, A.C. (2013). Nitrogen composition of interspecific hybrid and *Vitis Vinifera* wine grapes from the Eastern United States. *Doctoral Dissertation. Purdue University*, p. 227 [https://docs.lib.purdue.edu/dissertations/AAI3592130/].
39. Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D.A., Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: a review. *International Journal of Molecular Sciences, 15*(9), 15638–15678.
40. Touya, G., Reess, T., Pecastaing, L., Gibert, A., Domens, P. (2006). Development of subsonic electrical discharges in water and measurements of the associated pressure waves. *Journal of Physics D: Applied Physics, 39*(24), 5236–5244.
41. Trojanowska, A., Tsibranska, I., Dzhonova, D., Wroblewska, M., Haponska, M., Jovancic, P., Marturano, V., Tylkowski, B. (2019). Ultrasound-assisted extraction of biologically active compounds and their successive concentration by using membrane processes. *Chemical Engineering Research and Design, 147*, 378–389.
42. Vos, R. (2014). Stage of maturation, crop load, and shoot density affect the fruit quality of cold-hardy grape cultivars. *Doctoral Dissertation. Iowa State University, Aims*, IA, p. 120 [https://lib.dr.iastate.edu/etd/13812/].
43. Wang, T., Guo, N., Wang, S.X., Kou, P., Zhao, C.J., Fu, Y.J. (2018). Ultrasound-negative pressure cavitation extraction of phenolic compounds from blueberry leaves and evaluation

of its DPPH radical scavenging activity. *Food and Bioproducts Processing, 108*, 69–80.

44. Yan, L.G., Deng, Y., Ju, T., Wu, K., Xi, J. (2018). Continuous high voltage electrical discharge extraction of flavonoids from peanut shells based on “annular gap type” treatment chamber. *Food Chemistry*, *256*, 350–357.
45. Yang, J., Martinson, T.E., Liu, R.H. (2009). Phytochemical profiles and antioxidant activities of wine grapes. *Food Chemistry, 116*(1), 332–339.

Submitted: 23 July 2019. Revised: 11 November 2019 and 14 January 2020. Accepted: 31 January 2020. Published on-line: 02 March 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 113–123
DOI: 10.31883/pjfns/115461
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Effect of Enzyme-Assisted Vinification on Wine Phenolics, Colour Components, and Antioxidant Capacity

Ivana Generalić Mekinić[1], Živko Skračić[2], Ana Kokeza[1], Barbara Soldo[3], Ivica Ljubenkov[3], Mara Banović[4], Vida Šimat[5], Danijela Skroza[1]*

[1]*Department of Food Technology and Biotechnology, Faculty of Chemistry and Technology, University of Split, R. Boškovića 35, HR-21000 Split, Croatia*
[2]*Secondary School "Braća Radić", Put poljoprivrednika bb, HR-21217 Kaštel Štafilić, Croatia*
[3]*Department of Chemistry, Faculty of Science, University of Split, R. Boškovića 33, HR-21000 Split, Croatia*
[4]*Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, HR-10000 Zagreb, Croatia*
[5]*University Department of Marine Studies, University of Split, R. Boškovića 37, HR-21000 Split, Croatia*

Key words: Babica *cv.*, winemaking, enzyme-assisted vinification, phenolic compounds, colour, antioxidant activity

The aim of this study was to evaluate the impact of the macerating enzyme addition (Sihazym Extro and Vinozym Vintage) on the extraction of phenolics, colour components, and antioxidants in *Babica* wine. Spectrophotometry was used for determination of total phenolics, anthocyanins, and wine colour parameters (intensity, hue, and chromatic structure); the individual phenolics were detected using HPLC; while reducing and free radical scavenging activities of the samples were analysed using the FRAP and DPPH assays. The results indicate a more favourable effect of the Sihazym Extro on the extraction of phenolics, while both enzymes improved the extraction of anthocyanins during the maceration. The most abundant phenolic compounds were malvidin derivatives whose concentration continuously increased during the vinification and reached 82% of all anthocyanin derivatives in the control wine and 81% in both enzyme-treated wines. As expected, the antioxidant activity of the samples followed the trend of phenolics content growth and increased during the vinification, resulting in the higher activity of the enzyme-treated wines.

INTRODUCTION

Despite the devastation of natural potentials and strong deagrarization, the grape vine cultivation and wine production in Dalmatia has subsisted through the centuries. The representations of the individual grape varieties have changed significantly, but most of the indigenous species remained preserved. In the region of Central Dalmatia, especially in the coastal area from Kaštela to Trogir, *Babica* is the most abundant red grape cultivar. For a long time it was considered as synonym for the well-known Croatian red grape variety *Babić,* however, ampelographic and genetic identifications confirmed that *Babica* and *Babić* are two completely different varieties and that *Babica* is the direct descendant of the *Plavac mali* variety [Maletić *et al.*, 2009; Zdunić *et al.*, 2008]. According to the recent data, this grape cultivar occupies an area of approximately 18.5 ha, mostly in Kaštela-Trogir vineyards [Maletić *et al.*, 2015], where, due to its good features and resistance to the diseases, it is a leading grape variety for the production of different types of wine [Ozimec *et al.*, 2015].

Wine colour is an important element of wine quality and one of the most important features that influence its commercial acceptance. The compounds responsible for red wine colour and flavour are anthocyanins, that are accumulated in the grape skin, and their extraction during the maceration is an essential step during red wine production. In the traditional winemaking, during the classic maceration, only about 40% of total anthocyanins are extracted from the skins [El Darra *et al*., 2016], the maximal concentration is reached between 3 and 8 day of maceration and afterwards a slight decrease of anthocyanins could be noted [Ortega-Regules *et al*., 2006; Rio Segade *et al*., 2015; Romero-Cascales *et al*., 2012; Sacchi *et al*., 2005].

Today, the imperative of winemaking industry is the production of wines with a high biological value, expressed colour intensity, and improved stability during the aging so the addition of pectolytic enzymes often plays a fundamental role as their use offers quantitative (higher juice yield), qualitative (improved extraction and better organoleptic properties, colour stability during the wine aging, maturation and storage), and processing benefits (*e.g.* shorter maceration, filtration) [Claus & Mojsov, 2018; Kelebek *et al*., 2007; Mihnea *et al*., 2015; Mojsov, 2013; Romero-Cascales *et al*.,

* Corresponding Author: Tel.: +385 21 329 458;
E-mail: gene@ktf-split.hr (I. Generalić Mekinić)

2012]. The concentration of monomeric anthocyanins in red wines during the maturation declines constantly as a result of different mechanisms such as their adsorption by yeast, degradation and oxidation, precipitation with proteins, polysaccharides and/or condensed tannins, and the irreversible formation of more complex (oligomeric and polimeric) pigments which are crucial for colour stability [Boulton, 2001; He *et al*., 2012]. All these reactions are responsible for colour changes from the deep-purple colour of the young wines (due to presence of monomeric anthocyanins and reactions of self--association co-pigmentation), to the orange and brick-red tones as the wine ages [Marquez *et al*., 2013].

Numerous studies have been carried out into the effect of various pre-fermentative oenological practices on the yield and profile of extracted red wine phenolics, and the reported results are often contradictory. The results of our recent study on *Crljenak kaštelanski* variety [Generalić Mekinić *et al*., 2019] showed that the highest yield of phenolics was detected in wine produced without enzyme addition; no significant increase was reported in the study of El Darra *et al.* [2016], while studies of Pardo *et al*. [1999], Kelebek *et al.* [2007], and Romero-Cascales *et al.* [2012] reported a better extraction yield of phenolics by the use of enzyme preparations. In turn, Pardo *et al.* [1999], Kelebek *et al.* [2007], Mojsov *et al.* [2010], Bichescu *et al.* [2012], and Rio Segade *et al.* [2015] detected a higher content of anthocyanins in enzyme--treated wines, and regarding the wine chromatic and sensory characteristics Bautista-Ortin *et al.* [2005] reported opposite effects of two enzyme preparations, while Pardo *et al.* [1999], Kelebek *et al.* [2007], Soto-Vazquez *et al*. [2010], Bichescu *et al*. [2012], and Romero-Cascales *et al.* [2012] confirmed the positive effect of enzymes on these wine parameters.

The aim of this study was to evaluate the effect of pectolytic enzyme addition during winemaking of *Babica* cv. on the extraction and evolution of wine phenolic compounds, colour components, and antioxidants in order to draw conclusions on choosing the optimal procedure for the production of highly-coloured red wine rich in biologically active compounds.

MATERIALS AND METHODS

Chemicals and reagents

All standards, reagents, and solvents used in this research were of adequate analytical grade and were purchased from Kemika (Zagreb, Croatia), Alkaloid AD (Skopje, Macedonia), BDH Chemicals (London, UK), Fluka (Buchs, Germany), and Sigma-Aldrich (Steinheim, Germany).

Grape samples and vinification

The raw materials were hand-picked grapes from *Vitis vinifera* var. *Babica* (from the vineyard located in Kaštela, Croatia). After the harvest, about 100 kg of grapes for each experiment were transported to winery and processed as follows: a) by the traditional winemaking procedure (control sample), and by two procedures using enzymes (3 g/100 L); b) Vinozym Vintage® FCE (*Novozymes* A/S, Bagsvaerd, Denmark), and c) Sihazym Extro (Eaton Begerow Product Line, Langenlonsheim, Germany), respectively. The vinification procedure and sampling protocol were previously described by Generalić Mekinić *et al*. [2019]. Weighed and destemmed grapes were crushed with an MGM- 940 crusher (MIO, Osijek, Croatia) and pectolytic enzyme preparations were added to fermentation tanks. The prepared mashes were treated with potassium metabisulphite (10 g/100 L) and inoculated (15 g/100 L) (SIHA®, Aktiv Hefe 8, Burgundy Yeast, E. Begerrow GmbH & Co., Langenlonsheim, Germany). The maceration time was 5 days (at ~25 to 27°C), and the cap of grape solid was kept soaked using the mechanical barrier. After maceration, free run wine and the pressed wine were combined, and the pomace was removed. The produced wine was sealed with the tank's floating lid and paraffin oil. The dynamics of the extraction was monitored daily during the maceration and after the racking (approximately 40 and 160 days after the winemaking process started).

Phenolic compounds and wine colour parameters

Spectrophotometric measurements were performed on a Specord 200 spectrophotometer (Analytik Jena GmbH, Jena, Germany).

The total phenolics content in the samples was determined by the colorimetric Folin-Ciocalteu method [Singleton & Rossi, 1965] and the results are expressed as mg of gallic acid equivalents per litre (mg GAE/L).

The monomeric anthocyanins content was determined using the assay described originally by Amerine & Ough [1980]. The obtained results are expressed as mg of malvidin 3-*O*--glucoside equivalents per litre (mg M-3-gl/L).

Wine colour parameters as intensity (CI), hue (T), and the percentages of yellow (OD 420 = A 420 /CI × 100), red (OD 520 = A 520 /CI × 100), and blue (OD 620 = A 620 /CI × 100) were measured and calculated according to Alpeza *et al*. [2017].

HPLC analysis

The individual phenolic compounds were detected using a high-performance liquid chromatography (HPLC) system consisting of an autosampler, a binary pump, a vacuum degasser, a UV/VIS detector, and the Peltier column oven (all of Series 200) (Perkin Elmer, Walthamn, MA, USA). Separation, identification, and quantification of phenolic acids, flavonoids, and stilbene were performed on an UltraAqueous C18 column (250×4.6 mm, 5 μm, Restek, Bellefonte, PA, USA) while the analysis of anthocyanins was performed on the Kinetex core-shell C18 column (150×4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA).

Separation, identification, and quantification of the individual phenolics were performed by HPLC methods as described by Generalić Mekinić *et al*. [2019] using 0.2% phosphoric acid in water (solvent A) and 50% acetonitrile in methanol (solvent B). A gradient was applied as follows: from 96% A at 0 min to 50% A at 40 min, to 40% A at 45 min, to 20% A at 50 min, to 20% A at 53 min, then from 20 to 96% A at 54 min, and maintaining 96% A for 11 min (65 min). The flow rate was 0.8 mL/min and the injection volume was 20 μL. The detection of phenolic acids, flavonoids, and resveratrol was carried out at 280 nm on the column maintained at 25°C. The separated peaks were identified by comparing their

retention times with those acquired for corresponding standards, while quantification was performed using external calibration curves generated for each detected compound. Additionally, sample spiking was also used to assist confirmation of peak identity.

The anthocyanins were detected at 520 nm on the column maintained at 40°C. The elution solvents in this method were 0.3% perchloric acid in water (solvent A) and 0.3% perchloric acid in methanol (solvent B). The linear gradient was as follows: from 28% B to 51% B in 42 min, then to 69% in 3 min and to 80% B in 1 min 80% B for 3 min. The time of equilibration for the column to the initial gradient was 6 min. The flow rate was 0.6 mL/min and the injection volume was 10 μL. The peaks of anthocyanins were identified and quantified as described by Vanzo *et al*. [2008] and Budić-Leto *et al*. [2018]. The compounds were eluted in order of polarity which was used for their identification (according to the retention time of each peak at 520 nm), while quantifications (concentration in mg/L) were performed using the external standard curve generated for malvidin 3-*O*-glucoside.

Antioxidant activity

The reducing activity of the samples was detected as the ferric reducing antioxidant power (FRAP) [Benzie & Strain, 1996] and the results are expressed as micromoles of Trolox equivalents per litre (μmol TE/L).

The free radical-scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) was investigated according to the procedure described by Katalinić *et al*. [2013] and the results are expressed as inhibition percentage (%).

Statistical analysis

All analyses were carried out in triplicate and the data are given as the mean ± standard deviation. Statistical analysis was performed using STATGRAPHICS® Centurion XVI (StatPoint Technologies, Inc., The Plains, Virginia, USA). Differences between the investigated parameters were analysed by one-way ANOVA followed by Fisher's least significant difference test, while Pearson's correlation coefficient ($p<0.05$) was used to determine relations between the variables.

RESULTS AND DISCUSSION

Changes in concentrations of total phenolics and anthocyanins in *Babica* wine sampled during the vinification, with and without enzyme addition, are presented in Figure 1. The phenolics content was continuously increasing during the vinification, with slight decreases between the first and the second racking in the control sample and in the wine produced by the addition of Vinozym Vintage. At the end of winemaking, the highest concentration of phenolics was detected in the sample produced by the addition of Sihazym Extro (1771 mg GAE/L), while the two other wine samples contained the similar amount of phenolic compounds. Figure 1b presents results obtained for monomeric anthocyanins content and they are in agreement with those reported in our previous study [Generalić Mekinić *et al.*, 2019]. The majority of anthocyanins were extracted during the first few days of maceration, supporting the results reported by Ivanova *et al.* [2012] who demonstrated that extended maceration resulted in a slight decrease in the content of phenolic compounds and anthocyanins. The Vinozyme Vintage sample showed the highest value at day four (180 mg M-3-gl/L) and a slight decrease on the fifth day, while the anthocyanin content of the other two samples increased. Again, a decrease of anthocyanins from the first to the second racking can be noted in all samples with the final concentration being 144 mg M-3-gl/L in the control wine, 142 mg M-3-gl/L in the Vinozyme Vintage sample, and 152 mg M-3-gl/L in the Sihazym Extro sample. Statistical analysis confirmed a high correlation between the phenolic and anthocyanin content ($r=0.9348$, $p<0.0001$). If the results obtained for *Babica* are compared to those obtained for *C. kaštelanski*, it can be concluded that *C. kaštelanski* contains almost 2-fold higher concentration of total phenolics, while the content of anthocyanins is higher in *Babica* (especially in the samples prepared with Sihazym Extro). These results could be partially compared to those reported by Maletić *et al*. [2009] where two close relatives of *C. kaštelanski* (*Dobričić* and *Plavac mali*) contained more phenolics than *Babica*. However, these authors reported a high concentration of anthocyanins in *Babica* (200 mg/L). The highest concentration of anthocyanins was found in *Dobričić* while the lowest among all tested cultivars was detected in *Crljenak viški* (50.7 mg/L) and *Plavac mali* (81.5 mg/L). Results of their study confirmed also a negative correlation between contents of shikimic acid, which is important in the biosynthesis of anthocyanins, and anthocyanins.

The phenolic profiles of the investigated wines are shown in Table 1. Three phenolic acids (gallic, protocatechuic, and *p*-hydroxybenzoic acid), two flavonoids from the group of flavan-3-ols (catechin and its epimer), flavonol quercetin, and stilbene resveratrol were detected in all samples. The gallic acid was dominant among phenolic acids with the concentration range from 16.68 to 17.80 mg/L. The protocatechuic acid content was the highest in the control wine, while enzyme addition enhanced the extraction of *p*-hydroxybenzoic acid. Catechin and epicatechin are the most abundant compounds among the flavan-3-ols, which are usually detected in wines. These compounds are important as they interact with the anthocyanins by the process of co-pigmentation and play a crucial role in defining the sensory properties of red wines. The extraction of these flavan-3-ols is enhanced with prolongation of the maceration process and an increase of ethanol amount [Bautista-Ortin *et al*., 2007; Katalinić *et al*., 2004]. From the presented results it can be seen that both enzymes significantly affected extraction of catechin, while Sihazym Extro had a negative effect on epicatechin and quercetin contents. The concentration of resveratrol in the samples ranged from 1.86 mg/L to 2.82 mg/L with the maximum concentration found in the Vinozym Vintage wine sample. The detected concentrations of resveratrol are several times higher than those detected in *C. kaštelanski* wine [Generalić Mekinić *et al*., 2019].

Furthermore, 16 different anthocyanin derivatives (in the form of 3-*O*-glucosides, 3-*O*-acetylglucosides, (6-*O*-caffeoyl)glucosides, and (6-*O*-coumaryoyl)glucosides) were detected in the samples and the results are presented in Table 1, while the chromatograms are shown in Figure 2. Grape

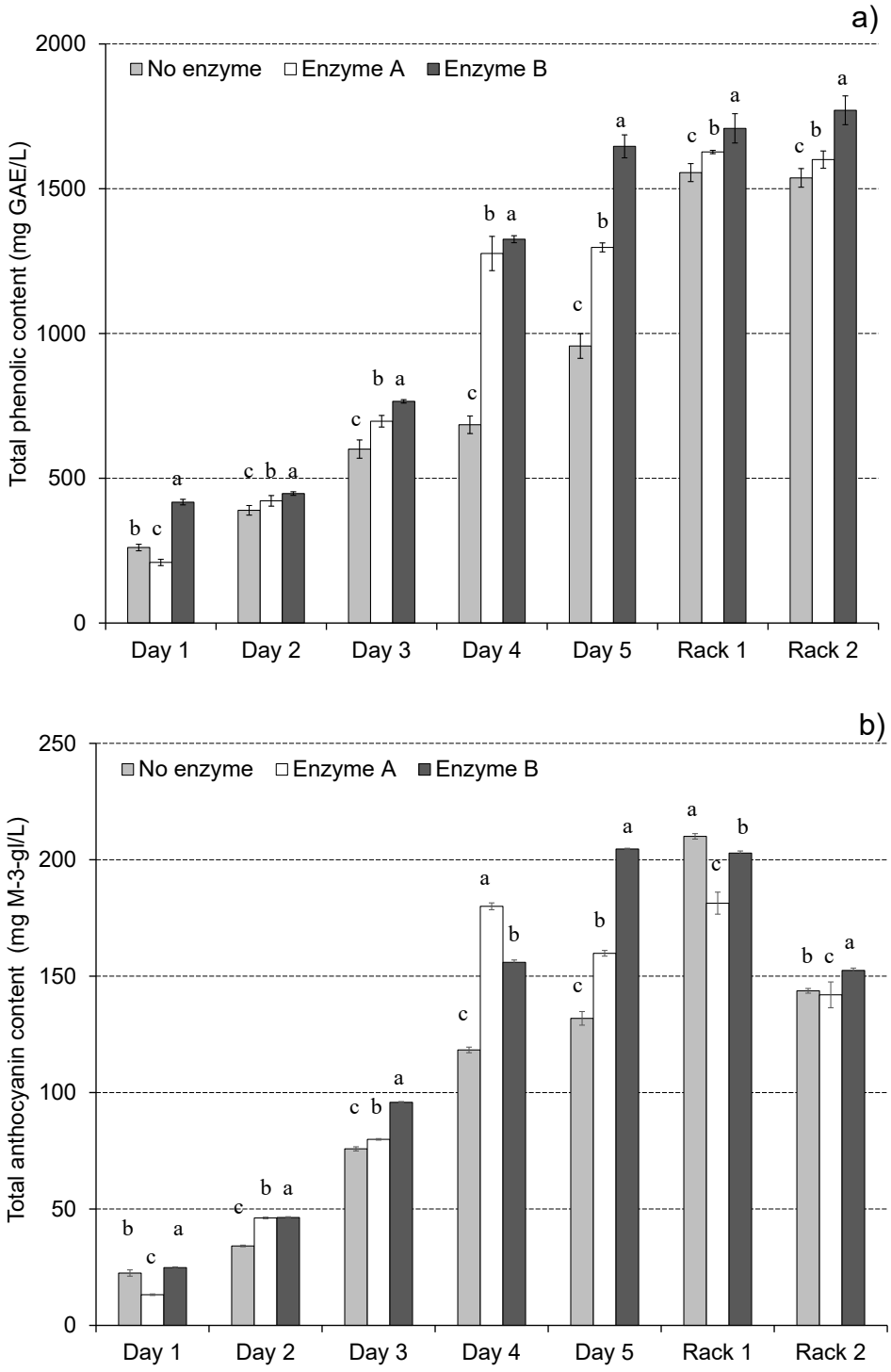


FIGURE 1. Changes of a) total phenolics and b) total anthocyanins in *Babica* wine during the vinification without enzyme addition and with the addition of enzyme A (Vinozym Vintage) or enzyme B (Sihazym Extro).

GAE- gallic acid equivalents, Enzyme A – Vinozym Vintage, Enzyme B – Sihazym Extro, M-3-gl- malvidin 3-*O*-glucoside, Rack 1/Rack 2- 40/160 day of winemaking process. Different letters (a-c) above bars denote statistically significant difference ($p<0.05$) among wine samples.

anthocyanidins are usually in the form of glycosides (primarily glucosides) in which the sugar component of the molecule increases their stability and solubility. The major forms of anthocyanins in *Babica* wine samples were 3-*O*-glucosides, with malvidin being the dominant and the most stable form. These results are consistent with those obtained in *C. kaštelanski* [Generalić Mekinić *et al*., 2019], but also with other results available in the literature [Alpeza *et al*., 2017; He *et al*., 2012; Maletić *et al*., 2009]. The proportion of malvidin derivatives in total anthocyanins reached 82% in the control wine and 81% in the enzyme-treated wines. The content of malvidin-3-*O*-glucoside after the first racking ranged from 91.62 to

TABLE 1. Phenolic compounds content of *Babica* young wine (mg/L) produced by traditional vinification without and with the addition of enzymes Vinozym Vintage (Enzyme A) or enzyme Sihazym Extro (Enzyme B).

Group	Phenolic compound	No enzyme	Enzyme A	Enzyme B
Phenolic acids	Gallic acid	16.68±0.08^{c}	17.80±0.07^{a}	17.39±0.23^{b}
	Protocatechuic acid	2.12±0.03^{a}	1.89±0.05^{b}	1.79±0.06^{c}
	p-Hydroxybenzoic acid	1.48±0.11^{c}	2.30±0.14^{a}	2.12±0.05^{b}
Flavonoids	Catechin	31.79±0.22^{c}	44.34±0.25^{a}	42.27±0.38^{b}
	Epicatechin	62.02±1.01^{b}	65.10±2.23^{a}	38.46±0.53^{c}
	Quercetin	1.39±0.02^{b}	1.65±0.02^{a}	1.24±0.02^{c}
Stilbenes	Resveratrol	1.86±0.01^{c}	2.82±0.04^{a}	2.17±0.02^{b}
Anthocyanins	Delphinidin-3-*O*-glucoside	3.27±0.01^{a}	3.22±0.02^{b}	2.66±0.04^{c}
	Cyanidin-3-*O*-glucoside	0.14±0.01^{a}	0.15±0.01^{a}	0.15±0.02^{a}
	Petunidin-3-*O*-glucoside	6.15±0.13^{a}	6.30±0.36^{a}	6.13±0.62^{a}
	Peonidin-3-*O*-glucoside	4.04±0.37^{b}	4.27±0.17^{ab}	4.47±0.21^{a}
	Malvidin-3-*O*-glucoside	59.90±0.25^{b}	58.86±0.02^{c}	61.91±0.20^{a}
	Delphinidin-3-*O*-acetylglucoside	0.82±0.05^{b}	0.56±0.00^{c}	1.10±0.09^{a}
	Cyanidin-3-*O*-acetylglucoside	0.03±0.00^{b}	0.03±0.01^{b}	0.07±0.00^{a}
	Petunidin-3-*O*-acetylglucoside	0.13±0.00^{b}	0.14±0.01^{a}	0.10±0.00^{c}
	Peonidin-3-*O*-acetylglucoside	0.25±0.00^{b}	0.27±0.01^{a}	0.23±0.00^{c}
	Petunidin-(6-*O*-caffeoyl)glucoside	0.11±0.00^{b}	0.10±0.00^{b}	0.24±0.07^{a}
	Malvidin-3-*O*-acetylglucoside	2.59±0.01^{c}	2.70±0.02^{b}	2.90±0.13^{a}
	Malvidin-(6-*O*-caffeoyl)glucoside	1.16±0.02^{a}	0.91±0.02^{c}	0.98±0.01^{b}
	Cyanidin-(6-*O*-coumaryoyl)glucoside	0.27±0.00^{b}	0.30±0.00^{a}	0.20±0.00^{c}
	Petunidin-(6-*O*-coumaryoyl)glucoside	0.04±0.00^{b}	0.05±0.00^{b}	0.06±0.00^{a}
	Peonidin-3-(6-*O*-coumaroyl)glucoside	0.58±0.00^{c}	0.72±0.02^{a}	0.65±0.01^{b}
	Malvidin-3-(6-*O*-coumaroyl)glucoside	4.30±0.00^{c}	4.82±0.03^{a}	4.37±0.02^{b}

Enzyme A – Vinozym Vintage; Enzyme B – Sihazym Extro. Different letters (a-c) in superscript in the row denote statistically significant difference ($p<0.05$) between concentrations of detected phenolics in wine samples.

95.13 mg/L (data not showed), while at the end of the vinification process those values significantly decreased. A significant correlation was observed between concentrations of malvidin-3-*O*-glucoside and monomeric anthocyanins ($r=0.5982$, $p=0.0042$).

The colour of red wine is influenced by numerous wine-growing and processing factors affecting transfer of pigments during wine-making. Moreover, during the maturation and aging, the red wine colour changes from an intense red hue characteristic for anthocyanins to a more red-orange hue which comes from different anthocyanin-derived compounds (pyranoanthocyanins) [He *et al*., 2012]. Wines with higher co-pigmentation and higher amount of acylated forms of non-malvidin compounds have deeper colour [Boulton, 2001]. Although these colour changes can be observed with the naked eye, they are usually easily determined spectrophotometrically by measuring such parameters as CI and T, as well as OD 420, OD 520, and OD 620.

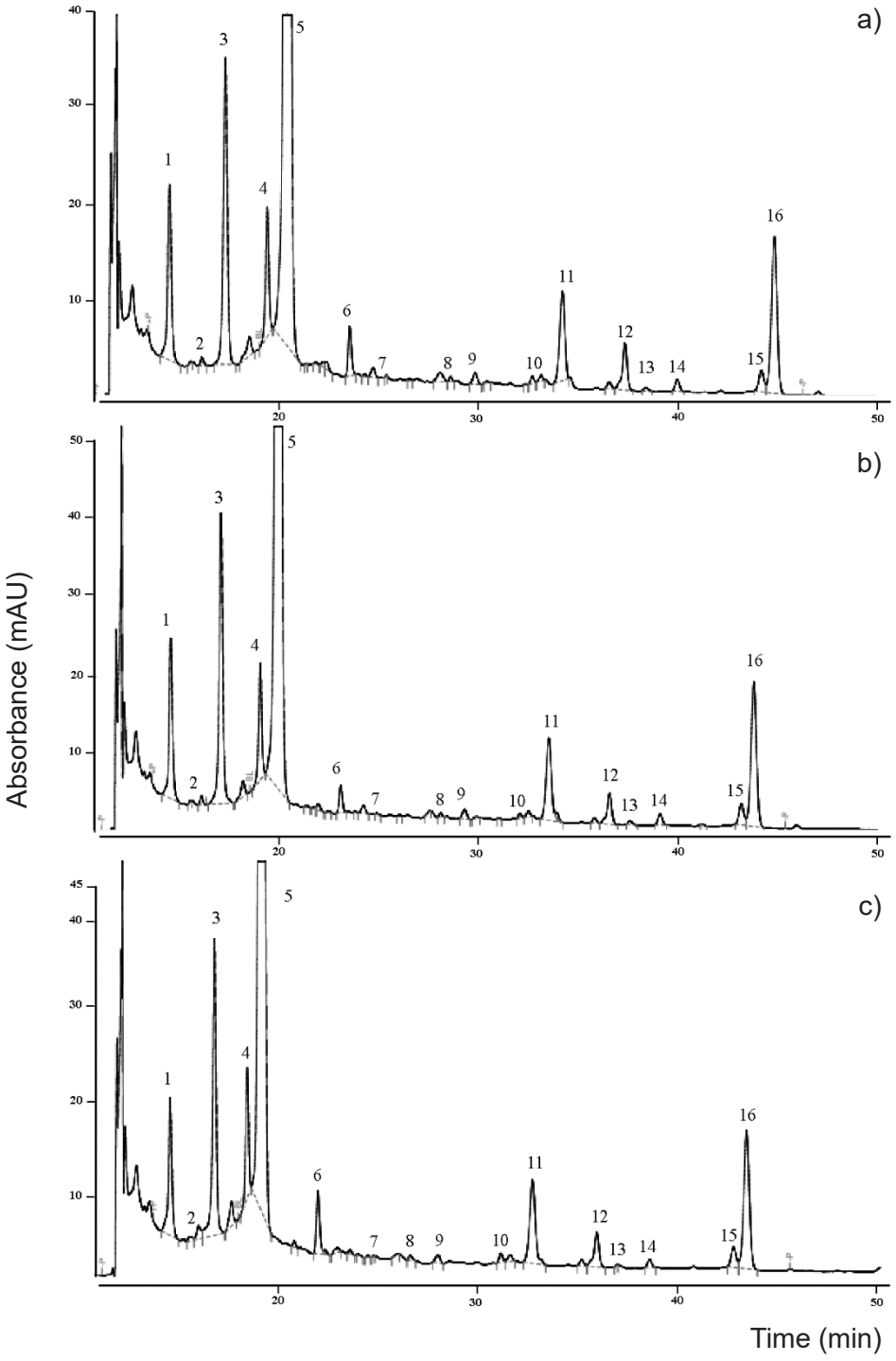


FIGURE 2. HPLC Chromatograms at 520 nm of *Babica* wine samples produced: a) without enzyme addition, b) with Vinozyme Vintage, and c) with Sihazym Extro.

Peaks are identified as follows: 1) Delphinidin-3-*O*-glucoside, 2) Cyanidin-3-*O*-glucoside, 3) Petunidin-3-*O*-glucoside, 4) Peonidin-3-*O*-glucoside, 5) Malvidin-3-*O*-glucoside, 6) Delphinidin-3-*O*-acetylglucoside, 7) Cyanidin-3-*O*-acetylglucoside, 8) Petunidin-3-*O*-acetylglucoside, 9) Peonidin-3-*O*--acetylglucoside, 10) Petunidin-(6-*O*-caffeoyl)glucoside, 11) Malvidin-3-*O*-acetylglucoside, 12) Delphinidin-(6-*O*-caffeoyl)glucoside, 13) Cyanidin-(6--*O*-coumaryoyl)glucoside, 14) Petunidin-(6-*O*-coumaryoyl)glucoside, 15) Peonidin-3-(6-*O*-coumaroyl)glucoside, and 16) Malvidin-3-(6-*O*-coumaroyl) glucoside.

CI, usually defined as the colour amount that indicates wine darkness, is a parameter that is mostly determined by the content and structure of the anthocyanins present in wine [Glories, 1984]. The CI of *Babica* wines was successively increasing and the highest values were detected in all samples after the second racking (Figure 3). Ivanova *et al*. [2012] investigated the effect of the maceration on the extraction of wine colour components and found a correlation between the concentration of total anthocyanins and the CI. In this study, a correlation was also detected between the value of this pa-

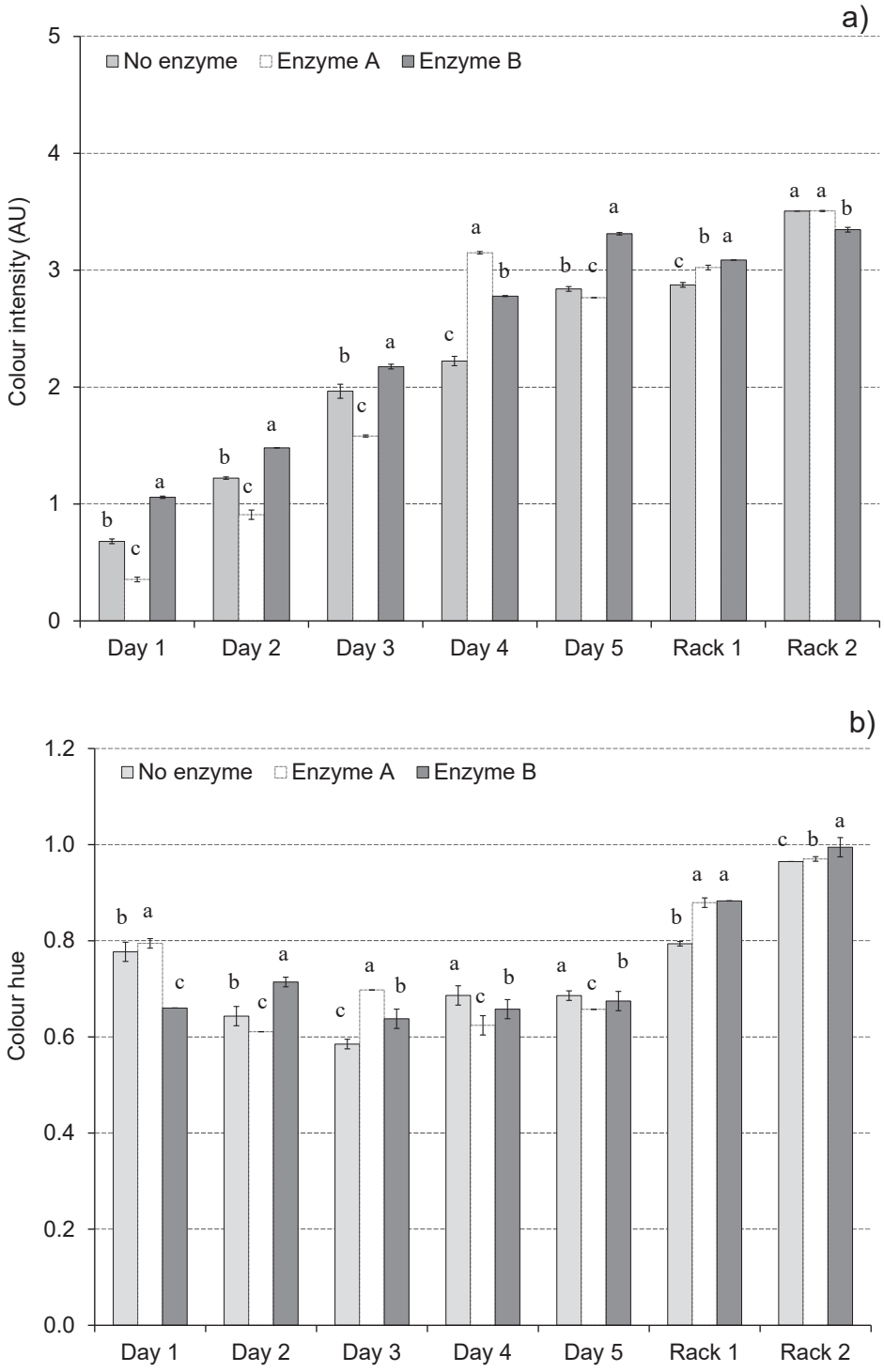


FIGURE 3. Colour parameters: a) colour intensity (CI) and b) colour hue (T) of *Babica* wine produced by traditional vinification and by the addition of enzymes.

Rack 1/Rack 2- 40/160 day of winemaking process. Different letters (a-c) above bars denote statistically significant difference ($p<0.05$) among wine samples.

rameter and total phenolics content ($r=0.9416$, $p<0.0001$) as well as between CI and monomeric anthocyanins content ($r=0.9150$, $p<0.0001$). T is a parameter that indicates the development of a colour towards orange tones and it increases during wine aging. Its highest values were recorded after the second racking (range from 0.96 to 0.99). Ribéreau-Gayon *et al*. [2006] reported that the normal T values for young red wines are between 0.5 and 0.7 but they increase during wine ageing up to 1.3. Figure 4 shows the shares of the three basic colour components (yellow, red and blue) in the overall *Babica* wine colour during the vinification without and with enzyme addition. The highest absorption was detected at the wavelength of 520 nm where red wines have the maximal absorption. According to Glories [1984], the most optimal ra-

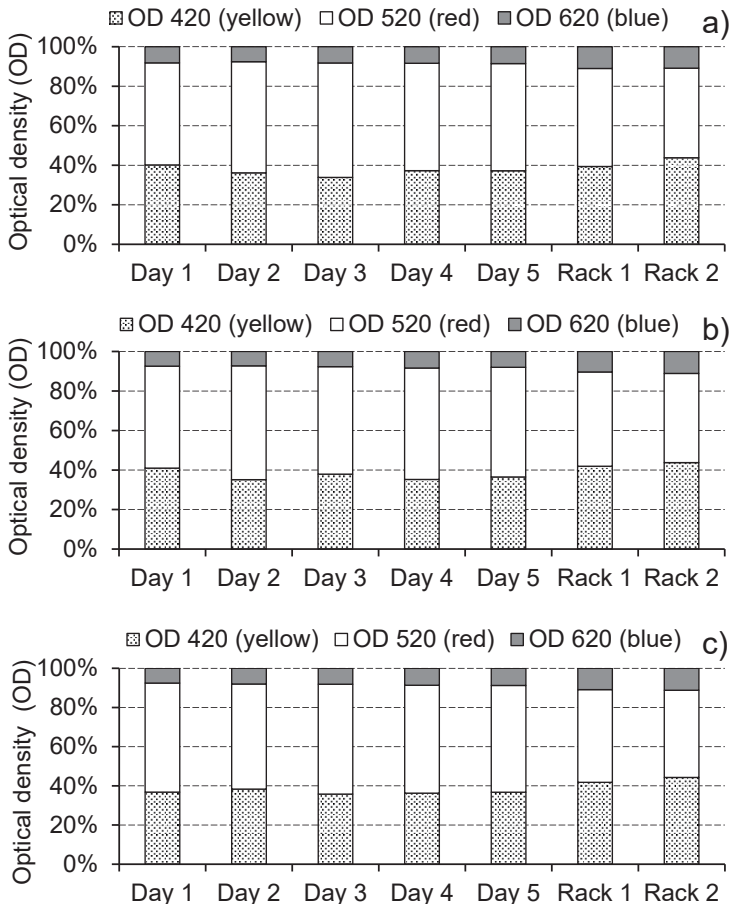


FIGURE 4. Colour composition of the three basic colour components in *Babica* wine during the vinification without enzyme addition (a) and with Vinozyme Vintage (b) and Sihazym Extro. Rack 1/Rack 2- 40/160 day of winemaking process.

tio of these three basic colour components in the overall wine colour is 35% of yellow, 55% of red and 10% of blue. Results presented in Figure 2. are in accordance with those optimal values as OD 520 ranged from 51.61% to 57.89% for all three samples and the maximal value was measured at the third day of maceration in the control sample. After that, these values decreased and after the second racking they were around 45%, the OD 420 was around 44% while OD 620 was around 11%.

Comparison of the antioxidant properties of *Babica* wine samples collected during the vinification with and without enzymes is shown in Figure 5. Two different methods were applied to get an insight into the antioxidant properties of the investigated wines during vinification, *i.e.* free radical scavenging ability using DPPH radical and reducing power assay by FRAP method. DPPH radical has been widely used for the determination of the antioxidant properties and the method is based on hydrogen atom transfer mechanism; while FRAP assay represents a method based on electron transfer reactions [Skroza *et al*., 2015]. A continuous growth of antioxidant properties during the vinification can be noted for the control wine and the wine produced by the addition of Sihazyme Extro, while a slight decrease of these parameters from the racking 1 to racking 2 has been detected for the Vinozym Vintage wine. However, the final results point out a good antioxidant activity of all samples, with the Sihazyme Extro wine being the most potent (FRAP value of 6763 μmol TE/L, DPPH inhibition of 24.4%). The total phenolics content showed a significant correlation with FRAP values ($r=0.9957$, $p<0.0001$) as well as with DPPH inhibition ($r=0.9642$, $p<0.0001$). Also, correlations were found between anthocyanins/malvidin 3-*O*-glucoside and FRAP ($r=0.9124$, $p<0.0001$ / $r=0.7585$, $p=0.0001$) and between anthocyanins/malvidin 3-*O*-glucoside and DPPH ($r=0.9154$, $p<0.0001$ / $r=0.7120$, $p=0.0003$).

CONCLUSION

The use of pectolytic enzyme preparations in winemaking is a well-established practice for improving wine quantity and quality. The results of this study contribute to a better understanding of the mechanism of enzyme actions and their effect on the phenolic profile, chromatic characteristics, and antioxidant properties of wine. Although there were differences in the extraction kinetic of phenolics between the control and the enzyme-treated wines, the use of enzymes, especially Sihazym Extro, improved the extraction of phenolics and anthocyanins. Wine colour properties, especially CI, correlated with the phenolics content and profile. Furthermore, all samples showed an increasing antioxidant activity during the vinification, with Sihazym Extro sample being the most potent. The results contribute to the general knowledge on the applied oenological practice for the production of highly coloured wines with the added health-promoting properties.

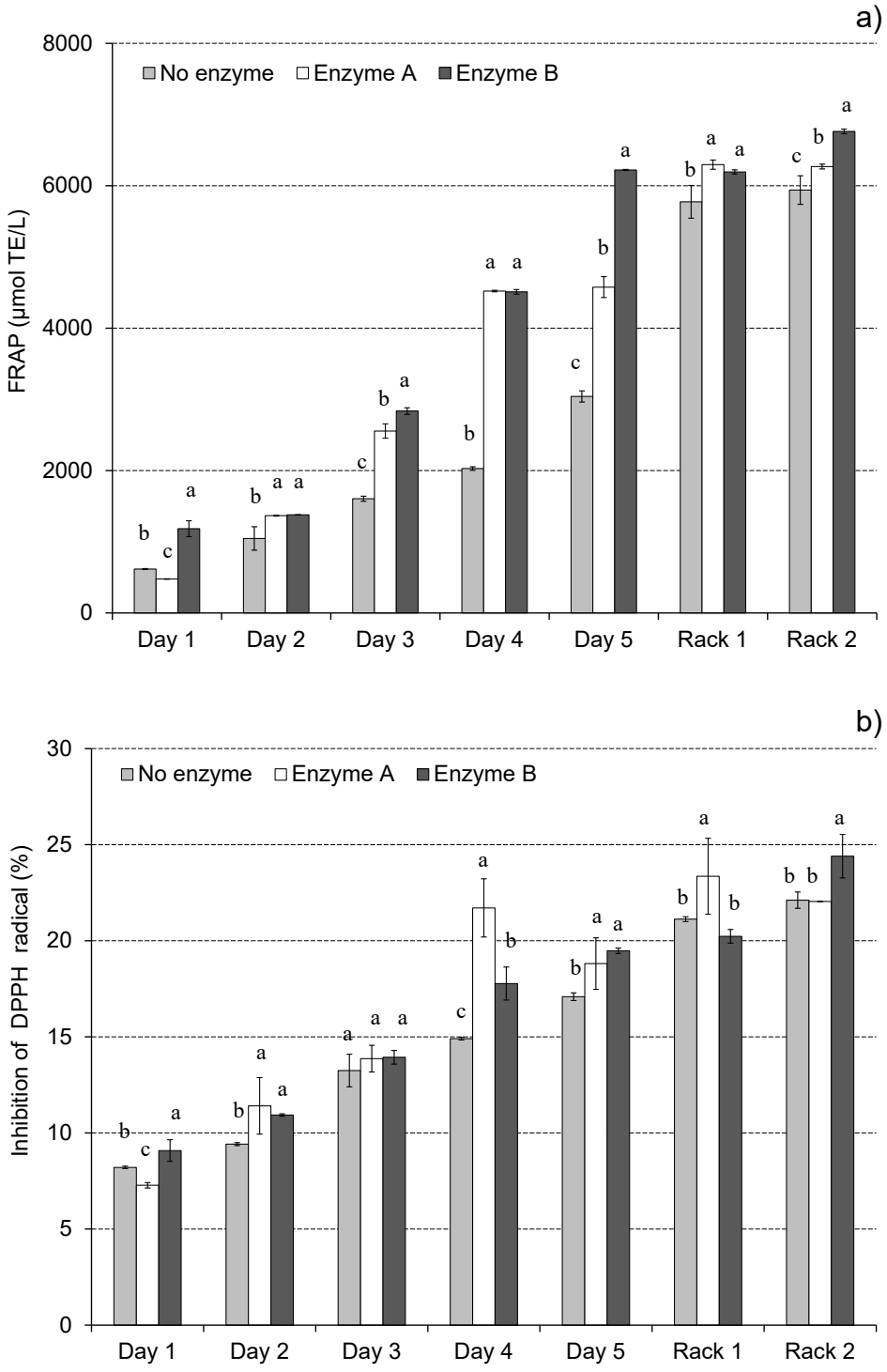


FIGURE 5. Comparison of the antioxidant properties of *Babica* wine samples with and without enzymes during vinification obtained by FRAP (a) and DPPH (b) method.

Enzyme A – Vinozym Vintage, Enzyme B – Sihazym Extro, TE- Trolox equivalents. Rack 1/Rack 2- 40/160 day of winemaking process. Different letters (a-c) above bars denote statistically significant difference ($p<0.05$) among wine samples.

RESEARCH FUNDING

This work has been partially supported by Croatian Science Foundation under the project IP-2013–11–8652.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

REFERENCES

1. Alpeza, I., Kovačević Ganić, K., Vanzo, A., Herjavec, S. (2017). Improved chromatic and sensory characteristics of Plavac mali wines – efficiency of maceration enzymes. *Czech Journal of Food Sciences, 35*(3), 236–245.
2. Amerine, M.A., Ough, C.S. (1980). *Methods for Analysis of Musts and Wines*. 2nd ed. New York: John Wiley & Sons, pp. 181–200.
3. Bautista-Ortín, A.B., Fernández-Fernández, J.I., López-Roca, J.M., Gómez-Plaza, E. (2007). The effects of enological practices in anthocyanins, phenolic compounds and wine colour and their dependence on grape characteristics. *Journal of Food Composition and Analysis, 20*(7), 546–552.
4. Bautista-Ortín, A.B., Martínez-Cutillas, A., Ros-García, J., López-Roca, J.M., Gómez-Plaza, E. (2005). Improving colour extraction and stability in red wines: the use of maceration enzymes and enological tannins. *International Journal of Food Science and Technology, 40*(8), 867–878.
5. Benzie, I.F.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as measurement of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76.
6. Bichescu, C., Bahrim, G., Stănciuc, N., Râpeanu, G. (2012). Color enhancement of Feteasc neagră wines by using pectolytic enzymes during maceration. *The Annals of the University Dunarea de Jos of Galati, Fascicle VI – Food Technology*, *36*(1), 18–25.
7. Boulton, R. (2001). The copigmentation of anthocyanins and its role in the colour of red wine: a critical review. *American Journal of Enology and Viticulture, 52*(2), 67–87.
8. Budić-Leto, I., Mucalo, A., Ljubenkov, I., Zdunić, G. (2018). Anthocyanin profile of wild grape *Vitis vinifera* in the eastern Adriatic region. *Scientia Horticulturae, 238*, 32–37.
9. Claus, H., Mojsov, K. (2018). Enzymes for wine fermentation: current and perspective applications. *Fermentation – Basel, 4*(3), art. no. 52.
10. El Darra, N.E., Turk, M.F., Ducasse, M.A., Grimi, N., Maroun, R.G., Louka, N., Vorobiev, E. (2016) Changes in polyphenol profiles and colour composition of freshly fermented model wine due to pulsed electric field, enzymes and thermovinification pretreatments. *Food Chemistry, 194*, 944–950.
11. Generalić Mekinić, I., Skračić, Ž., Kokeza, A., Soldo, B., Ljubenkov, I., Banović, M., Skroza, D. (2019). Effect of winemaking on phenolic profile, colour components and antioxidants in Crljenak kaštelanski (sin. Zinfandel, Primitivo, Tribidrag) wine. *Journal of Food Science and Technology, 56*(4), 1841–1853.
12. Glories, Y. (1984). La couleur des vins rouges. 2e partie: mesure, origine et interpretation. *Journal International des Sciences de la Vigne et du Vin, 18*, 253–271 (in French).
13. He, F., Liang, N.N., Mu, L., Pan, Q.H., Wang, J., Reeves, M.J., Duan, C.Q. (2012). Anthocyanins and their variation in red wines II. Anthocyanin derived pigments and their colour evolution. *Molecules, 17*(2), 1483–1519.
14. Ivanova, V., Vojnoski, B., Stefova, M. (2012). Effect of winemaking treatment and wine aging on phenolic content in Vranec wines. *Journal of Food Science and Technology, 49*(2), 161–172.
15. Katalinić, V., Milos, M., Modun, D., Musić, I., Boban, M. (2004). Antioxidant effectiveness of selected wines in comparison with (+)-catechin. *Food Chemistry, 86*(4), 593–600.
16. Katalinić, V., Smole Možina, S., Generalić, I., Skroza, D., Ljubenkov, I., Klančnik, A. (2013). Phenolic profile, antioxidant capacity and antimicrobial activity of crude leaf extracts of six *Vitis vinifera* L. varieties. *International Journal of Food Properties, 16*(1), 45–60.
17. Kelebek, H., Canbas, A., Cabaroglu, T., Selli, S. (2007). Improvement of anthocyanin content in the cv. Öküzgözü wines by using pectolytic enzymes. *Food Chemistry, 105*(1), 334–339.
18. Maletić, E., Kontić, J.K., Preiner, D., Jeromel, A., Patz, C.D., Dietrich, H. (2009). Anthocyanin profile and antioxidative capacity of some autochthonous Croatian red wines. *Journal of Food, Agriculture and Environment, 7*(1), 48–51.
19. Maletić, E., Pejić, I., Karoglan Kontić, J., Zdunić, G., Preiner, D., Šimon, S., Andabaka, Ž., Žulj Mihaljević, M., Bubola, M., Marković, Z., Stupić, D., Mucalo, A. (2015). Ampelographic and genetic characterization of Croatian grapevine varieties. *Vitis, 54*, SI, 93–98.
20. Marquez, A., Dueñas, M., Serratosa, M.P., Merida, J. (2013). Identification by HPLC-MS of anthocyanin derivatives in raisins. *Journal of Chemistry*, *2013,* art. no. 274893.
21. Mihnea, M., González-San José, M.L., Ortega-Heras, M., Pérez-Magariño, S. (2015). A comparative study of the volatile content of Mencía wines obtained using different prefermentative maceration techniques. *LWT – Food Science and Technology, 64*(1), 32–41.
22. Mojsov, K. (2013). Use of enzymes in wine making: A review. *International Journal of Marketing and Technology, 3*(9), 112–127.
23. Ortega-Regules, A., Romero-Cascales, I., Ros-García, J.M., López-Roca, J.M., Gómez-Plaza, E. (2006). A first approach towards the relationship between grape skin cell-wall composition and anthocyanin extractability. *Analytica Chimica Acta, 563*(1–2), 26–32.
24. Ozimec, R., Karoglan Kontić, J., Maletić, M., Matotan, Z., Strikić, F. (2015). Tradicijske sorte i pasmine Dalmacije. Program Ujedinjenih naroda za razvoj, Tiskara Zelina d.d., 142–154 (in Croatian).
25. Pardo, F., Salinas, M.R., Alonso, G.L., Navarr, G., Huerta, M.D. (1999). Effect of diverse enzyme preparations on the extraction and evolution of phenolic compounds in red wines. *Food Chemistry*, *67*(2), 135–142.
26. Ribéreau-Gayon, P., Glories, Y., Maujean, A., Dubourdieu, D., Donèche, B. (2006). *Handbook of Enology*, Volume 2, The Chemistry of Wine, Stabilization and Treatments. 2nd ed. Wiley, Chichester, pp. 178–181.
27. Río Segade, S., Pace, C., Torchio, F., Giacosa, S., Gerbi, V., Rolle, L. (2015). Impact of maceration enzymes on skin softening and relationship with anthocyanin extraction in wine grapes with different anthocyanin profiles. *Food Research International, 71*, 50–57.
28. Romero-Cascales, I., Ros-García, J.M., López-Roca, J.M., Gómez-Plaza, E. (2012). The effect of a commercial pectolytic

enzyme on grape skin cell wall degradation and colour evolution during the maceration process. *Food Chemistry, 130*(3), 626–631.
29. Sacchi K.L., Bisson, L.F., Adams, D.O. (2005). A review of the effect of winemaking techniques on phenolic extraction in red wines. *American Journal of Enology and Viticulture, 56*(3), 197–206.
30. Singleton, V.L., Rossi, J. (1965). Colourimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.
31. Skroza, D., Generalić Mekinić, I., Svilović, S., Šimat, V., Katalinić, V. (2015). Investigation of the potential synergistic effect of resveratrol with other phenolic compounds: A case of binary phenolic mixtures. *Journal of Food Composition and Analysis, 38*, 13–18.
32. Soto Vázquez, E., Río Segade, S., Orriols Fernández, I. (2010). Effect of the winemaking technique on phenolic composition and chromatic characteristics in young red wines. *European Food Research Technology, 231*(5), 789–802.
33. Vanzo, A., Terdoslavich, M., Brandoni, A., Torres, A.M., Vrhovsek, U., Passamonti, S., (2008). Uptake of grape anthocyanins into the rat kidney and the involvement of bilitranslocase. *Molecular Nutrition & Food Research, 52*(10), 1106–1116.
34. Zdunić, G., Pejić, I., Karoglan Kontić, J., Vukičević, D., Vokurka, A., Pezo, I., Maletić, E. (2008). Comparison of genetic and morphological data for inferring similarity among native Dalmatian (Croatia) grapevine cultivars (*Vitis vinifera* L.). *Journal of Food, Agriculture and Environment, 6*(2), 333–336.

Submitted: 12 July 2019. Revised: 9 September, 15 November, and 2 December 2019. Accepted: 18 December 2019. Published on-line: 30 January 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 125–137
DOI: 10.31883/pjfns/113533
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Buckwheat as an Interesting Raw Material for Agricultural Distillate Production

Szymon Ługowoj, Maria Balcerek, Katarzyna Pielech-Przybylska*

Lodz University of Technology, Faculty of Biotechnology and Food Sciences, Institute of Fermentation Technology and Microbiology, Wolczanska 171/173, 90–924 Lodz, Poland

Key words: buckwheat, reducing sugars, starch content, ethanol fermentation, agricultural distillate, volatile compounds

The objective of this study was to assess the suitability of buckwheat grain for agricultural distillate production. The effects of the method of starch liberation on the physicochemical composition of the prepared mashes, fermentation results, the concentration of volatile compounds in the obtained distillates, and their organoleptic features, were investigated. The raw materials used were two cultivars of buckwheat grains, Panda and Kora. Both cultivars were characterized by similar starch content, however Panda cultivar had a significantly higher content of reducing sugars. Fermentation of the Kora cultivar-based mashes resulted in a higher fermentation efficiency (up to approximately 85% of the theoretical yield) compared to the Panda cultivar-based mashes (up to approx. 75%). Of the tested methods of starch liberation, the pressure-thermal treatment was revealed as superior, especially in the case of the Panda cultivar. In the case of the Kora cultivar, both the pressure-thermal method and the pressureless method of starch liberation resulted in a high process efficiency (up to 85% of the theoretical). Supplementation of the buckwheat mashes with $(NH_4)_2HPO_4$ improved fermentation results. The highest scores in sensory assessment were given to distillates from mashes prepared with the pressure-thermal treatment, which contained relatively low concentrations of undesirable compounds, such as acetaldehyde and methanol, and revealed pleasant organoleptic features.

INTRODUCTION

The alcohol industry is a rapidly developing sector of the economy in many countries. In order to ensure the highest quality of spirit, distilleries strive to reduce undesirable substances that may be found in agricultural distillates (raw spirits). The distilling industry is also investigating unconventional raw materials, with high ethanol efficiency yet interesting flavor and aroma properties for ethanol production. Today, distilleries use conventional cereals such as rye, wheat, and corn – namely raw materials with a high starch content, which translates into a high ethanol efficiency, *i.e.* above 40 L per 100 kg. Potatoes are another widely used raw material, despite a much lower starch content in comparison to grains, at only 14.5% to 24.3%, which allows producing only 10 L of ethanol per 100 kg [Banerjee & Kundu, 2013].

Given its rapidly growing popularity all around the world, low soil requirements, and comparable starch content to widely used cereal grains, buckwheat could potentially be a new and valuable raw material for agricultural distillate production. In some countries, buckwheat is already used for the production of alcoholic beverages. For example, in France and the United States distillers use it in whisky production, while in Japan buckwheat grain is processed to make an alcoholic beverage called *Soba Shōchū* [Haros & Sanz-Penella, 2017].

Buckwheat does not belong to the grass family (*Poaceae*), as cereals do, and differs also in its grain structure. It is therefore referred to as a pseudo-cereal. There are fifteen species of buckwheat, nine of which are used in agriculture, but only two are grown for food purposes – *Fagopyrum esculentum* and *Fagopyrum tataricum* [Bonafaccia *et al*., 2003; Dziadek *et al.*, 2016]. Buckwheat has a similar chemical composition to plants usually considered as cereals. The overall composition of buckwheat grains depends on such conditions as the cultivars used, the kind of soil that the buckwheat grows on, and the kind of fertilizer used. The average starch content in buckwheat grain amounts to about 50% d. m. The content of proteins is 12%, lipids 4%, soluble saccharides 2%, dietary fiber 7%, ash 2%, and other substances 18% [Im *et al*., 2003].

Buckwheat is often used in the manufacture of products intended for consumers suffering from celiac disease. It is used as a buckwheat malt in the production of gluten-free beer [Deželak *et al*., 2014]. In the case of distilled alcohols, the absence of gluten proteins in buckwheat grains is of little interest, because all spirits are gluten-free, regardless of whether gluten proteins are present in the raw material or not. The processes employed in the production of cereal-based distillates involve cereal processing, including extraction of starch from the cereals and its conversion to fermentable sugars, fermentation, and distillation of alcohol and other volatiles from the fermented mashes. The nature of the manufacturing process makes it unlikely that significant levels of allergenic proteins, peptides or fragments will be carried over into the distillate during a properly con-

* Corresponding Author: E-mail: maria.balcerek@p.lodz.pl (Prof. M. Balcerek)

trolled distillation process [EFSA, 2004]. Interest in using buckwheat grains for the production of spirit drinks is due rather to the favorable starch content and aroma profile of its compounds, which open the possibility for efficient production of spirit with interesting, original sensory qualities, which could be used in the preparation of new spirit drinks (*e.g.* grain brandy, vodka, and others).

Sensory analysis is an integral part of the development process of products that fulfill consumer expectations. Aroma is of particular importance in satisfying consumer preferences. Sensory assessment is often complemented by determination of the concentration of volatile compounds in food and beverages, as this enables a better understanding of the effect of food components on the overall aroma intensity and/or perception of the final product [Starowicz *et al*., 2018]. Janes *et al.* [2009] identified the following aroma--active compounds responsible for the common organoleptic features of buckwheat: (E, E)- 2,4-decadienal, (E)-2-nonenal, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, 2-methoxy-4-vinylphenol, and 2-phenylacetaldehyde. According to Aoki & Koizumi [1986], nonanal, octanal, and hexanal are also important aroma compounds in buckwheat. Various studies have investigated the key aroma compounds in buckwheat--based alcohol beverages. In the samples of the Japanese distilled beverage shochu, 22 potent odorants were identified, with ethyl cinnamate being the most intense aroma-active component [Sakaida *et al.,* 2003]. A representative aroma compound in buckwheat-based alcoholic beverages, such as mead and beer, was found to be 2-phenylethanol [Wintersteen *et al.,* 2005; Deželak *et al.,* 2014]. A wide range of esters have also been identified, among which isoamyl acetate showed high aroma thresholds in buckwheat mead, shochu, and beer [Sakaida *et al*., 2003; Wintersteen *et al.,* 2005; Deželak *et al.,* 2014].

A high number of reports in the literature on the use of buckwheat for the production of various food products, including alcoholic beverages [Starowicz *et al*., 2018], and the direct interest of Polish producers of spirit drinks in the production of new, original beverages, prompted us to undertake research on the use of buckwheat grain for the production of agricultural distillate, which, after appropriate treatment (*e.g.* re-distillation with the separation of the head and tail fractions, or ageing in the presence of wood) could be used for the production of spirit drinks. The aim of the present study was therefore to investigate the suitability of buckwheat grain for agricultural distillate production, including the influence of the method of starch liberation on the physicochemical composition of the prepared mashes, fermentation results, the chemical composition of the obtained distillates, and their organoleptic features.

MATERIALS AND METHODS

Raw materials

Studies were conducted with using two cultivars of buckwheat (*Fagopyrum esculentum* Moench), Panda and Kora, classified as traditional cultivars of crop species grown in Poland [COBORU, 2019]. The raw material was purchased from the Plant Breeding Station (Palikije, Poland).

Enzymatic preparations

Liquefaction and saccharification of starch were carried out using the enzymatic preparations Termamyl SC (α-amylase from *Bacillus stearothermophilus*, EC 3.2.1.1) and SAN Extra (glucan 1,4-α-glucosidase from *Aspergillus niger*, EC 3.2.1.3), purchased from Novozymes A/S (Bagsværd, Denmark). A supportive enzymatic preparation Viscoferm® (a multienzyme complex containing cellulase, EC 3.2.1.4; xylanase (endo-1,4-), EC 3.2.1.8; β-glucanase (endo-1,3(4)-), EC 3.2.1.6) (Novozymes A/S, Denmark) was used during the mashing process.

Sweet mash production

Prior to the alcoholic fermentation, sweet mashes were prepared from grains from the two buckwheat cultivars, using two methods of starch liberation commonly used in distilleries [Balcerek *et al.,* 2016]: pressureless liberation of starch (PLS), and pressure-thermal treatment (steaming).

In the case of PLS, 5 kg of milled buckwheat grain was mixed with an adequate volume of water (2.5 L/kg of raw material) and the mixture was transferred into a stainless--steel tank with a water heating mantle. The mixture was heated to a temperature of 90°C with continuous stirring and Termamyl SC and Viscoferm preparations were added. The conditions for starch liquefaction were maintained for 1 h. The mash was then cooled to approximately 65°C and digested with the saccharifying SAN Extra preparation. The sweet mash was finally cooled to fermentation temperature (approximately 30°C).

For pressure-thermal treatment, 5 kg of whole buckwheat grain was placed in a tapered cylindrical steamer, filled previously with 17.5 L of water. Steaming was performed at 150°C and 0.4 MPa for 30 min, with periodical circulation of the content. After steaming, the content of the steamer was transferred into a steel-mashing vessel equipped with a heating/cooling coil and a thermometer. The steamed mass was cooled to a temperature of 90°C and mashed according to the same process as in the PLS method, but without Viscoferm preparation addition. Experiments were carried out on a semi-technical scale in the mini distillery of the Department of Spirit and Yeast Technology (Institute of Fermentation Technology and Microbiology, Lodz University of Technology, Poland). Figure 1 presents a scheme of the processes of buckwheat mashes preparation.

Fermentation of buckwheat mashes

Fermentation of buckwheat mashes was carried out in 10 L flat-bottomed flasks, each containing 6 L of mash with the pH adjusted to 4.8 (using 25% w/v H_2SO_4 solution), supplemented or not with mineral nutrients for yeast (an aqueous solution of $(NH_4)_2HPO_4$ at a dose of 0.3 g/L mash). Ethanol Red® dry distillery yeast (*S. cerevisiae*), dedicated for fermentation of mashes prepared from starchy raw materials (Fermentis, Division S.I. Lesaffre, France), was used. Prior to fermentation, the yeast slurry was prepared by suspending an appropriate amount of dry yeast in tap water (0.5 g/L sweet mash; yeast count 2.8×10^6 cfu/mL sweet mash) and disinfected by acidification using a sulfuric (VI) acid solution (the final pH of the yeast slurry was set at 2.5). The yeast slurry was kept at room temperature for 15 min to eliminate

weaker yeast cells and undesirable bacterial cells. It was then added to the sweet mash samples. The inoculated mashes were carefully mixed prior to fermentation. The flasks were closed with stoppers equipped with fermentation pipes containing glycerol, and fermentation was performed at 35±2°C for 72 h. The fermentation process was controlled gravimetrically, by determining the decrease in the mass of the mashes related to the emission of CO_2 (*i.e.*, periodic measurement of the weight of the flat-bottomed flasks containing the fermenting mash).

Distillation

Once fermentation was complete, ethanol from the mashes was distilled using a unit consisting of a distillation flask, a Liebig cooler, a flask for collecting ethanol, and a thermometer. Distillates containing 20–25% vol. ethanol were strengthened to ethanol contents of approximately 42–43% vol. in a glass distillation apparatus with a special dephlegmator/condenser, according to the method provided in a work by Young [1922].

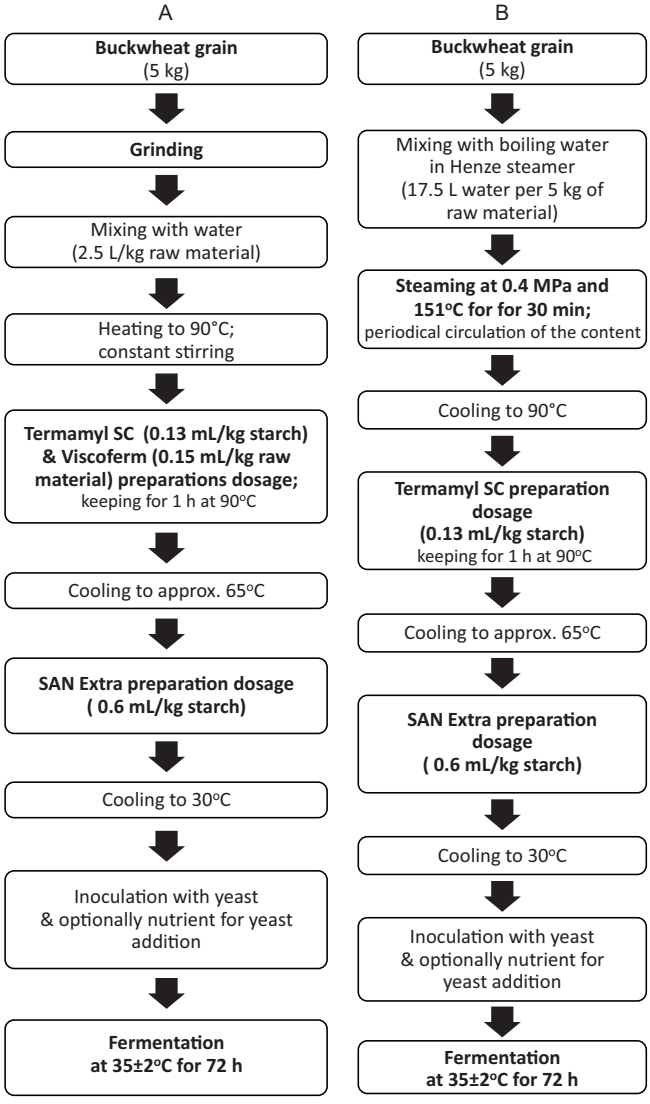


FIGURE 1. Scheme of buckwheat mash preparation: A – pressureless liberation of starch (PLS) method; B – pressure-thermal starch liberation method.

Analytical methods

Buckwheat grains

The raw material was analyzed for contents of: starch using the Ewers method [PN-EN ISO 10520, 2002], reducing and total sugars (after acid hydrolysis) using DNS reagent [Miller, 1959], moisture with the use of a WPS-305 Radwag weighing dryer (105°C), and total nitrogen according to the Kjeldahl method, calculated as protein (N×5.7) and expressed as the percentage of dry weight [AOAC, 1995].

Sweet and fermented mashes

Both the sweet and fermented mashes were analyzed for: pH; extract – the concentration of dissolved solids, mostly sugar (in the sweet mashes, total extract; in the fermented mashes, apparent extract in the presence of alcohol, and real extract after distillation of alcohol), using an aerometer with a scale in g/L [Balcerek *et al*., 2016]; the concentration of reducing sugars and total sugars [Miller, 1959]; dextrin concentration calculated as the difference between total sugars and reducing sugars, using a conversion coefficient of 0.9; and ethanol content using an areometer with a scale in percentage of ethanol by volume, after distillation from fermented mashes in a Super Dee digital distilling unit (Gibertini, Novate Milanese, Italy).

Distillates

Chromatographic analysis of the volatile compounds in the obtained distillates was carried out using gas chromatography (GC apparatus) (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) with a mass spectrometer (Agilent MSD 5975C, USA). An Agilent VF-WAX MS polar capillary column (60 m × 0.50 μm×0.32 mm) was used to separate the compounds. The GC oven temperature was programmed to increase from 40 (6 min) to 80°C at a rate of 2°C/min, and then to 220°C at a rate of 10°C/min (hold time 5 min). The flow rate of the carrier gas (helium) through the column was 1.2 mL/min. The temperature of the injector (split/splitless) was 250°C. Direct injections of the tested distillates (1 μL) were made in the split mode (1:40). The conditions for MS were as follows: ion source temperature 230°C; transfer line temperature 250°C; quadrupole temperature 150°C; ionization energy 70 eV. Identification of the volatile components was based on a comparison of their mass spectra with the mass spectra in the NIST/EPA/NIH Mass Spectra Library (2012; Version 2.0g.). Retention indices (RIs) were also compared with reference compounds and literature data [Chida *et al*., 2004]. The RIs were calculated relative to a homologous series of *n*-alkanes from pentane to octadecane. Quantification of the volatile compounds was performed using calibration curves in the selected ion monitoring mode (SIM). Six calibration solutions, containing different concentrations of each standard compound, were prepared with 4-heptanone, which was added to a concentration of 45 mg/L of absolute alcohol of the analyzed samples as an internal standard, to monitor instrument response and retention time stability. Quantitative analysis was conducted using Agilent MassHunter software (Agilent Technologies, Santa Clara, CA, USA). The concentrations of the determined volatile compounds were expressed in mg/L alcohol 100% vol..

Sensory analysis

Samples of the buckwheat distillates were subjected to sensory evaluation by a panel of six qualified assessors, who possess knowledge of spirits and their quality requirements. Sensory assessment was performed using the Buxbaum model of positive ranking [Tešević *et al.,* 2005]. This model is based on four sensory experiences, rated with a maximum of 20 points overall. Each judge gives a score for color, 0–2; clearness, 0–2; aroma (odor), 0–4; and taste, 0–12.

Calculations

Fermentation efficiency and total sugar intake were calculated to evaluate the fermentation process. The intake of sugars was calculated as the ratio of sugars utilized during fermentation to their initial content in the mash and expressed as a percentage. Fermentation efficiency was calculated according to the stoichiometric Gay-Lussac equation in relation to total sugars and expressed as a percentage of the theoretical yield [Nicol & Rum, 2003].

Statistical analysis

All fermentation variants were prepared and analyzed in triplicate. The results were tested statistically by analysis of variance with a significance level $p \leq 0.05$ using Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Chemical composition of raw material

Based on the analysis of the raw material (Table 1), the moisture levels of both buckwheat cultivars were found to be similar ($p>0.05$). The protein content in the raw materials was between 98.5±0.2 g/kg (Panda) and 107.5±0.8 g/kg (Kora). The greatest differences between the buckwheat cultivars were in terms of reducing sugars content which was significantly higher in the Panda cultivar (133.6±8.1 g glucose/kg), compared to the second cultivar (29.1±0.4 g glucose/kg). Despite the lower content of reducing sugars, the Kora cultivar had a similar ($p>0.05$) starch content (493.5±51.7 g/kg, *i.e.* 561.3±58.9 g/kg d.m) to the Panda cultivar (452.3±51.9 g/kg, *i.e.* 515.0±59.2 g/kg d.m.). If these results are compared with the available literature [Stempińska & Soral-Smietana, 2006], it can be concluded that both cultivars used in our study had a lower starch content (Panda, 642.3 g/kg d.m., Kora, 635.4 g/kg d.m.), but similar values of the other analyzed parameters. Using appropriate agrotechnical measures, it is possible to significantly affect the yield and quality of cereal grains. Stankiewicz [2004] showed that the total protein content in triticale grain increased with sowing density, while the starch content decreased only at the highest sowing density (750 grains per m^2). The recommended dose of selective herbicides also caused an increase in the total protein content and a reduction in starch level in the grain.

TABLE 1. Chemical composition of buckwheat cultivars used in the study.

Components	Kora cv.	Panda cv.
Moisture (g/kg)	120.9±8.7^a	121.9±8.0^a
Protein (Nx6.25) (g/kg d.m.)	107.5±0.8^a	98.5±0.2^b
Reducing sugars (g glucose/kg)	29.1±0.4^b	133.6±8.1^a
Starch (g/kg)	493.5±51.7^a	452.3±51.9^a

Results expressed as mean values±SE (n=3); values with different superscript letters in the same row are significantly different ($p<0.05$).

Physicochemical composition of sweet mashes

After preparation of all the variants of sweet mashes, their physicochemical analysis was conducted (Table 2), including measurement of pH and determination the concentration of soluble solids (expressed as total extract), reducing sugars, and dextrin.

The pH of sweet mashes prepared from both buckwheat cultivars with the use of pressure-thermal treatment ranged from 5.7±0.1 to 5.9±0.1 and was lower ($p<0.05$) than in analogous samples prepared using the pressure starch liberation method (between 6.2±0.1 and 6.4±0.1). Before inoculation with yeast slurry, the pH value of all the mashes was adjusted to 4.8.

The total extract content in the sweet mashes differed depending on the buckwheat cultivars used. There were no significant differences ($p>0.05$) in the value of this parameter compared to the starch liberation method. The total extract of mashes prepared from Kora cultivar was similar (130.1±0.3 g/L) after the use of both the pressure-thermal method and the PLS method. Mashes prepared from the second buckwheat cultivar (Panda) had a higher ($p<0.05$) soluble solids content than those made of the Kora cultivar. Most likely, this difference was due to the higher total

TABLE 2. Chemical composition of sweet buckwheat mashes.

Parameter	Kora cv.		Panda cv.	
	Pressure-thermal treatment	Pressureless starch liberation	Pressure-thermal treatment	Pressureless starch liberation
pH	5.9±0.1^b	6.2±0.1^a	5.7±0.1^b	6.4±0.1^a
Extract (g/L)	129.8±0.3^c	133.1±0.3^b	140.9±0.5^a	140.8±0.6^a
Reducing sugars (g glucose/L)	36.3±0.5^c	58.1±1.2^b	36.5±0.8^c	65.8±1.5^a
Dextrin (g/L)	61.4±2.1^b	37.8±1.7^d	76.8±0.6^a	45.6±1.7^c

Results expressed as mean values±SE (n=3); values with different superscript letters in the same row are significantly different ($p<0.05$).

sugars content (the sum of reducing sugars and hydrolyzed starch) in the Panda cultivar. However, taking into consideration the starch liberation method, the total extract of Panda cultivar-based mashes did not show significant differences and was similar (140.9±0.5 g/L) ($p>0.05$) for the samples prepared with both methods.

The initial content of reducing sugars in the sweet mashes from both buckwheat cultivars was higher in the mashes prepared with the PLS method. For the Panda cultivar, it was 65.8±1.5 g glucose/L mash, whereas the mashes prepared from the Kora cultivar had a lower reducing sugars content, *i.e.* 58.1±1.2 g glucose/L mash. In turn, the concentrations of reducing sugars in the mashes prepared using the pressure-thermal method were significantly ($p<0.05$) lower than in the samples prepared with the PLS method, but similar for both cultivars (36.3±0.5 g glucose/L mash). In terms of dextrin content, the samples prepared with the steaming method contained from 61.4±2.1 g/L mash (Kora cultivar) to 76.8±0.6 g/L mash (Panda cultivar). The PLS method led to a significantly lower ($p<0.05$) dextrin content in the mashes before fermentation, *i.e.* 37.8±1.7 g/L mash (Kora cultivar) and 45.6±1.7 g/L mash (Panda cultivar). The relatively low starch saccharification level may due to the simultaneous saccharification and fermentation, without a dedicated starch hydrolysis stage. However, this technology is commonly used in industry, because it has a positive influence on yeast fermentation activity, by eliminating osmotic stress caused by a high sugar content in mashes [Russell, 2003].

Physicochemical composition of fermented mashes

Physicochemical analysis of the fermented mashes was carried out in order to determine whether the course of the process and its efficiency were correct (Table 3). During any fermentation, yeast secretes H^+ ions, causing a decline in the pH value of the medium. The pH value of most of the fermented mashes ranged between 4.30±0.01 and 4.55±0.01, which is consistent with literature data and confirms the correct duration of the fermentation process [Russell, 2003]. Mashes prepared from the Kora cultivar using the PLS method showed lower pH, from 3.94±0.01 to 4.08±0.01. Often, sharp decreases in pH are due fermentation medium contamination by lactic acid bacteria (LAB). This is undesirable for the course of fermentation and results in a reduced spirit yield, since the sugars consumed by the bacteria are no longer available to the yeast for ethanol production [Balcerek *et al.*, 2016; Russell, 2003]. Moreover, one of the Kora cultivar-based mashes was not supplemented with nutrient for yeast. Gravimetric monitoring of the course of fermentation indicated a lower decrease in the mass of this sample, resulting from lower CO_2 liberation, which, in turn, is connected with slower fermentation compared to mash with $(NH_4)_2HPO_4$ (data not shown).

The factor used in the distilling industry to assess the apt course of the fermentation process is the apparent extract content, measured in the presence of ethanol. In the case of well-fermented distillery mashes with an initial extract content of approximately 180 g/L, the apparent extract content should not exceed 10–15 g/L [Kotarska *et al.,* 2006]. The apparent extract content in all fermented mashes varied widely between 3.00±0.01 g/L in the case of Kora cultivar-based mashes (prepared by steaming and supplemented with mineral nutrient for yeast) and 12.00±0.01 g/L for mashes prepared from Panda cultivar with the PLS method, without supplementation with minerals. The mashes prepared from Panda cultivar with the PLS method and supplemented with $(NH_4)_2HPO_4$ also showed the highest real extract content (43.14±0.01 g/L), while the lowest value of this parameter (27.52±0.01 g/L) was observed in the samples prepared with the pressure-thermal method and supplemented with $(NH_4)_2HPO_4$ (where the Kora cultivar was used).

The alcohol content in the samples prepared from Kora buckwheat cultivar ranged from 4.99±0.15% vol. to 5.89±0.35% vol. In terms of alcohol biosynthesis, the preferred method of buckwheat starch liberation was found to be the pressure-thermal treatment. Moreover, fermentation medium supplementation with mineral nutrients for yeast was found advisable, irrespective of the method of mash preparation. The lowest ethanol content was found in the sample of mash prepared with the PLS method and fermented without additional mineral nutrient for yeast. This sample simultaneously showed the lowest pH (3.94±0.01) after fermentation. Muthaiyan *et al.* [2011] demonstrated that the successive

TABLE 3. Chemical composition of fermented buckwheat mashes.

Parameter	Kora cv.				Panda cv.			
	Pressure-thermal treatment		Pressureless starch liberation		Pressure-thermal treatment		Pressureless starch liberation	
	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$
pH	4.41±0.01^{c}	4.34±0.01^{d}	4.08±0.01^{f}	3.94±0.01^{g}	4.52±0.01^{b}	4.55±0.01^{a}	4.30±0.01^{e}	4.39±0.01^{c}
Apparent extract (g/L)	5.00±0.01^{f}	3.00±0.01^{g}	10.40±0.01^{b}	10.40±0.01^{b}	9.20±0.01^{c}	5.50±0.01^{e}	8.80±0.01^{d}	12.00±0.01^{a}
Real extract (g/L)	27.52 ±0.01^{g}	29.13±0.01^{f}	40.50±0.01^{b}	35.52±0.01^{c}	33.52±0.01^{d}	33.54±0.01^{d}	29.51±0.01^{e}	43.14±0.01^{a}
Alcohol (% vol.)	5.89±0.35^{ab}	5.27±0.21^{cd}	5.50±0.21^{bc}	4.99±0.15^{de}	5.96±0.18^{a}	4.47±0.13^{f}	4.86±0.11^{e}	3.80±0.21^{g}
Reducing sugars (g glucose/L)	0.52±0.03^{g}	0.82±0.01^{f}	1.52±0.20^{e}	1.23±0.12^{e}	1.81±0.06^{d}	2.12±0.14^{c}	2.83±0.15^{b}	3.43±0.14^{a}
Dextrin (g/L)	3.81±0.22^{e}	4.23±0.25^{e}	6.33±0.11^{c}	5.92±0.13^{d}	3.83±0.41^{ef}	3.22±0.24^{f}	7.34±0.22^{b}	7.72±0.13^{a}

Results expressed as mean values±SE (n=3); values with different superscript letters in the same row are significantly different ($p<0.05$).

production of acids during fermentation shortens the lifespan of yeast by up to 60%, while the synergistic action of acetic and lactic acids decreases the yeast growth rate, the glucose consumption rate, and ethanol yield. Alcohol contents varied widely in the fermented mashes from Panda cultivar. However, the relationship between the alcohol content, the method of starch liberation, and supplementation with minerals was analogous to that observed in the case of Kora cultivar-based mashes. The lowest alcohol concentration (3.80±0.21% vol.) was determined in the sample prepared using the PLS method, without supplementation with mineral nutrients, whereas the highest one (5.96±0.18% vol.) was determined in the mash from steamed raw material and supplemented with $(NH_4)_2HPO_4$) ($p<0.05$). The lower ethanol concentration may due to the microbiological infection in the medium prepared with the PLS method. At the temperature of 90°C usually applied in this process most viable vegetative forms of microorganisms are inactivated, except for spores which are heat resistant. Moreover, secondary microbial contamination is possible in subsequent stages of processing, due to the presence of microorganisms in the water, air, yeast, and distillery equipment [Narendranath *et al.,* 2003]. In this study, the absence of added nutrients for yeast may have been the reason for the weak condition of the yeast in some mashes, which may have resulted in incomplete fermentation [Pielech-Przybylska *et al.*, 2017].

Reducing sugars content differed the least in the mashes prepared with the same method of starch liberation. For mashes from Kora cultivar prepared by steaming the raw material, it ranged from 0.52±0.03 to 0.82±0.01 g glucose/L mash. Meanwhile, in those prepared with the use of the PLS method, it was from 1.23±0.12 to 1.52±0.20 g glucose/L mash. The mashes from Panda cultivar contained on average higher amounts of reducing sugars ($p<0.05$); in the steamed samples, the reducing sugars content was between 1.81±0.06 and 2.12±0.14 g glucose/L mash, whereas in mashes prepared with the PLS method it ranged from 2.83±0.15 to 3.43±0.14 g glucose/L mash.

Relatively higher concentrations of non-hydrolyzed dextrin (between 5.92±0.13 and 7.72±0.13 g/L mash) were found to remain in the buckwheat mashes prepared with the PLS method than in samples in which the buckwheat grains were subjected to pressure-thermal treatment in a Henze steamer. Under a water vapor pressure of 0.4 MPa (150°C), the cellular structure of cereal grain is destroyed, enabling the release and accessibility of starch to enzymes. Moreover, gelatinization of starch, which takes place in the pressure-thermal method, facilitates the hydrolysis of this polysaccharide by amylolytic enzymes [Balcerek *et al*., 2016].

Fermentation factors

Sugar intake by the yeast during fermentation was calculated on the basis of the sugar content in the sweet and fermented mashes. The percentage value of sugar consumption is shown in Figure 2. In the mashes prepared from both the Kora and Panda cultivars, the highest sugar consumption (from 95.03±1.90% to 95.32±1.91%) ($p<0.05$) was determined in the samples prepared by the pressure-thermal treatment. The addition of $(NH_4)_2HPO_4$ was not observed to have an effect on the sugar intake. On the contrary, lower sugar consumption (between 90.13±1.83% and 92.31±1.85%) ($p>0.05$) was observed in the mashes prepared with the PLS method. Supplementation with minerals was also found to have no effect. Sugar intake during fermentation of the buckwheat mashes was comparable with that for fermented mashes prepared from conventional raw materials (cereals) used in ethanol production [Balcerek *et al.*, 2016; Pielech-Przybylska *et al.*, 2017].

The efficiency of ethanol biosynthesis (expressed as a percentage of the theoretical amount) was also calculated to

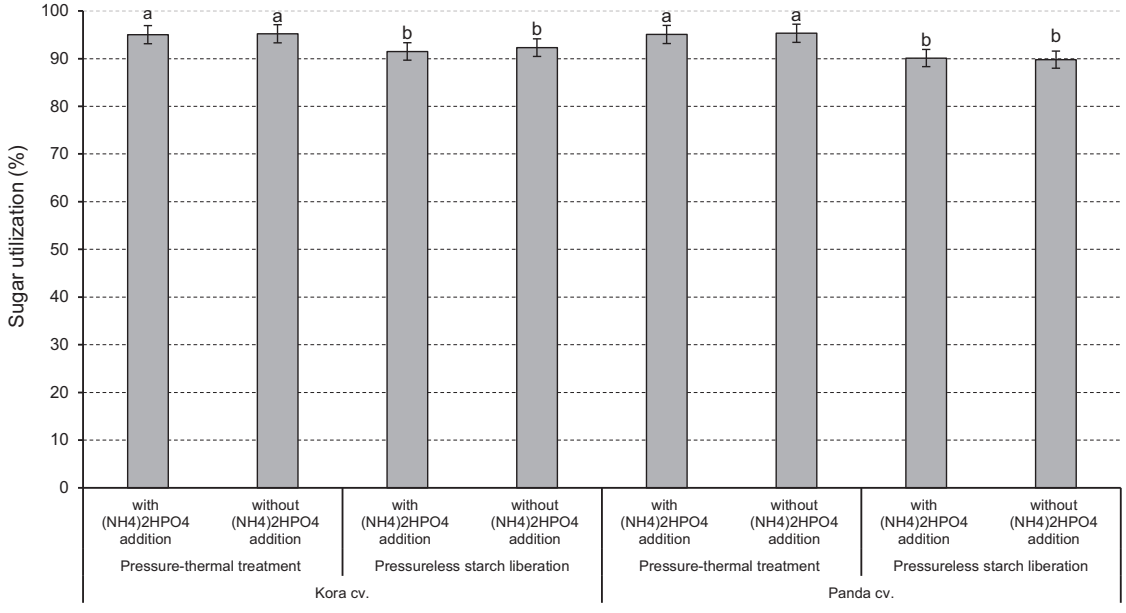


FIGURE 2. Sugar consumption in distillery mashes prepared from buckwheat grain. Different letters indicate significant differences ($p<0.05$) between mean values.

evaluate the fermentation results (Figure 3). For the mashes prepared from Kora cultivar, the highest value of this parameter, at 84.88±2.5% of theoretical efficiency, was registered for the fermentation variant based on the PLS method and supplemented with mineral nutrient for yeast. Fermentation of mashes with initial pressure-thermal treatment (both with and without the addition of $(NH_4)_2HPO_4$) resulted in similar fermentation efficiencies (from 81.68±2.45% to 82.84±2.34%) ($p>0.05$). The lowest efficiency of ethanol biosynthesis (77.01±2.31% of the theoretical yield) was obtained for mashes prepared with the PLS method, without supplementation with minerals. These results suggest that deficiency of $(NH_4)_2HPO_4$ was responsible for lower ethanol production.

After fermentation of the mashes prepared from Panda buckwheat cultivar, fermentation efficiency was significantly lower than in the mashes prepared from Kora cultivar. Large differences in the fermentation efficiency were also observed between individual variants (Figure 3). The mashes prepared by steaming and supplemented with $(NH_4)_2HPO_4$ were characterized by the highest fermentation efficiency (75.54±2.27% of the theoretical). The rest of the prepared samples of mashes were characterized by considerably ($p<0.05$) lower fermentation efficiency, which ranged between 50.40±1.51% and 64.46±1.93% of the theoretical yield. Taking into consideration the chemical composition of the mashes in which the low ethanol yield was obtained, there are no simple reasons to explain these results. The pH values of the mashes were similar to those observed in the samples with high fermentation efficiency, which eliminates the possibility of a high level of microbial contamination. It is possible that the relatively high initial content of reducing sugars in the mashes, and the lack of nutrients for yeast in selected samples, could cause osmotic stress, and as a consequence lower fermentation efficiency.

When the results of this study are compared with literature data [Balcerek *et al.*, 2016; Pielech-Przybylska *et al.*, 2017], it can be stated that the mashes prepared from Kora cultivar showed similar fermentation efficiency to mashes prepared from conventional raw materials, such as rye or wheat. The exceptions were samples made of the Panda cultivar, which were characterized by much lower fermentation efficiency compared to both the Kora cultivar and mashes made of other popular raw materials (rye, barley).

Characteristics of the obtained distillates

During the fermentation process, yeast produces ethanol and CO_2. Simultaneously, the synthesis of many volatile compounds occurs, such as carbonyl compounds, alcohols, esters, and organic acids, which determine the flavor and aroma of alcoholic beverages [Stewart, 2017]. Evaluation of the chemical composition of the obtained distillates revealed that the buckwheat cultivar, the method of sweet mash preparation, and the supplementation of the fermentation medium with nutrients for yeast, all had an effect (Table 4).

Carbonyl compounds, represented by aldehydes and ketones, are intermediates in the decarboxylation of alpha-keto acids to alcohols as well as in the synthesis and oxidation of alcohols. Their concentrations in agricultural distillates depend on the quality of the raw material, its chemical composition, the conditions of the technological process, and microbial contamination. Aldehydes are often observed to have a negative influence on the quality characteristics of spirits [Plutowska *et al.,* 2010]. In the spirits obtained from buckwheat mashes, the highest concentration of acetaldehyde (606.73±20.67 mg/L alcohol 100% vol.) was determined in the distillate obtained from the Kora cultivar with the use of steaming for starch liberation and without supplementation with minerals ($p<0.05$). The lowest content of this compound (208.71±9.71 mg/L alcohol 100% vol.) occurred in the distil-

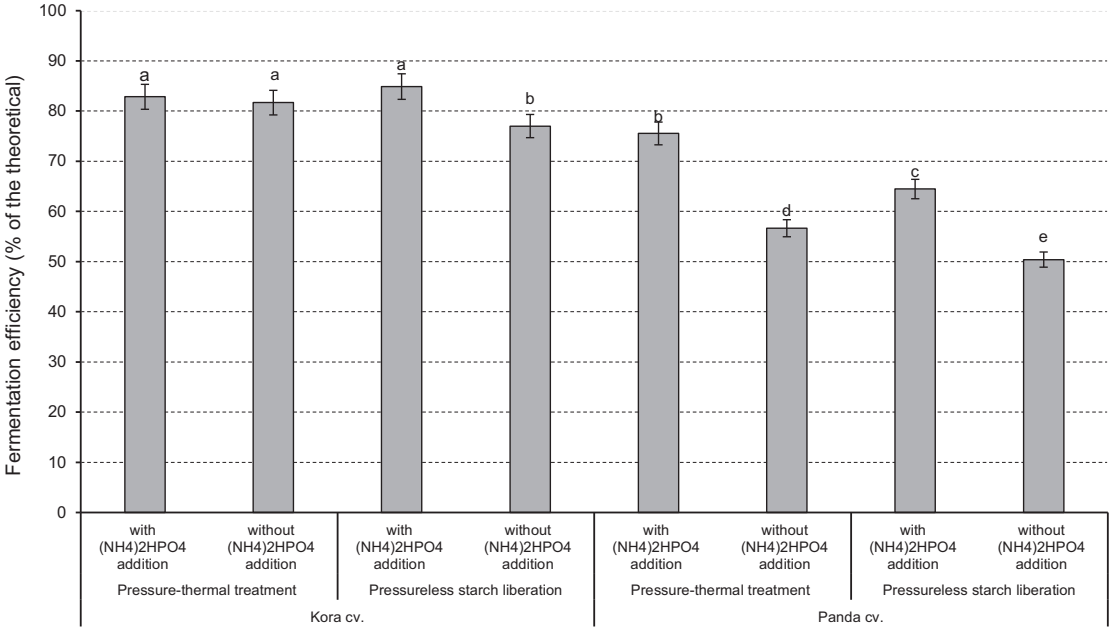


FIGURE 3. Fermentation efficiency in distillery mashes prepared from buckwheat grain. Different letters indicate significant differences ($p<0.05$) between mean values.

TABLE 4. Volatile compounds concentrations in the obtained distillates (mg/L alcohol 100% vol.).

Volatile compounds	Kora cv.				Panda cv.			
	Pressure-thermal treatment		Pressureless starch liberation		Pressure-thermal treatment		Pressureless starch liberation	
	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$
Carbonyl compounds								
Acetaldehyde	274.90±12.49^{d}	606.73±20.67^{a}	492.09±19.21^{b}	260.30±11.03^{d}	275.69±12.57^{d}	535.81±24.58^{b}	404.50±13.45^{c}	208.71±9.87^{e}
Furfural	6.12±0.31^{f}	22.94±1.29^{a}	7.55±0.56^{e}	17.95±1.12^{bc}	10.42±1.04^{d}	18.81±1.24^{b}	10.91±0.79^{d}	15.66±1.27^{c}
5-Methyl-2-furfural	2.73±0.17^{d}	5.51±0.25^{a}	3.23±0.22^{c}	5.17±0.32^{a}	3.43±0.14^{c}	4.38±0.24^{b}	3.31±0.13^{c}	4.29±0.23^{b}
Acetone	n.d.	n.d.	n.d.	n.d.	137.26±9.73^{b}	298.93±12.89^{a}	n.d.	n.d.
Diacetyl	186.13±9.61^{h}	1635.83±26.58^{a}	606.00±12.60^{g}	902.92±17.30^{e}	843.85±14.39^{f}	1523.83±15.38^{b}	1061.72±16.17^{c}	1021.00±12.10^{d}
Esters								
Ethyl acetate	159.67±5.97^{e}	253.55±9.36^{a}	220.88±8.09^{b}	192.88±8.29^{c}	175.50±7.55^{d}	199.40±10.94^{c}	174.97±7.50^{d}	232.01±13.20^{b}
Isoamyl acetate	5.82±0.18^{b}	5.65±0.27^{bc}	n.d.	4.74±0.17^{e}	7.43±0.34^{a}	n.d.	5.26±0.23^{cd}	5.02±0.22^{de}
Ethyl hexanoate	1.39±0.08^{a}	0.18±0.02^{b}	n.d.	1.39±0.09^{a}	n.d.	n.d.	n.d.	n.d.
Ethyl lactate	n.d.	3.66±0.17^{d}	2.01±0.13^{e}	82.06±3.21^{a}	1.24±0.08^{f}	4.07±0.24^{c}	3.50±0.15^{d}	76.94±2.69^{b}
Ethyl octanonate	3.18±0.22^{c}	3.00±0.25^{c}	1.46±0.15^{e}	5.10±0.31^{a}	4.13±0.31^{b}	2.28±0.23^{d}	4.55±0.46^{ab}	5.43±0.52^{a}
Ethyl decanoate	3.18±0.12^{c}	2.33±0.13^{d}	3.51±0.15^{b}	3.64±0.16^{b}	3.38±0.14^{bc}	n.d.	6.26±0.33^{a}	3.21±0.12^{c}
Alcohols								
Methanol	164.51±10.45^{c}	646.43±34.64^{a}	639.37±33.98^{a}	268.08±16.81^{b}	175.84±9.58^{c}	689.38±38.94^{a}	688.72±38.87^{a}	294.56±19.46^{b}
1-Propanol	49.54±2.95^{d}	58.18±3.82^{c}	75.01±3.50^{a}	52.75±2.28^{c}	63.06±3.31^{b}	68.18±3.82^{b}	57.97±2.47^{c}	52.31±2.83^{c}
2-Methyl-1-propanol	1528.97±52.90^{b}	876.93±27.69^{d}	883.28±28.33^{d}	1223.49±62.34^{c}	1708.54±70.85^{a}	847.80±44.78^{d}	825.24±42.52^{d}	1493.45±49.35^{b}
1-Butanol	7.13±0.21^{b}	8.38±0.36^{a}	9.14±0.41^{a}	4.97±0.26^{d}	7.28±0.33^{b}	8.78±0.38^{a}	6.20±0.22^{c}	5.09±0.21^{d}
3-Methyl-1-butanol	2453.93±65.39^{a}	1758.98±35.90^{c}	1648.14±34.81^{d}	2128.23±22.82^{b}	2471.47±27.15^{a}	1574.92±17.49^{e}	1785.04±18.50^{c}	2168.36±26.84^{b}
Phenylethyl alcohol	1588.88±48.89^{b}	1025.09±22.51^{e}	755.89±15.60^{g}	1720.88±32.08^{a}	1193.76±19.38^{c}	974.32±27.43^{f}	1141.49±21.15^{d}	1581.76±48.18^{b}
Acids								
Acetic acid	43.98±3.43^{d}	32.23±2.22^{e}	71.10±4.11^{c}	138.56±7.86^{a}	84.30±5.43^{b}	72.37±4.24^{c}	130.84±8.08^{a}	142.60±8.26^{a}
Isobutyric acid	5.54±0.35^{e}	1.74±0.07^{g}	99.33±3.93^{b}	492.78±19.28^{a}	6.58±0.06^{d}	n.d.	2.84±0.08^{f}	10.01±0.12^{c}
2-Methylhexanoic acid	7.54±0.25^{b}	5.23±0.15^{d}	3.32±0.08^{f}	8.66±0.47^{a}	6.19±0.22^{c}	3.94±0.08^{e}	3.29±0.13^{f}	7.38±0.34^{b}
Octanoic acid	7.91±0.29^{a}	8.16±0.32^{a}	4.89±0.24^{d}	3.13±0.15^{e}	7.19±0.32^{b}	5.65±0.27^{c}	8.38±0.34^{a}	6.83±0.28^{b}

Results expressed as mean values±SE (n=3); values with different superscript letters in the same row are significantly different ($p<0.05$); n.d. – not detected.

late obtained from mash prepared from the Panda cultivar, prepared with the PLS method with $(NH_4)_2HPO_4$ supplementation. It was observed that spirits from previously steamed raw material and supplemented with nutrients for yeast contained significantly ($p<0.05$) lower concentrations of acetaldehyde than those without the addition of $(NH_4)_2HPO_4$ (Table 4). It can be hypothesized that the pressure-thermal treatment of buckwheat grain leads to an impoverishment of the fermentation medium with nutrients, and that in order to prevent the suppression of the enzymatic activity of yeast (especially alcohol dehydrogenase activity responsible for the reduction of acetaldehyde to ethyl alcohol) it is advisable to supplement the fermentation medium with minerals. The opposite relation was observed in the composition of distillates from mashes prepared with the PLS method. The distillates from mashes with the addition of nutrients for yeast contained higher concentrations of acetaldehyde than the those from non-supplemented fermented samples ($p<0.05$).

Furfural and 5-methyl-2-furfural, which are mainly formed during the dehydration of pentoses and hexoses at elevated temperatures (*e.g.* during pressure thermal-treatment of starchy raw materials), are among the heterocyclic aldehydes occurring in agricultural distillates [Lee *et al*., 2001]. Furfural also arises during distillation involving Maillard reactions, hence its synthesis in the heated pot still is probably a fundamental factor causing its successive increase. Furfural, with an aroma resembling that of grain, also occurs at concentrations of as much as 20–30 mg/L in Scotch malt

whiskies [Lyons, 2003]. A study conducted by Pielech-Przybylska *et al.* [2017] showed high concentrations of furfural, between 79.70±2.21 and 225.81±6.48 mg/L alcohol 100% vol., in the distillates obtained from barley grain of the Karakan variety used as raw material and barley Munich malt as a source of amylolytic enzymes for starch hydrolysis. In turn, when the hydrolysis of barley starch was carried out using enzyme preparations, the concentrations of the furfural were lower and ranged from 43.53±1.23 to 95.50±2.42 mg/L alcohol 100% vol.

In the distillates obtained in our study, the concentrations of furfural were lower and ranged from 6.12±0.31 to 22.94±1.29 mg/L alcohol 100% vol. Both limit values determined in samples of the distillates obtained from mashes prepared by pressure-thermal treatment of buckwheat grain from the Kora cultivar, were differentiated only by the addition or not of nutrients for yeast. Despite statistically significant differences in the furfural content (Table 4), pressure-thermal treatment was observed to have no clear effect on its concentrations. The content of 5-methyl-2-furfural was much lower compared to the amounts of furfural ($p<0.05$), and ranged from 2.73 ±0.17 to 5.51 ±0.25 mg/L alcohol 100% vol.. All the distillates from the samples supplemented with nutrients for yeast contained significantly ($p<0.05$) lower concentrations of furfural and 5-methyl-2-furfural compared to the spirits from mashes without the addition of $(NH_4)_2HPO_4$.

The presence of acetone was only observed in the distillates obtained from the mashes based on Panda buckwheat cultivar, prepared by steaming the raw material. The main substrate for acetone synthesis is acetyl-CoA, produced by yeast and bacteria from acetic acid which, in turn, is a result of Maillard reactions that occur when mashes are prepared by pressure-thermal treatment of raw material [Pielech-Przybylska *et al.,* 2019].

Another ubiquitous compound in alcoholic beverages is vicinal diketone, 2,3-butanedione (diacetyl) with a buttery aroma [Stewart, 2017]. Diacetyl is formed as a by-product of valine biosynthesis in *Saccharomyces* yeast. The amount formed varies greatly depending on the yeast strain used, wort quality (*e.g*., free amino nitrogen (FAN) content, pH, and valine content), and fermentation conditions (*e.g*., temperature, pressure, and yeast pitching rate) [Kobayashi *et al*., 2005]. The distillates obtained in our study contained various concentrations of diacetyl, ranging from 186.13±9.61 mg/L alcohol 100% vol. to 1635.83±26.58 mg/L alcohol 100% vol.. Significantly higher ($p<0.05$) concentrations of diacetyl were determined in the majority of distillates from mashes prepared by pressure-thermal treatment and without supplementation with $(NH_4)_2HPO_4$. Kobayashi *et al.* [2005] reported that fermentation conditions promoting rapid yeast growth enhance diacetyl production if the free amino nitrogen content of the wort is insufficient. This may explain the higher amounts of diacetyl in the distillates from mashes not supplemented with $(NH_4)_2HPO_4$.

The concentrations of diacetyl were higher in the tested buckwheat distillates than in the samples of spirits obtained from other starchy raw materials, *e.g.* from rye and barley grains, and the corresponding malts (from approx. 3 to 5 mg/L alcohol 100% vol.) [Balcerek *et al.,* 2016]. Sensory analysis of buckwheat-based beer carried out by Phiarais *et al.* [2010] showed a very distinct "buttery" flavor, which is imparted by vicinal diketones, *i.e.* 2,3-butanedione and 2,3-pentanedione. Although these compounds are commonly considered undesirable in terms of the quality of beer, the results showed that buckwheat beer was acceptable for all attributes (aroma, purity of taste, and bitterness). Taking into consideration the results of our study and findings reported by Phiarais *et al.* [2010], it can be hypothesized that diacetyl is characteristic for buckwheat fermented (including distilled) beverages.

Esters are an important group of flavor compounds found in spirits. They are largely formed during the active phase of fermentation by the enzymatic condensation of organic acids with alcohols. Aroma constituents of major importance include: ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), phenylethyl acetate (roses and honey aroma), ethyl hexanoate (sweet apple aroma), and ethyl octanoate (sour apple aroma) [Stewart, 2017]. Grain distillates of agricultural origin also contain other esters, such as ethyl propanoate, ethyl butyrate, ethyl pentanoate, ethyl heptanoate, ethyl nonanoate, ethyl decanoate, ethyl undecanoate, and ethyl dodecanoate [Plutowska *et al.,* 2010]. In the analyzed distillates, the predominant ester was ethyl acetate, which was present in concentrations from 159.67±5.97 to 253.55±9.36 mg/L alcohol 100% vol. The distillates had also small amounts of isoamyl acetate, as well as esters of higher carboxylic acids and ethanol, *i.e*., ethyl hexanoate, ethyl octanoate, and ethyl decanoate. The significant differences observed in the concentrations of these esters were not strictly associated with the use of different buckwheat cultivars or processing conditions. The profiles of esters and their concentrations in the buckwheat-based distillates were similar to those determined in other cereal distillates [Balcerek *et al*., 2016; Pielech-Przybylska *et al*., 2017].

Higher alcohols are an important group of fermentation by-products, in terms of the quantity but also the sensory characteristics of agricultural distillates. They are represented mainly by *n*-propanol, isobutanol, and amyl alcohol (with its isomers, *i.e*., 2-methyl-1-butanol and 3-methyl-1-butanol). Regulation of the biosynthesis of higher alcohols is complex, since they are either produced as by-products of amino acid metabolism or *via* pyruvate and ethanol produced during carbohydrate metabolism [Russell, 2003]. These compounds play an important role in the formation of flavor qualities in spirits including whisky and others. Malt Scotch whiskies are rich in higher alcohols, with contents often well over 2 g/L [Lyons, 2003]. According to the recommendations of the Polish Standard [PN-A-79523, 2002], the maximum concentration of higher alcohols in agricultural distillates used for Starka production is 5 g/L absolute alcohol. In the buckwheat-based raw spirits obtained in our study, the most abundant higher alcohol was 3-methyl-1-butanol. In addition, 2-methyl-1-propanol and phenylethyl alcohol were found at relatively high concentrations, compared with the other cereal-based distillates [Balcerek *et al*., 2016; Pielech-Przybylska *et al.,* 2017]. Previous studies have identified 2-phenylethanol as a representative aroma compound in buckwheat-based alcoholic

TABLE 5. Sensory assessment of buckwheat distillates.

Sensory attributes	Kora cv.				Panda cv.			
	Pressure-thermal treatment		Pressureless starch liberation		Pressure-thermal treatment		Pressureless starch liberation	
	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$	with $(NH_4)_2HPO_4$	without $(NH_4)_2HPO_4$
Color (max. 2 pts)	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}
Clearness (max. 2 pts)	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}
Odor (max. 4 pts)	3.0 ± 0.5^{a}	2.5 ± 0.5^{ab}	2.0 ± 0.3^{bc}	1.5 ± 0.2^{c}	3.5 ± 0.5^{a}	3.0 ± 0.5^{a}	2.5 ± 0.2^{b}	2.5 ± 0.2^{b}
Taste (max. 12 pts)	8.0 ± 0.5^{a}	7.5 ± 0.5^{a}	4.5 ± 0.5^{c}	4.0 ± 0.5^{c}	8.0 ± 0.5^{a}	8.5 ± 0.5^{a}	5.5 ± 0.2^{b}	5.5 ± 0.2^{b}
Overall (max. 20 pts)	15.0 ± 0.5^{ab}	14.0 ± 0.5^{b}	10.5 ± 0.4^{d}	9.5 ± 0.4^{e}	15.5 ± 0.4^{a}	15.5 ± 0.4^{a}	12.0 ± 0.2^{c}	12.0 ± 0.2^{c}

Results expressed as mean values±SE (n=3); values with different superscript letters in the same row are significantly different ($p<0.05$); pts – points.

beverages including mead [Wintersteen *et al.,* 2005] and beer [Deželak *et al.,* 2014].

In our analysis of the qualitative and quantitative composition of volatile compounds in the buckwheat distillates, attention was also given to the relationship between the chemical composition of the sweet mashes (the content of reducing sugars) (Table 2) and the concentration of volatiles (Table 4). As a result, it was observed that the distillates from mashes prepared by pressure-thermal treatment, with initially lower fermentable sugars content, contained lower amounts of aldehydes and diacetyl, but higher levels of 2-methyl-1-propanol and 3-methyl-1-butanol. Kłosowski *et al.* [2015] observed the effect of the availability of sugars released during hydrolysis of starchy raw materials on the concentration of higher alcohols during the fermentation process. Their results indicate that the highest glucose content in maize mashes resulted in significantly higher isobutanol and 2-methyl-1-butanol contents during the initial stage of fermentation. Moreover, the maize-based distillates contained higher final total content of fusel alcohols compared to rye and amaranth-based distillates.

One of the undesirable compounds in spirit distillates is methanol, which is generated through the hydrolysis of methylated pectins present in plants and fruits. While methanol does not directly affect the flavor of the distillate, it is subjected to restrictive controls, owing to its high toxicity [Adam & Versini, 1996]. The tested distillates in our study contained methyl alcohol at concentrations between 164.51±10.45 and 689.38±38.94 mg/L alcohol 100% vol. There were no clear effects related to the buckwheat cultivar, the method of starch liberation or supplementation with nutrients for yeast on methanol content. The results of our previous study showed that the agricultural distillates obtained from rye and barley grains and the corresponding malts contained methanol in concentrations between 42.5±6.5 mg/L and 198.1±17.3 mg/L alcohol 100% vol. [Balcerek *et al.*, 2016]. In turn, Pielech-Przybylska *et al.* [2017] observed methanol contents in barley distillates at between approx. 33 and 73 mg/L alcohol 100% vol. Moreover, they were higher in the samples obtained from mashes prepared by the pressure-thermal than with the PLS method. While EU Regulation no. 110/2008 [Regulation (EC) No 110/2008] defines acceptable concentrations of methanol in ethyl alcohols of agricultural origin (rectified spirit), wine spirits, and fruit spirits, it does not set any limits on the content of this compound in distillates of agricultural origin.

The final group of volatiles found in the tested distillates were volatile acids, among which acetic acid was predominant. Contaminating bacteria can alter the normal profiles of organic acids. Lactobacilli are very ethanol-tolerant and are capable of very rapid growth in distillery mashes. Increases in lactic and acetic acids are observed when there is contamination and these acids can inhibit the growth of yeast if produced in excess. Concentrations of lactic acid over 0.8% (w/v) and of acetic acid over 0.05% (w/v) negatively affect the growth of yeast. In addition, low pH can affect glucoamylase activity against residual dextrin. Bacterial contamination always means loss of ethanol. During fermentation, it has been observed that there is an increase in C6--C10 and a considerable decrease in C12-C18:3 acids as consequence of yeast metabolism [Russel, 2003]. The application of pressureless treatment in our study resulted in significantly higher concentrations, especially of acetic acid ($p<0.05$), compared to the pressure-thermal method. In the majority of distillates obtained by the pressure-thermal treatment of raw material, the concentrations of this compound did not exceed the recommended limit for agricultural distillates from cereals, *i.e*., 0.1 g/L alcohol 100% vol. [Polish Standard PN--A-79523, 2002]. In contrast, the majority of distillates from both cultivars of buckwheat processed with the PLS method exceeded the permissible content of acetic acid by approx. 40%. The results of chromatographic analysis also showed the presence of other acids, such as isobutyric, 2-methylhexanoic, and octanoic. It is important to note the very high concentrations of isobutyric acid in the distillates obtained after the fermentation of Kora cultivar-based mashes, prepared with the PLS method, which were characterized as having a lower pH after completed fermentation (Table 3). Isobutyric acid was at the level of 99.33±3.93 mg/L alcohol 100% vol. in the distillate from mash with the addition of $(NH_4)_2HPO_4$, whereas in the distillate without supplementation its concentration reached the very high level of 492.78±19.28 mg/L alcohol 100% vol. ($p<0.05$). Thus, the results obtained in our study indicate that the pressureless method of preparing dis-

tillery mashes carries the risk of microbial contaminations, which may reduce the quality of the distillates.

Sensory analysis of distillates

The results of sensory evaluation of the tested buckwheat distillates are presented in Table 5. All of the tested samples were assessed by the judges as being clear and colorless, as is characteristic of fresh distillates, and obtained the highest scores for these visual properties (2 pts). Significant differences were observed in terms of odor ($p<0.05$). The least pleasant, pungent odor was attributed to the distillate from the Kora buckwheat cultivar, which was previously treated with the PLS method, and without the addition of $(NH_4)_2HPO_4$ (this mash had the lowest pH after fermentation). It received only 1.5±0.2 of the possible 4 points for aroma. The distillate from the same cultivar, also processed with the PLS but supplemented with $(NH_4)_2HPO_4$, was assessed similarly. These samples were also judged to have an acrid taste and unharmonized aroma, as evidenced by the lowest taste ratings compared to other samples. Their overall sensory quality was the lowest and varied between 9.5±0.4 and 10.5±0.4 points (on a 20-point scale). The distillates obtained from the Panda cultivar using the PLS method were rated as not being very pleasant in smell and taste, although they obtained statistically significantly higher scores than analogous distillates from the Kora cultivar. The best rated distillates were those obtained from both cultivars when the mashes were prepared by pressure-thermal treatment. They were characterized by a pleasant aroma (odor) and a well-harmonized taste, characteristic of cereal distillates.

Despite the differences in the sensory quality of the buckwheat distillates, correlated mainly with the raw material processing method, the tasting panel considered that the majority of the buckwheat-based distillates were characterized by interesting organoleptic features and a specific aroma and flavor, defined as characteristic of the processed raw material. It was suggested that the application of additional treatments, such as re-distillation with separation of the head and tail fractions, and possibly ageing with wood, could allow producing interesting, original spirit beverages.

CONCLUSION

This study has demonstrated that to obtain a high yield of buckwheat distillate with appropriate organoleptic features, it is necessary to select the appropriate method of raw material pretreatment, including starch liberation, and enzymes for hydrolysis, possibly including supportive enzyme preparations, such as xylanase, as well as to supplement the mashes with mineral nutrients for yeast.

The chemical compositions of two buckwheat cultivars, Kora and Panda, were assessed to determine their suitability for use in the production of agricultural distillate (raw spirit). Both cultivars contained similar amounts of starch, while the second contained a significantly higher content of reducing sugars. However, the sweet mashes obtained after digestion of grain from the Panda cultivar with amylolytic enzyme preparations had a higher content of dextrin, which may indicate more difficult starch hydrolysis than in the case of the Kora cultivar. Of the tested methods of starch liberation, pressure-thermal treatment, was found to be more efficient in terms of ethanol biosynthesis, especially for the Panda cultivar. A beneficial effect on fermentation efficiency was also observed in the case of mashes supplemented with $(NH_4)_2HPO_4$. In order to boost fermentation efficiency and improve the quality of the obtained distillates, further research is necessary to study the conversion of buckwheat grain into agricultural distillate, taking into consideration the conditions of mashing and the fermentation process, as well as antimicrobial protection. Moreover, separation of the head and tail fractions which contain undesirable compounds in terms of the sensory qualities of the buckwheat distillates should be carried out.

There is increasing interest in niche craft products from both micro-distilleries and large spirit plants, which are considering the use of new raw materials, while taking into account social attachments and tradition. Selection of the factors in buckwheat distillation identified in this study opens the way for using grain from this pseudo-cereal in the production of original distillates with a specific aroma, flavor, and raw material identity.

REFERENCES

1. Adam, L., Versini, G. (1996). A study on the possibilities to lower the content of methyl-alcohol in eaux-de-vie de fruits; European Commission: Brussels, Belgium, pp. 1–8, [https://op.europa.eu/en/publication-detail/-/publication/0b908be6–2673–45a5–8c2f-b3b6abc1aa37] (accessed on 12 September 2019).
2. AOAC (1995). Official Methods of Analysis of AOAC International. Method 960.52, 16th edition. AOAC International, Maryland, USA.
3. Aoki, M., Koizumi, N. (1986). Organoleptic properties of the volatile components of buckwheat flour and their changes during storage after milling. *Nippon Shokuhin Kogyo Gakkaishi – Journal of the Japanese Society for Food Science and Technology, 33*(11), 769–772.
4. Balcerek, M., Pielech-Przybylska, K., Dziekońska-Kubczak, U., Patelski, P., Strąk, E., (2016). Fermentation results and chemical composition of agricultural distillates obtained from rye and barley grains and the corresponding malts as a source of amylolytic enzymes and starch. *Molecules, 21*(10), art. no. 130.
5. Banerjee, S., Kundu, D. (2013). A comparative overview of ethanol production from cereal grains and potato by enzymatic treatment. *International Journal of Research in Engineering & Advanced Technology*, *1*(2), 1–11.
6. Bonafaccia, G., Marocchini, M., Kreft, I. (2003). Composition and technological properties of the flour and bran from common and tartary buckwheat. *Food Chemistry*, *80*(1), 9–15.
7. Chida, M., Sone, Y., Tamura, H. (2004). Aroma characteristics of stored tobacco cut leaves analyzed by a high vacuum distillation and canister system. *Journal of Agricultural and Food Chemis*try, *52*(26), 7918–7924.
8. COBORU (2019). Centralny Ośrodek Badania Odmian Roślin Uprawnych (Krajowy Rejestr (KR), [http://www.coboru.pl/Polska/Rejestr/rejestr_KR.aspx] (accessed on 10 September 2019).
9. Deželak, M., Zarnkow, M., Becker, T., Košir, I.J. (2014). Processing of bottom-fermented gluten-free beer-like beverages based

on buckwheat and quinoa malt with chemical and sensory characterization. *Journal of the Institute of Brewing*, *120*(4), 360–370.
10. Dziadek, K., Kopeć, A., Pastucha, E., Piątkowska, E., Leszczyńska, T., Pisulewska, E., Witkowicz, R., Francik, R. (2016). Basic chemical composition and bioactive compounds content in selected cultivars of buckwheat whole seeds, dehulled seeds, and hulls. *Journal of Cereal Science*, *69*, 1–8.
11. EFSA Journal (2004). Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to a notification from CEPS on distillates made from cereals pursuant to Article 6 paragraph 11 of Directive 2000/13/EC. 130, 1–6.
12. Haros, C.M., Sanz-Penella, J.M. (2017). Food uses of whole pseudocereals. In C.M. Haros, R. Schonlechner (Eds.), Pseudocereals: Chemistry and Technology, Wiley-Blackwell, Hoboken, New Jersey, USA, pp. 163–192.
13. Im, J.S., Huff, H.E., Hsieh, F.H. (2003). Effect of processing conditions on the physical and chemical properties of buckwheat grit cakes. *Journal of Agricultural and Food Chemistry*, *51*(3), 659–666.
14. Janes, D., Kantar, D., Kreft, S., Prosen, H. (2009). Identification of buckwheat (*Fagopyrum esculentum* Moench) aroma compounds with GC-MS. *Food Chemistry*, *112*(1), 120–124.
15. Kłosowski, G., Mikulski, D., Macko, D., Miklaszewska, B., Kotarska, K., Czupryński, B. (2015). Influence of various yeast strains and selected starchy raw materials on production of higher alcohols during the alcoholic fermentation process. *European Food Research and Technology*, *240*(1), 233–242.
16. Kobayashi, K., Kusaka, K., Takahashi, T., Sato, K.(2005). Method for the simultaneous assay of diacetyl and acetoin in the presence of α-acetolactate: Application in determining the kinetic parameters for the decomposition of α-acetolactate. *Journal of Bioscience and Bioengineering*, *99*(50), 502–507.
17. Kotarska, K., Czupryński, B., Kłosowski, G. (2006). Effect of various activators on the course of alcoholic fermentation. *Journal of Food Engineering*, *77*(4), 965–971.
18. Lee, M.K.-Y., Paterson, A., Piggott, J.R., Richardson, G.D. (2001). Origins of flavour in whiskies and a revised flavour wheel: A review. *Journal of the Institute of Brewing*, *107*(5), 287–313.
19. Lyons, T.P. (2003). Production of Scotch and Irish whiskies: Their history and evolution. In K.A. Jacques, T.P. Lyons, D.R. Kelsall (Eds.), The Alcohol Textbook, 4th ed., Alltech Inc., Nicholasville, KY, USA, pp. 193–206.
20. Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determining reducing sugars. *Analytical Chemistry*, *31*(3), 426–428.
21. Muthaiyan, A., Limayem, A., Rücke, S. (2011). Antimicrobial strategies for limiting bacterial contaminants in fuel bioethanol fermentations. *Progress in Energy and Combustion Science*, *37*, 351–370.
22. Narendranath, N.V. (2003). Bacterial contamination and control in ethanol production. In K.A. Jacques, T.P. Lyons, D.R. Kelsall (Eds.), The Alcohol Textbook, 4th ed., Alltech Inc., Nicholasville, KY, USA, pp. 287–291.
23. Nicol, D.A. (2003). Rum. In A.G.H. Lea, J.R. Piggott (Eds.), Fermented Beverage Production; Kluwer Academic/Plenum Publishers, New York, NY, USA, p. 271.
24. Phiarais, B.P.N., Mauch, A., Schehl, B.D., Zarnkow, M., Gastl, M., Herrmann, M., Zaninni, E., Arendt, E.K. (2010). Processing of top fermented beer brewed from 100% buckwheat malt with sensory and analytical characterization. *Journal of the Institute of Brewing*, *116*(3), 265–274.
25. Pielech-Przybylska, K., Balcerek, M., Dziekońska-Kubczak, U., Pacholczyk-Sienicka, B., Ciepielowski, G., Albrecht, Ł., Patelski, P. (2019). The role of *Saccharomyces cerevisiae* yeast and lactic acid bacteria in the formation of 2-propanol from acetone during fermentation of rye mashes obtained using thermal-pressure method of starch liberation. *Molecules*, *24*(3), art. no. 610.
26. Pielech-Przybylska, K., Balcerek, M., Nowak, A., Wojtczak, M., Czyżowska, A., Dziekońska-Kubczak, U., Patelski, P. (2017). The effect of different starch liberation and saccharification methods on the microbial contaminations of distillery mashes, fermentation efficiency, and spirits quality. *Molecules*, *22*(10), art. no. 1647.
27. Plutowska, B., Biernacka, P., Wardencki, W. (2010). Identification of volatile compounds in raw spirits of different organoleptic quality. *Journal of the Institute of Brewing*, *116*(4), 433–439.
28. Polish Standard PN-A-79523 (2002). Agricultural Distillate. Polish Committee for Standardization, Warsaw, Poland, pp. 1–7.
29. Polish Standard PN-EN ISO 10520 (2002). Native starch. Determination of starch content. Ewers Polarimetric Method. Polish Committee for Standardization: Warsaw, Poland, pp. 1–13.
30. Regulation (EC) No 110/2008 of the European Parliament and of the Council of 15 January 2008 on the Definition, Description, Presentation, Labelling and the Protection of Geographical Indications of Spirit Drinks and Repealing Council Regulation (EEC) No 1576/89; Official Journal of the European Union: Luxembourg, p. L 39/16
31. Russell, I. (2003). Understanding yeast fundamentals. In K.A. Jacques, T.P. Lyons, D.R. Kelsall (Eds.), The Alcohol Textbook, 4th ed., Alltech Inc., Nicholasville, KY, USA, pp. 85–120.
32. Sakaida, H., Nakahara, N., Watashi, N., Kai, T., Nakashima, Y., Sakakibara, Y., Nishiyana, K., Fukuda, N., Suiko, M. (2003). Characteristic flavor of buckwheat Shochu and comparison of volatile compounds from variety cereal Shochu. Nippon shokuhin Kagaku Kogaku Kaishi – *Journal of the Japanese Society for Food Science and Technology,* 50(12), 555–562.
33. Stankiewicz, C. (2004). Effect of sowing density, herbicides and harrowing application on yield and content of total protein and starch in 'Wanad' spring triticale kernels. *Acta Scientiarum Polonorum, Agricultura*, *3*(2), 77–88 (in Polish).
34. Starowicz, M., Koutsidis, G., Zieliński, H. (2018). Sensory analysis and aroma compounds of buckwheat containing products – a review. *Critical Reviews In Food Science And Nutrition*, *58*(11), 1767–1779.
35. Stempińska K., Soral-Smietana M. (2006). Chemical compounds and physicochemical estimation of buckwheat grains – Comparison of three Polish varieties. *Żywność. Nauka. Technologia. Jakość*, *2*(47), Suppl., 348–359 (in Polish; English abstract).
36. Stewart, G.G. (2017). The production of secondary metabolites with flavour potential during brewing and distilling wort fermentations. *Fermentation – Basel*, *3*(4), art. no. 63.
37. Tešević, V., Nikićević, N., Jovanović, A., Djoković, D., Vujisić, L., Vučković, I., Bonić, M. (2005) Volatile components from old plum brandies. *Food Technology and Biotechnology*, *43*(4), 367–372.

38. Wintersteen, C.L., Andrae, L.M., Engeseth, N.J. (2005). Effect of heat treatment on antioxidant capacity and flavor volatiles of mead. *Journal of Food Science*, *70*(2), 119–126.
39. Young, S. (Ed.) (1922). Distillation Principles and Processes. Macmillian and Co., London, pp. 58, 146, 190, 220 [http://www.archive.org/details/distillationprin00younrich] (accessed on 10 September 2018).

Submitted: 24 June 2019. Revised: 19 September 2019. Accepted: 29 October 2019. Published on-line: 17 December 2019.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 139–150
DOI: 10.31883/pjfns/113532
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Hybrid Approach in the Analysis of Bovine Milk Protein Hydrolysates as a Source of Peptides Containing Di- and Tripeptide Bitterness Indicators

Anna Iwaniak, Piotr Minkiewicz, Monika Hrynkiewicz, Justyna Bucholska, Małgorzata Darewicz*

University of Warmia and Mazury in Olsztyn, Faculty of Food Science,
Chair of Food Biochemistry, Pl. Cieszyński 1, 10–719 Olsztyn-Kortowo, Poland

Key words: bitter peptides, BIOPEP-UWM database, protein hydrolysates, hybrid approach

The aim of this study was to employ a hybrid approach combined with a fragmentomic idea of research used to analyze bovine milk protein hydrolysates as a source of peptides with a potential bitter taste. Firstly, selected sequences of bovine milk proteins were *in silico* hydrolyzed using bromelain, ficin, papain, and proteinase K. Hydrolysis was simulated using the BIOPEP-UWM "Enzyme(s) action" tool. Potentially released peptides (called parent peptides) were analyzed for the presence of shorter peptide regions with bitter taste. Some of them were defined as peptide bitterness indicators. Then, *in silico* results were verified in the *in vitro* experiments with the use of a bovine milk protein concentrate (MPC) as a substrate. The verification included the MPC hydrolysis and identification of peptides in MPC hydrolysates using RP-HPLC and RP-HPLC-MS/MS, respectively.

The hybrid analysis of bovine milk protein hydrolysates showed that all released peptides contained fragments with bitter taste and some of them were bitterness indicators, which could potentially determine the taste of a whole sequence. However, the results of *in silico* and *in vitro* hydrolysis were divergent. It was also reflected by the ranking of enzymes acting *in silico* and *in vitro*. Despite above discrepancies, our predictions concerning the release of peptides that may affect the bitter taste of a hydrolysate, contribute to bringing more insights into the taste of foods, especially if unwanted. However, before introducing a food product to the market, sensory studies are required to confirm (or not) its taste.

LIST OF ABBREVIATIONS

BSA, bovine serum albumin; B, bromelain; B-MPC, bromelain hydrolysate of milk protein concentrate; F, ficin; F-MPC, ficin hydrolysate of milk protein concentrate; MLR, multivariate linear regression; MPC, milk protein concentrate; O-MPC, non-hydrolyzed milk protein concentrate; P, papain; P-MPC, papain hydrolysate of milk protein concentrate; PK, proteinase K; PK-MPC, proteinase K hydrolysate of milk protein concentrate; RP-HPLC, reversed-phase high performance liquid chromatography; RP-HPLC-MS/MS, reversed--phase high performance liquid chromatography and mass spectrometry; Rcaf., the ratio of caffeine (the threshold concentration for 1 mM caffeine solution as a standard (Rcaf. = 1.0); $t_{R\ predicted}$, theoretical retention time; $t_{R\ experimental}$, experimental retention time; α_{s1}, casein; α_{s2}-CN, α_{s2}- casein; α-La, α–-lactalbumin; β-Lg, β– lactoglobulin; β-CN, β– casein; κ-CN, κ-casein; and TFA, trifluoracetic acid.

INTRODUCTION

Peptides derived from food proteins exhibit a variety of biological functions, *e.g.*, they contribute to the reduction of blood pressure, glucose and cholesterol levels as well as act as antioxidative, antibacterial, antithrombotic, immunomodulating *etc.* agents [Li *et al.*, 2019]. There are five taste sensations, *i.e.*, bitter, salty, sour, sweet, and umami, and some of them are more attributable to peptides, which is due to their specific amino acids [Ding *et al.*, 2017]. It especially concerns sweet, bitter, and umami peptides [Temussi, 2012].

Milk and dairy products represent sources of valuable nutrients like proteins, sugar (lactose), fat, micro- and macroelements [Guetouache *et al.*, 2014]. It is also well-known that bovine milk proteins are precursors of biopeptides and some of them are components of functional foods aiming to, *e.g.*, reduce blood pressure [Sánchez & Vázquez, 2017]. Despite this fact, hydrolysis of milk proteins leads to the release of bitter-tasting peptides [Kilara & Panyam, 2003]. Bitterness can be then regarded as a problematic property of peptides representing an additional, *e.g.*, health-beneficial function [Iwaniak *et al.*, 2016a]. Thus, the choice of an appropriate debittering method that would not compromise the particular bioactivity of a peptide is a challenge for food scientists and technologists producing protein hydrolysates [Lafarga & Hayes, 2017].

Loads of information on the physiological functions of compounds, including peptides, can be found in biological and chemical databases [Minkiewicz *et al.*, 2013; Bucholska *et al.*, 2018]. Sequences found in databases as well as the computer software dedicated to peptide analyses are helpful in predicting, *e.g.*, the possible mechanisms of peptide

* Corresponding Author: Tel./Fax: +48 89 523 37 22;
E-mail: ami@uwm.edu.pl (Prof. Anna Iwaniak)

action, the structure-bioactivity relationship [Iwaniak *et al.*, 2015], and bioactivity [Mooney *et al.*, 2012]. The analysis of bioactive peptides based on computer predictions is called an *in silico* approach and, according to Udenigwe [2014], it is one of the preferred methodological approaches when studying peptides derived from food proteins. Another approach that combines *in silico* studies with classical ones (*i.e*., experimental) is called an integrated or hybrid approach [Udenigwe, 2014; Iwaniak *et al.*, 2019a]. This term was firstly introduced by Udenigwe [2014] who described the limitations of classical and bioinformatic approaches used "separately" to analyze bioactive peptides. The integrated approach may offer an efficient solution to problems encountered during, *e.g*., detection of bioactive peptides using sets of data provided in databases in terms of identification of some structural motifs associated with already "known" bioactivities [Udenigwe, 2014]. It can also be referred to the presence of specific amino acids in a peptide sequence which may determine its bitter taste. For example, peptides composed of phenylalanine (F) and tyrosine (Y) were found as bitter-tasting [Kim & Li-Chan, 2006]. Pripp & Ardö [2007] reported that the bitter taste of peptides depends on the presence of N-terminal basic and bulky residue as well as C-terminal amino acid with the hydrophobic side chain. To conclude, the motif(s) assigned to the particular (*i.e*., known) peptide bioactivity found in a fragment with the "unknown" function may define its biological activity. This way of establishing the function of an unknown fragment is consistent with the fragmentomic idea introduced by Zamyatnin [2009]. Taking into account the fact that such a rule can also be applicable to peptides, we have advanced a novel idea of introducing the bitter-tasting indicators defined as shorter motifs with known bitterness, which when found in the sequences of peptides may potentially determine their taste. Thus, the aim of this study was to employ the hybrid approach to identify peptides likely to be bitter due to the presence of bitter peptidic fragments, especially those called bitter-tasting indicators, in bovine milk protein hydrolysates.

MATERIALS AND METHODS

In silico analysis

Sequences of proteins and bitter-tasting peptides including peptide indicators

The following sequences of bovine (*Bos taurus*) milk proteins were derived from the UniProt database (http://www.uniprot.org/uniprot) [The UniProt Consortium, 2019] (accessed July 2018): αs_1-casein (P02662; 199), αs_2-casein (P02663; 207), β-casein (P02666; 209), κ-casein (P02668; 169), β-lactoglobulin (P02754; 162), α-lactalbumin (B6V3I5; 123), and serum albumin (P02769; 583). Their UniProt accession numbers and number of amino acid residues in the chain (excluding signal peptide), respectively, are provided in brackets. The sequences of bitter-tasting di- and tripeptides were found in the BIOPEP-UWM database (formerly BIOPEP) of sensory peptides and amino acids (51 dipeptides and 51 tripeptides; accessed in July 2018) [Minkiewicz *et al.*, 2008]. They were subjected to analysis according to the multivariate linear regression (MLR) protocol described by Iwaniak *et al.* [2019b]. Based on MLR results [Iwaniak *et al.*, 2019b], di- and tripeptides whose predicted measure of bitterness approximated that of the experimental ones achieved the status of bitter-tasting indicators. The measure of bitterness was bitterness intensity (the ratio of caffeine – Rcaf) value defined as follows [Otagiri *et al.*, 1983]:

$$Rcaf = 1mM/TV$$

TV is defined as detection threshold value of a substance, (the lowest concentration causing detectable bitterness), expressed in mM. The threshold value of caffeine is 1 mM.

Finally, the following twenty dipeptides: LG(0.05/0.1), VD(0.08/0.12), AD(0.17/0.12), IG(0.22/0.14), VI(0,17/0.19), VE(0.17/0.19), RG(0.13/0.21), VL(0.17/0.21), FG(0.17/0.23), GV(0.22/0.23), EY(0.25/0.21), YG(0.33/0.27), VY(0.33/0.29), LE(0.33/0.35), GY(0.33/0.37), LL(0.40/0.37), FP(0.67/0.58), IF(0.67/0.62), FL(0.67/0.67), and FF(0.83/0.70); and nineteen tripeptides: GLG(0.10/0.16), PGR(0.04/0.05), PGP(0.11/0.09), GGL(0.1/0.13), VVV(0.22/0.08), LGL(0.2/0.16), PPG (0.11/0.26), FGG(0.22/0.24), GVV(0.22/0.23), FPK(0.33/0.22), KPK(0.33/0.43), PPP(0.50/0.49), YGG(0.43/0.53), GGF (0.67/0.57), PIP(0.70/0.79), GLL(0.67/0.75), LLL(0.83/0.79), GRP(1.25/1.25), and GYY(2.50/2.41), were the bitterness indicators. Their experimental/predicted Rcafs. are given in brackets.

Theoretical hydrolysis and identification of bitter-tasting indicators

Milk proteins were theoretically hydrolyzed using: bromelain (EC 3.4.22.33), ficin (EC 3.4.22.3), papain (EC 3.4.22.2), and proteinase K (EC 3.4.21.64). Hydrolysis was performed with the BIOPEP-UWM tool called "Enzyme(s) action" [Minkiewicz *et al.*, 2008]. This option is provided when opening the "Analysis" tab of the BIOPEP-UWM engine. The hydrolysis was carried out by selecting the option "one substrate *(i.e.* milk protein sequence): one enzyme". Each product of potential proteolysis (*i.e.* peptide fragment; single amino acids were excluded) was then copied and pasted to the window called "For your sequence" found in the "Profiles of potential biological activity" tab (see "Analysis" panel of BIOPEP-UWM tool). This way, all potentially released fragments were searched for the presence of: bitter-tasting indicators, bitter peptides with no such status as well as their additional bioactivity (if any).

Theoretical retention times

Theoretical (*i.e.* predicted) retention times ($t_{R\ predicted}$) of peptides to be then identified using LC-MS/MS were calculated using the Sequence Specific Retention Calculator (SSRCalc). Correction of retention time predictions was firstly introduced by Dziuba *et al.* [2011] and included corrections made taking into account the type of column, apparatus, and mobile phase composition used for the experimental part of analyses being different from these used to construct reference dataset in SSRCalc software [Spicer *et al.*, 2007]. The following equation [Darewicz *et al.*, 2014] was used for peptide retention time prediction:

$$t_{Rpredicted} = 0.0002 \times t_{R\ SSRCalc}{}^{3} - 0.0085 \times t_{R\ SSRCalc}{}^{2} + 1.0415 \times t_{R\ SSRCalc} + 8.6434$$

where: $t_{RSSRCalc}$ – retention time (min) calculated with the Sequence Specific Retention Calculator (SSRCalc, available at: http://hs2.proteome.ca/SSRCalc/SSRCalc.html, accessed: December 2018) [Spicer *et al.*, 2007].

To calculate the retention times with SSRCalc, peptides were provided in a one-letter code and implemented to the software. The following parameters were set up: a = 2.02, *i.e.* the retention time of the substance not adsorbed on the column; and b = 0.94, *i.e.* the parameter dependent on the acetonitrile gradient (0.66% per min) [Dziuba *et al.*, 2011]. The mathematical algorithm provided by the SSRCalc software was adjusted to the column, the same was done with separation parameters like pore diameter: 100 Å, column: C18, and TFA concentration: 0.1% [Krokhin *et al.*, 2004; Krokhin, 2006].

In vitro analyses

Materials and reagents

A commercial milk protein concentrate (MPC) called TMP80 (containing 80% protein in a proportion of casein to whey proteins at 8:2 (w/w)) was produced by Milei GmbH (Leutkirch, Germany) and was donated by Nordmann, Rasmann, Poland Ltd. (Warsaw, Poland). Bromelain (EC 3.4.22.32, Sigma-Aldrich No. B5144; 5–15 units/mg protein), proteinase K from *Tritiarachium album* (EC 3.4.21.64, Sigma-Aldrich No. P2308; ≥30 units/mg protein), papain (EC 3.4.22.2, Sigma-Aldrich No. P4762; 10 units/mg protein), ficin (EC 3.4.22.3, Sigma-Aldrich No. F4125; ≥1 unit/mg protein), trifluoroacetic acid (TFA), acetonitrile, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (Bis-Tris), 2-mercaptoethanol, and urea were purchased from Sigma-Aldrich® (Poznań, Poland). All chemicals were of analytical grade. Water used to formulate solutions and buffers was prepared using a Milli-Q PLUS system (Millipore Corp., New York, NY, USA).

Hydrolysis of milk proteins

MPC was dissolved in distilled water to obtain 5 separate solutions containing 3% protein (w/v) each [Cheung *et al.*, 2015] and having the non-adjusted pH of 6.9±0.1. All MPC solutions were continuously and gently stirred as well as preheated for 5 min using an Heidolph Unimax Modular Incubator 1010 (Heidolph Instruments GmbH & CO. KG, Schwabach, Germany). Pre-incubation temperatures were typical of enzyme activities specified by manufacturers, *i.e.* 50°C (bromelain; B), 37°C (proteinase K; PK), 65°C (papain; P), and 50°C (ficin; F). Afterwards, 3-h hydrolysis of four samples of MPC solutions was carried out [Cheung *et al.*, 2015; Lacroix *et al.*, 2016] under continuous stirring and the enzyme-to-substrate ratio (protein) of 1:100 (w/w) [Al-Shamsi *et al.*, 2018]. The pH values of MPC solutions taken for proteolysis were typical of enzyme activity, *i.e.* 7.0 (B, P, PK) and 6.5 (F). After the hydrolysis, the mixtures were heated at 90°C for 15 min to inactivate the enzymes and then freeze-dried [Lacroix *et al.*, 2016]. Four MPC hydrolysates (bromelain-, ficin-, papain-, and proteinase-K MPC hydrolysates, named B-MPC, F-MPC, P-MPC, and PK-MPC, respectively) as well as one non-hydrolyzed MPC (O-MPC; reference sample) were prepared in duplicate.

Separation of MPC and products of its hydrolysis by reversed-phase high performance liquid chromatography (RP-HPLC)

The reversed-phase high performance liquid chromatography (RP-HPLC) was used to separate MPC and products of its hydrolysis. The Shimadzu® system (Tokyo, Japan) was used for sample separation. It comprised of: two LC-20AD pumps, an SIL-20AC HT autosampler, a CBM-20A controller, a CTO-10AS VP thermostat, an SPD-M20A photodiode detector, and a DGU-20A5 degasser. The Jupiter Proteo Phenomenex® column (Torrance, CA, USA) with the following parameters: 250×2 mm, particle diameter – 4 μm and pore diameter – 90Å, was used. Mobile phase consisted of solvent A – 0.01% (v/v) TFA solution in water and solvent B – 0.01% (v/v) TFA dissolved in acetonitrile. The gradient of solvent B increased from 0 to 40% during 60 min. Then, the column was washed with solvent B in the mobile phase as follows: 40–100%, 60–65 min; 100%, 65–70 min, 100–0%, 70–71 min; 0%, 71–80 min. The injection volume was 30 μL, flow rate was 0.2 mL/min, and column temperature was 30°C [Bucholska & Minkiewicz, 2016]. Chromatograms were acquired at the wavelength of 220 nm [Visser *et al.,* 1991]. Data was analyzed using Lab Solution (LC Solution) software provided by Shimadzu®. RP-HPLC analyses were performed in duplicate.

The samples of MPC and its enzymatic hydrolysates were prepared as follows: 2 mg of a freeze-dried sample was dissolved in 300 μL of a buffer containing 0.1 M Bis-Tris and 4 M urea. Then, 20 μL of 2-mercaptoethanol were added and the mixture was vortexed and next incubated at a room temperature for 1 h. After the incubation, 680 μL of 6 M urea solution in a mixture of acetonitrile and water (at a ratio of 1: 9 (v/v); pH 2.2 adjusted by the addition of TFA) were added to the sample and stirred. Samples were then centrifuged (10 min, 10,000×g) (Hermle Z 233, M-2, HERMLE LaborTechnik GmbH, Wehingen, Germany) [Visser *et al.,* 1991; Dziuba *et al.,* 2011].

Identification of peptides using liquid chromatography and mass spectrometry (RP-HPLC-MS/MS)

The samples of MPC hydrolysates were prepared identically like for RP-HPLC analysis. The only difference was the weight of the freeze-dried hydrolysate taken for sample preparation (10 mg instead of 2 mg).

LC-MS/MS identification analysis was carried out using the VARIAN® 500-MS (Agilent Technologies, Santa Clara, CA, USA) ion trap mass spectrometer with an electrospray ion source and an HPLC assembly comprising two 212-LC pumps, a ProStar 410 autosampler, and a Degassit degasser (MetaChem Technologies®, Torrance, CA, USA) as well as a nitrogen generator (Parker Domnick Hunter Scientific®, Gateshead, UK). Gradient of the mobile phase, column type, and column parameters were identical as those described in the subchapter above. Data was registered between 5 and 60 min. The other parameters for mass spectrometry were as follows: needle and shield voltages: 5000 and 600 V re-

spectively; spraying and drying gas (nitrogen) pressure: 55 and 30 psi, respectively; drying gas temperature 390°C; and flow rate of damping gas (helium) 0.8 mL/min. The other parameters were as follows: positive polarity with current ionization 600 V, capillary voltage 100 V, retardation factor loading 100%, isolation window 3.0, excitation storage level m/z = 100–2000 Da, flow rate 0.2 mL/min, injection volume 15 μL, frequency data recording 0.05–0.07 Hz single scan averaged from five microscans, options such as: use of air segment, headspace pressure and alarm buzzer were included [Darewicz *et al.,* 2014; Bucholska & Minkiewicz, 2016]. For peptide retention times determination, all chromatograms were smoothed using Savitzky & Golay method [1964] implemented from MS WorkStation v. 6.9 software. All identification analyses were performed in duplicate.

Mass to charge ratios of fragment ions were theoretically calculated using the Fragment Ion Calculator available at: http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html, accessed: December 2018. After loading the peptide of interest into a window "Peptide:" the following software options were ticked: "+1", "+2", and "+3" (function called "Charge state") referring to mono-, double-, and triple-ionized ions, respectively as well as "A, B, C, X, Y, Z" [Roepstorff & Fohlman, 1984; Paizs & Suhai, 2005]. Submitted results included sequences of peptides to be potentially identified, their monoisotopic masses, and m/z of fragment ions (A, B, C, X, Y, Z).

RESULTS AND DISCUSSION

There are several aspects to consider when thinking about bitter taste of food resulting from the presence of peptides. They include, *e.g*., amino acid composition of peptides, physicochemical properties of amino acids constituting the peptide, conditions of hydrolysis of food proteins, enzymes used for proteolysis, taste-taste interactions between peptides, and methodologies applied to evaluate the taste of foods [Iwaniak *et al.*, 2016a]. It is also well-known that tastant peptides exhibit a variety of biological functions. It especially concerns short sequences (di- and tripeptides). Among them, inhibition of proteolytic enzymes is the main activity correlated with food taste [Iwaniak *et al.*, 2016a]. The most extensively studied so far has been the correlation between ACE inhibition and bitterness of peptides [Daskaya-Dikmen *et al.*, 2017]. The latest studies showed also that bitter peptides derived from a pepsin hydrolysate of peeled shrimp (*Litopenaeus vannamei*) exhibited lymphocyte and lysozyme activity contributing to the improvement of fish survival in aquaculture [Deng *et al.*, 2019].

The above-mentioned aspects related to bitterness of peptides indicate them to be the best-described group of tastant peptides. It can also be reflected by the number of bitter peptides in the BIOPEP-UWM database of sensory peptides and amino acids [Iwaniak *et al.*, 2016b]. Currently, 483 sequences of sensory peptides (including some amino acids) can be found in this database. This number includes 305 bitter peptides (excluding bitter amino acids), *i.e.* ~63% of all sensory sequences (accessed: October 2019). To recapitulate, considering the methodological aspects of analysis of peptides derived from food proteins, which is costly and time consuming, bitter peptides are in the focus of interests of scientists trying to develop new methods that may contribute to the extension of knowledge about food bitterness. These new methods involve also bioinformatic analyses. According to Gallego *et al*. [2019], the bioinformatic-assisted approach affords the opportunity to predict the production of bitter peptides from food sources in a cheaper and faster way. Our idea of employing predictive (*i.e. in silico*) methods combined with experimental analysis, which is presented in this paper, inscribes into this trend.

Initially (data not shown), 15 proteases were taken for the *in silico* hydrolysis of bitter peptides. Taking into account the number of peptides predicted to be released from the individual milk protein sequence by an enzyme, four proteases were reported as the "potentially most effective" ones when producing peptidic motifs. They were as follows: bromelain, papain, ficin, and proteinase K. According to literature data, these enzymes were used for the production of protein hydrolysates as well as peptides exhibiting the biological activity. For example, Choopinham *et al.* [2015] used papain to produce hydrolysates with ACE-inhibiting and antioxidative properties derived from gelatin extracted from tilapia skin. Arihara [2006] used papain for the hydrolysis of porcine actin, which allowed identifying a DAQEKLE peptide exhibiting the antioxidative activity. In turn, bromelain was expected to release from clam proteins peptides rich in cysteine which induces the antibacterial effect [Zambrowicz *et al.*, 2013]. Antibacterial peptides were also derived from goat casein hydrolyzed by ficin [Esmaeilpour *et al.*, 2016]. In turn, proteinase K was used for the hydrolysis of beef proteins to release a GFHI sequence with the ACE-inhibiting effect [Ryan *et al.,* 2011].

Our results of *in silico* hydrolysis of milk protein sequences revealed that many peptides exhibiting various biological and physiological activities, including the bitter-tasting activity, can be released using B, F, P, and PK. However, we focused on the results of simulated proteolysis which showed such peptide products that were composed of at minimum 4 residues and were not defined by BIOPEP-UWM database search options as "bitter/bioactive". They were defined as parent (*i.e.* precursor peptides) and were planned to be identified in the experimental samples of MPC hydrolysates. Moreover, released precursor peptides were searched for the presence of bitterness indicators as well as bitter peptides without such a status (see Methods). This strategy was consistent with Zamyatnin's [2009] fragmentomic idea (see above). Our results are shown in Table 1.

The number of potentially released fragments fulfilling the above criteria ranged from 0 (β-lactoglobulin treated with PK) to 27 (serum albumin hydrolyzed using B). Bromelain was the most effective enzyme in terms of production of parent peptides, while proteinase K was the least effective one. Comparable results were obtained for papain. The effectiveness of enzymes in releasing the highest number peptides was ranked in the following order: bromelain>papain>ficin>proteinase K. The total number of potentially released peptides from all milk proteins treated with four enzymes was 226. All peptides released from the milk proteins contained shorter fragments with a documented bitter taste.

TABLE 1. Peptide fragments released *in silico* from bovine milk proteins containing bitterness indicators (**in bold**) or peptides without the status of the indicator (normal font).

Milk protein	Bromelain	Ficin	Papain	Proteinase K
κ-Casein	VESTVA/VE IPPK/**PP, PK** VLSRY/**VL** TEIPTINTIA/**EI** DERFFSDK/**FF**, RF TLEDSPEVIESPPEINTVQVTSTA/ **PP, VI**, LE, **EI** LINNQFLPY/**FL**, LI QILQWQVLSNTVPA/**VL**, IL	IPPK/**PP, PK** IESPPEIN/**PP, EI** TEIPTIN/ **EI** DERFFSDK/**FF**,RF	IPPK/**PP, PK** VLSRY/**VL** FFSDK/**FF** LINNQFLPY/**FL,** LI QILQWQVLSNTVPA/**VL**, IL	ASGEP/GE KKNQDKTEI/**EI** RCEKDERF/RF
αs_1-Casein	VNELSK/EL VNQELA/EL VPLG/**LG** EPMIG/**IG** ESISSSEEIVPNSVEQK/VE, IV**, EI** PSFSDIPNPIG/**IG** VPQLEIVPNSA/IV, LE, **EI** LEQLLRLK/LE, LL TTMPLW/PL, LW PFPEVFG/FG, FP, VF, PF, PFP PELFRQFY/LF, EL LPQEVLNENLLRFFVA/**FF**, RF, FV, **VL**, LL	EPMIG/**IG** TTMPL/L ESISSSEEIV/IV, **EI** PFPEV/FP, PF, PFP RFFV/**FF**, RF, FV	VFG/FG, **VF** VLNE/**VL** IVPNSA/IV VPQLE/LE IVPNSVE/VE, IV PFPE/**FP,** PF, PFP NLLR/LL PMIG/**IG** PSFSDIPNPIG/**IG** FFVA/**FF**, FV TTMPLW/PL, LW	TDAP/DA KHQGL/GL KEGI/**GI**, EG SSSEEI/**EI** NQEL/EL
αs_2-Casein	NEEEY/EYFP ITVDDK/**VD** FPQY/**FP** VIPY/**VI** PWIQPK/**PK** VPITPTLNREQLSTSEENSK/EEN TVDMESTEVFTK/**VF, VD** NRLNFLK/**FL** NTMEHVSSSEESIISQETY/II LNEINQFY/**EI** PIVLNPWDQVK/**VL, IV**	STSEEN/EEN EEEY/EY FPQY/**FP** PWIQPK /**PK** SSSEESIISQETY/II	ITVDDK/**VD** TVDME/**VD** VFTK/**VF** SIISQE/II FPQY/**FP** VIPY/**VI** PWIQPK/**PK** LNFLK/**FL** PIVLNPWDQVK/**VL, IV**	TEEEKNRL/EE, EE STSEENSKKTV/EEN RNANEEEY/EY
β-Casein	PVRG/RGFP PFPG/**FP**, PF, PFP VLPVPQK/**VL** QEPVLG/**VL**, LG IHPFA/PF EMPFPK/**PK, FP**, FPK, PF, PFP RELEELNVPG/LE, EL, EL EIVESLSSSEESITRINK/VE, **IV, EI** FLLY/**FL**, LL PFPIIV/PFPIIV, **FP,** II PIPNSLPQNIPPLTQTPVVVPPFLQPEVMG/ **PP, PP, FL**, PF, PPF, VVV, **PIP**, VPPFL PVEPFTESQSLTLTDVENLHLPLPLLQSWM HQPHQPLPPTVMFPPQSVLSLSQSK/ **PP, PP, FP**, FPP, PF, **VL**, VE, VE, LL	PPTV/**PP** PIPN/**PIP** PFPG/**FP**, PF, PFP IPPL/**PP** EMPFPK/**PK, FP**, FPK, PF, PFP EPFTESQSL/PF MFPPQSV/**PP, FP**, FPP PPFL/**PP, FL**, PF, PPF PFPIIV/PFPIIV, **FP,** II	PFTE/PF PVLG/**VL, LG** PFPG/**FP**, PF VLPVPQK/**VL** QTQSLVY/VY, LV MPFPK/**PK, FP**, FPK, PF, PFP SQSLTLTDVE/VE FLLY/**FL**, LL PFPIIV/PFPIIV, **FP,** II QPLPPTVMFPPQS VLSLSQSK/**PP, PP, FP**, FPP, **VL** PIPNSLPQNIPPLTQTPVVVPPFLQPE/ **PP, PP**, FL, PF, PPF, VVV, **PIP**, VPPFL LPLPLLQSWMH/LL	–

TABLE 1. Continued...

Milk protein	Bromelain	Ficin	Papain	Proteinase K
β-Lactoglobulin	PTPEG/EGFP VEELK/VE, EL LDIQK/LD EPEQSLA/SLA PLRVY/VY CQCLVRTPEVDDEA/**VD**, LV LIVTQTMK/LI, **IV** SDISLLDA/LL, LD, DA VLVLDTDY/**VL, VL**, LD, DY LLFCMENSA/LL, LF DLEILLQK/LL, LE, IL, DL, **EI** LPMHIRLSFNPTQLEEQCHI/LE	PTPEG/EG	LDIQK/LD ILLQK/IL, LL LIVTQTMK/LI, **IV** LSFNPTQLE/LE SDISLLDA/LL, LD, DA VLVLDTDY/**VL, VL**, LD, LV LLFCME/LL, LF	DAQSAP/DA
α-Lactalbumin	ILDK/LD, ILFP IVQNNDSTEY/**IV, EY** CEVFRELK/**VF**, EL ILFHA/LF, IL LDQWLCEK/LD FLDDDLTDDIMCVK/**FL**, LD, DL MMSFVSLLLVG/FV, LL, LL, LLL, LV	MMSFV/FV DSTEY/**EY**	IVQNNDSTE/**IV** ILDK/LD, IL LFQINNK/LF ILFH/LF, IL LDQWLCE/LD FLDDDLTDDIMCVK/**FL**, LD, DL MMSFVSLLLVG/FV, LL, LL, LLL, LV	HTSGY/GY QNNDSTEY/**EY** SCDKF/KF
Serum albumin	HRFK/RF FP SRRHPEY/RR, **EY** RRHPY/RR DELCK/EL DDSPDLPK/**PK**, DL VFQECCQA/**VF** STVFDK/**VF** QEPERNECFLSHK/**FL** CLLPK/**PK**, LL LVTDLTK/LV, DL EFVEVTK/FV, VE, EF LQQCPFDEHVK/PF DLLECA/**DLL**, LE, LL, DL CCTESLVNRRPCFSA/RR, RP, LV LIVRY/IV, LI HLVDEPQNLIK/**VD**, LV, LI PDPNTLCDEFK/EF SLHTLFG/FG, LF LVLIA/**VL**, LV, LI TVMENFVA/FV PELLY/**ELL,** LL, EL LFTFHA/LF LVELLK/VE, **ELL**, LL, LV, EL IPENLPPLTA/**PP** VPQVSTPTLVEVSRSLG/VE, **LG**, LV VSVLLRLA/**VL**, LL LVNELTEFA/LV, EL, EF	SRRHPEY/RR, **EY** RRHPY/RR CDEFK/EF DDSPDL/DL RRPCFSA/RR, RP QQCPFDEHV/PF ECFL/**FL**	FVDK/FV, VD SLVNR/LV DDSPDLPK/**PK**, DL PQNLIK/LI STVFDK/VF LVVSTQTA/LV LIVR/**IV**, LI NFVA/FV CLLPK/**PK**, LL LVTDLTK/LV, DL CFLSH/**FL** DLLE/**DLL**, LE, LL, DL LCVLH/**VL** PLLE/LL, LE LQQCPFDE/PF NLPPLTA/**PP** VSVLLR/**VL**, LL VPQVSTPTLVE/VE, LV LSLILNR/LI, IL LVLIA/**VL**, LV, LI SFLY/**FL** LFTFH/LF	GTRCCTKP/KP ARRHP/RR KECCDKP/KP KHKP/KP NRRP/RR, RP AKEY/**EY** ADCCEKQEP/AD DTHKSEI/**EI** KADEKKF/KF, AD, ADE HKECCHGDL/DL ADESHAGCEKSL/AD, ADE GERAL/GE GEEHF/GE QEAKDAF/AF, DA ECADDRADL/AD, AD, DL CDEF/EF

Some of them, mainly dipeptides, had the status of bitterness indicators. Tripeptide bitterness indicators were present occasionally in the parent peptides. They were typical of precursor peptides composed of at minimum 5 amino acids (*e.g.* DLLECA containing **DLL** indicator and PELLY with **ELL**; sources: B hydrolysate of serum albumin). One of the exceptions was precursor PIPN with **PIP** indicator in its structure (F hydrolysate of β-casein). According to Iwaniak & Dziuba [2011], the shorter the peptide chain, the higher the probability of finding it in their protein precursor. This rule can also be referred to our study concerning parent peptides with the potential to be bitter. Another observation made by Iwaniak & Dziuba [2009] regarding the impact of the length of a parent sequence on the higher probability of finding the functional fragment in it (briefly, the longer the precursor, the higher the probability of detecting the shorter motif in it) was not applicable to our study. Although some theoretically released peptides were composed of over a dozen of amino acids, the bitterness indicators detected in them were not as numerous as expected. Thus, the potential of parent peptide to be the source of bitterness indicators was evaluated considering the frequency of occurrence of a bitterness indicator in a precursor (data not shown). It was defined as A and was used for, *e.g.*, *in silico* evaluation of animal and plant proteins as the precursors of bioactive peptides [Iwaniak & Dziuba, 2009]. The A is calculated automatically by BIOPEP-UWM using the algorithm A=a/N, where: a is the number of peptides with a given activity, and N is the number of amino acids in a parent protein (in our case – parent peptide). The higher the A is, the better source of bitterness indicators the parent peptide is. According to A, the best potential sources of peptide indicators were, *e.g.*, IPPK (source: B, F, and P hydrolysates of κ-casein containing **PP** and **PK**), PPFL with **PP**, **FL** indicators (from F hydrolysate of β-casein) or VLSLSQSK with indicators: **PP**, **PP**, **FP**, and **VL** (from P hydrolysate of β-casein). For parent peptides, *i.e.* IPPK, PPFL, and VLSLSQSK, found in the *in silico* hydrolysates of κ- and β-caseins (first and other two sequences, respectively) A was 0.5. For example, A calculated for 30-mer parent peptide PIPNSLPQNIPPLTQTPVVVPPFLQPEVMG (from B hydrolysate of β-casein) containing **PP**, **PP**, **FL**, and **PIP** indicators was 0.133. To summarize, all bovine milk proteins had the potential to release peptides containing bitter peptides, including bitterness indicators. Among them, β-casein seemed to be the richest source of parent peptides likely to be bitter due to the presence of bitter-tasting peptides with and without the status of an indicator. Bumberger & Belitz [1993] isolated and then hydrolyzed bovine β-casein using trypsin and found regions of this sequence (fragments: 49–97, 203–209) as the sources of shorter fragments with confirmed bitterness (*e.g.* segment 49–68). According to BIOPEP-UWM computations (data not shown), the majority of potentially released parent peptides were also present in the above-mentioned regions of β-casein.

Results concerning the *in silico* prediction of "bitterness potential" of parent peptides released due to the action of proteases were the premise to verify them in laboratory conditions. Then, MPC protein solutions (3%; w/v) were hydrolyzed using the above-mentioned enzymes (see Methods). Such concentrations of milk protein solutions, enzyme-to-substrate ratio, and proteolysis time were used by Cheung *et al.* [2015] for the analysis of milk whey protein hydrolysates as sources of ACE (*i.e.* angiotensin converting enzyme; EC 3.4.15.1) inhibitors as well as for the sensory evaluation of the obtained hydrolysates after additional exopeptidases treatment. Lacroix *et al.* [2016] successfully applied such parameters for the hydrolysis of commercial milk whey protein isolate to analyze the hydrolysates as sources of ACE and DPP IV (dipeptidyl peptidase IV; EC 3.4.14.5) inhibitors. Our O-MPC solution as well as respective 4 hydrolysates were analyzed using RP-HPLC to separate MPC and products of its hydrolysis. The results are shown in Figure 1 and Table 2. When looking at the chromatograms of O-MPC and its hydrolysates (Figure 1a and 1b-e, respectively), two retention time intervals referring to the process of molecules separation can be distinguished, *i.e.*: 14.00–39.99 min and 40.00–60.00 min. The first, biggest peak that was eluted at about 10 min, is the so-called "injection peak" representing

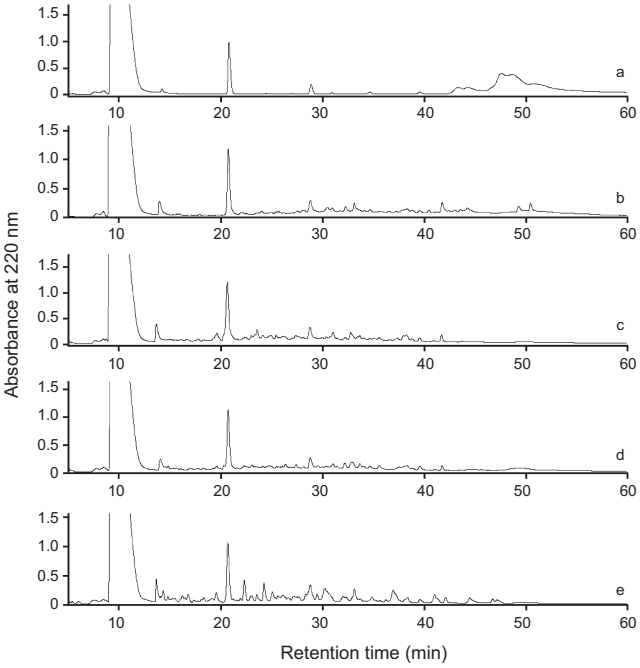


FIGURE 1. RP-HPLC chromatograms of milk protein concentrate (MPC) and its hydrolysates: a) non-hydrolyzed MPC; b) bromelain hydrolysate of MPC; c) ficin hydrolysate of MPC; d) papain hydrolysate of MPC; e) proteinase K hydrolysate of MPC.

TABLE 2. Relative peak areas (%)[1] of RP-HPLC separations of milk protein concentrate and its enzymatic hydrolysates.

Time interval (min)	O-MPC	B-MPC	F-MPC	P-MPC	PK-MPC
14.00–39.99	1.2	18.0	21.4	16.1	22.8
40.00–60.00	20.5	10.7	1.1	1.9	1.1

[1]Area of all peaks between 0 and 60 min is 100%.
O-MPC – non-hydrolyzed milk protein concentrate; B-MPC, F-MPC, P-MPC, PK-MPC – milk protein concentrate hydrolyzed by bromelain, ficin, papain and proteinase K, respectively.

non-retained substances like, *e.g.*, components of buffers used to dissolve proteins or peptides [Bucholska & Minkiewicz, 2016]. Such compounds could also be present in the peak eluted between 20.00 and 20.99 min. Thus, peaks eluted between 00.00–14.00 and 20.00–20.99 min were not subjected to further interpretation. The highest number of peaks was observed in the time interval between 14.00 and 39.99 min and they were characteristic for all hydrolysates. Some peaks eluted between 40.00 and 60.00 min appeared only in O-MPC chromatogram (Figure 1a) and can be considered as typical of the high molecular mass molecules, like proteins. They disappeared in the chromatograms of hydrolysates. Changes in peaks distribution during the RP-HPLC separation were also confirmed by the relative peak areas in the above-specified time intervals (Table 2). The changes in the distribution of peak areas confirmed that MPC was hydrolyzed by 4 enzymes. The content of compounds with long retention times (above 40.00 min) in a bromelain hydrolysate was higher than in the other hydrolysates. Long retention times may be tentatively attributed to intact proteins and/or polypeptides being products of proteolysis. If there is no significant difference in hydrophobicity, peptides with longer chains have usually longer retention times than these with shorter chains [Krokhin *et al.,* 2004; Krokhin, 2006; Spicer *et al.*, 2007; Dziuba *et al.*, 2011]. In the light of results presented in Figure 1 and Table 2, bromelain seems to be less efficient in the hydrolysis of proteins form milk powder than other enzymes used in this experiment.

Identification of parent peptides was based on the analysis of their fragment ions, according to the nomenclature introduced by Roepstorff & Fohlman [1984]. Peptides were shown as the groups of ions detected at the same retention time. Fragmentation of peptides may occur due to, *e.g.*, the non-sequential charge-directed pathway. It is related to the formation of fragment ions involving neutral loss of neutral molecules like water or ammonia [Paizs & Suhai, 2005]. The following requirements had to be fulfilled to enable identification of a specific peptide in a hydrolysate sample: the presence of fragment ions detected in a specific retention time, and the difference between predicted ($t_{R\ predicted}$) and experimental ($t_{R\ experimental}$) retention times of *ca*. ±10% [Bucholska & Minkiewicz, 2016].

An example of a chromatogram with the identified peptide is shown in Figure 2. It concerns the parent sequence KEGI identified in α_{s1}-casein. KEGI was identified in PK hydrolysate of MPC. The *m/z* of precursor ion $[M+H]^+$ was 446.26. Eight fragment ions: Y_3^+, Z_4^+, X_1^+, C_1^+, B_4^+, B_3^+, A_4^+, and A_3^+, were eluted between 13.27 and 14.00 min of the separation process. Based on the presence of the group of fragment ions eluted at the same time interval as well as their intensity expressed in thousands of counts (kCounts), KEGI was classified to the group of identified parent peptides. The differences between $t_{R\ predicted}$ (14.15 min) and $t_{R\ experimental}$ (13.64 min – average value from these presented above) was 5.6%.

Results concerning the identification of other peptides in MPC hydrolysates are summarized in Table 3. Twenty eight peptides were identified in MPC *in vitro* hydrolysates, which accounted for 12.38% of the total number of parent sequences "identified" *in silico* (226; see Table 1). Ficin and papain were

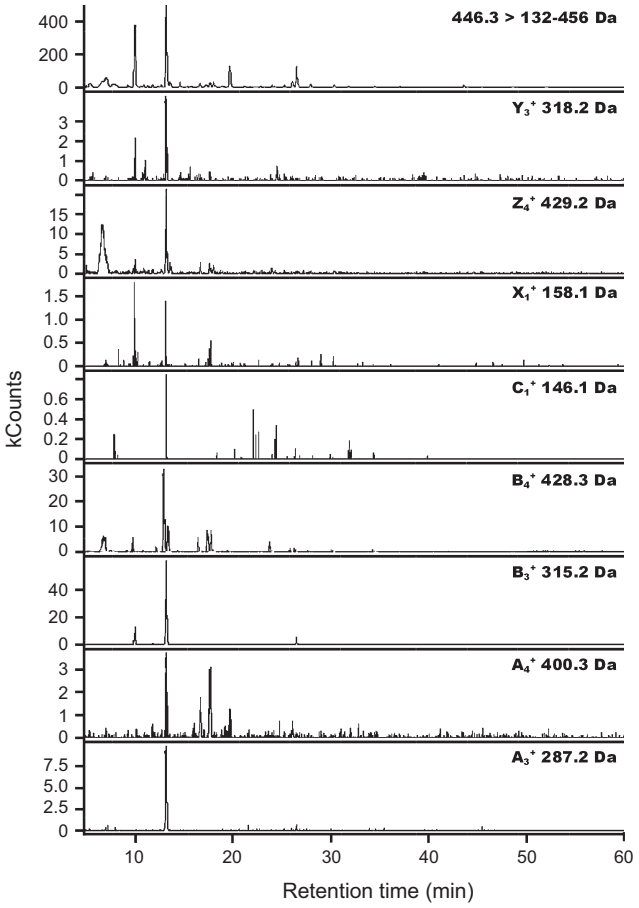


FIGURE 2. Exemplary LC-MS/MS chromatogram of peptide KEGI (from proteinase K hydrolysate of milk protein concentrate) containing: *m/z* of precursor ion $[M+H]^+$ (the top of the chromatogram), *m/z* range of investigated fragment ions (the top of the chromatogram), and types of fragment ions including their *m/z* (chromatograms 2–9).

the most effective enzymes *in vitro* – they both released 9 parent peptides. Bromelain and proteinase K released 5 such peptides both. Thus, enzyme effectiveness in releasing the highest number of peptides could be ranked as follows: ficin = papain > bromelain > proteinase K, and it differed from the ranking based on *in silico* predictions (bromelain > papain > ficin > proteinase K). The fact that hydrolysis of milk proteins by papain and ficin hydrolysis led to obtaining more identified peptides of interest than the hydrolysis by proteinase K is consistent with *in silico* predictions. The discrepancy between the predicted and the determined effectiveness of bromelain in producing oligopeptides may be explained taking into account incomplete hydrolysis of proteins by this enzyme (Figure 1 and Table 2). Although, both rankings of enzymes were different, β-casein was the protein in which the highest number of peptides was detected during *in silico* experiment. The highest number of peptides referring to those present in β-casein (8) were also identified in MPC hydrolysates. All *in vitro* identified peptides contained shorter bitter fragments, including bitterness indicators. For example, PFPIIV peptide was known as bitter itself, and contained the following fragments, including two indicators (in bold): PFP, PF, IV, **FP, II**, PFPIIV. This peptide was identified in B, F, and P hydrolysates of MPC. Two identified parent peptides, *i.e.* TTMPLW (P-hy-

TABLE 3. Peptides identified in hydrolysates of milk protein concentrate (MPC) using LC-MS/MS.

MPC hydrolysate	Identified peptide	$[M+H]^+$ (*m/z*)	$t_{R\ predicted}$ (min)	$t_{R\ experimental}$ (min)	Bitter peptide	Protein source
B-MPC	VLPVPQK (antioxidative)	780.50	24.6	26.80 -27.81	**VL**	β-casein
	EIVESLSSSEESITRINK	2021.04	35.43	38.99–41.00	VE, **IV, EI**	
	FLLY	555.32	39.05	34.80 – 35.70	**FL**, LL	
	PFPIIV	685.43	40.60	40.28 – 40.90	PFP,PF, IV, **FP, II**, PFPIIV	
	SDISLLDA	833.43	34.71	38.10 -39.88	LL, LD, DA	β-lactoglobulin
F-MPC	TTMPL	562.29	27.11	28.67 -28.10	**FP,** PF, PFP	α_{s1}-casein
	ESISSSEEIV	1079.51	28.15	30.20 – 31.46	PL, LW	
	FPQY	554.26	25.62	24.98 – 25.20	FP	α_{s2}- casein
	PPFL	473.28	32.99	34.57 – 35.20	**PP, FL**, PF, PPF	β-casein
	PFPIIV	685.43	40.60	40.28 – 40.90	PFP,PF, IV, **FP, II**, PFPIIV	
	IESPPEIN	898.45	26.45	26.04 – 26.10	**PP, EI**	κ-casein
	DERFFSDK	1043.48	27.77	23.53 – 25.30	**FF**, RF	
	MMSFV	614.27	34.00	33.83 -34.10	FV	α-lactalbumin
	PTPEG	500.24	11.70	12.87 -13.01	EG	β-lactoglobulin
P-MPC	TTMPLW (opioid, immunomodulating, ACE inhibitor)	748.37	38.39	39.83 -40.10	PL, LW	α_{s1}-casein
	MPFPK	619.33	27.49	30.23 -33.11	**PK, FP**, FPK, PF, PFP	β-casein
	PFPIIV	685.43	40.60	40.28 – 40.70	PFP,PF, IV, **FP, II,** PFPIIV	
	VLSR	474.30	17.00	15.50–16.10	**VL**	κ-casein
	SDISLLDA	833.43	34.71	38.10 -39.88	LL, LD, DA	β-lactoglobulin
	LLFCME	755.35	39.05	40.01 – 40.60	LL, LF	
	NLPPLTA	725.42	29.09	32.28- 32.66	**PP**	serum albumin
	SFLY	529.27	34.00	34.62–34.80	**FL**	
	LCVLH	584.32	27.39	24.69 -25.20	**VL**	
PK-MPC	KHQGL	582.34	11.80	9.62 – 10.80	GL	α_{s1}-casein
	KEGI	446.26	14.15	13.27 – 14.00	**GI**, EG	
	KKNQDKTEI	1103.61	14.34	15.77 -16.24	**EI**	κ-casein
	DAQSAP	588.26	9.78	9.96 – 10.20	DA	β-lactoglobulin

B-MPC – milk protein concentrate hydrolyzed by bromelain, F-MPC – milk protein concentrate hydrolyzed by ficin, P-MPC – milk protein concentrate hydrolyzed by papain, PK-MPC – milk protein concentrate hydrolyzed by proteinase K; additional biological activity of peptide (if any) was given in brackets; peptide bitterness indicators (**bold**) and bitter peptides without the status of the indicator (normal font).

drolysate of MPC) and VLPVPQK (B-hydrolysate of MPC), served additional biofunctions. According to data found on these sequences in the BIOPEP-UWM database, the first acted as an opioid (ID 3127) as well as an immunomodulating (ID 8172) and ACE-inhibiting (ID 3530) agent, while the second one exhibited antioxidative activity (ID 7877). More detailed information about additional bioactivities of these peptides can be found in the BIOPEP-UWM database under the accession numbers provided in the brackets. The fact that peptides with health-beneficial effects may have an undesired taste may be considered important by food manufacturers and scientists when producing foods attractive for the consumers [Iwaniak *et al.*, 2018]. Additionally, the process of food production like, *e.g.*, production of milk protein hydrolysates, may require additional technological procedures aiming to reduce/debitter the unwanted taste [Lafarga & Hayes, 2017]. Our idea of research based on the fragmentomic approach enables predicting whether, due to the presence of specific bitter-tasting fragments, the released biopeptides may have the potential to taste bitter.

The number of parent peptides successfully identified in protein sequences during *in silico* and in *vitro* experiment varied. The discrepancies between predictions and actual results are the common fact in the literature [Mallick *et al*., 2007]. Firstly, the experiment carried out in laboratory conditions has its own specificity. It takes *e.g.* time for reagent and sample preparation, time involved in experiment, and time for results' analysis. *In silico* hydrolysis is relatively easy and cost-effective to perform but peptides produced with this method match those already present in the database. Moreover, the *in silico* prediction may differ when made during different time intervals [Udenigwe, 2014]. The BIOPEP-UWM is a curated database, *i.e.* systematically updated by the experienced staff in the field of bioactive peptides. No new bitter peptide sequences have been found in the literature and uploaded to the BIOPEP-UWM database between running the experiment and submission of this article. Thus, "not-updating" the database, could have not been the reason of *in silico* and *in vitro* discrepancies in peptides identification. Nevertheless, the necessity of the regular update of databases is a very important aspect that should be considered when comparing the results of *in silico* and *in vitro* analyses [Udenigwe, 2014].

According to Bucholska & Minkiewicz [2016], the following factors may be found responsible for the unsuccessful identification of peptides: the absence of a detectable amount of peptide in the hydrolysate (*e.g.* if some peptide bonds predicted to be cleaved are actually resistant to proteolysis), and the absence of a detectable fragmentation in an ion trap mass spectrometer. Moreover, possible peptides to be identified are defined as proteotypic peptides and may vary depending on the mass spectrometer used (*e.g.* matrix-assisted laser desorption ionization – MALDI or electrospray) [Bucholska & Minkiewicz, 2016]. To recapitulate, there is no method that would enable identifying all products of protein hydrolysis [Bucholska & Minkiewicz, 2016].

The differences between *in silico* and *in vitro* results may also stem from the issue referring to the hydrolysis of protein. According to Panjaitan *et al.* [2018], results of theoretical and practical analyses of proteases may not always be comparable. Programs for *in silico* hydrolysis are based on the specificity of enzymes. Moreover, theoretical hydrolysis assumes that peptide bonds of a substrate are 100% cleavable by the enzyme [Panjaitan *et al.*, 2018]. Complete enzyme characteristics is more vast than the information about the peptide bonds cleaved by an appropriate protease [Vermeirssen *et al.*, 2004]. Moreover, during the *in silico* hydrolysis, the peptide bond is cleaved by the enzyme relatively easily, especially when some amino acids in a substrate (protein) are not modified, *e.g.* by glycosylation. Under experimental conditions, glycosylated residues may impede the cleavage of the peptide bond [Khaldi, 2012]. Additionally, the complexity of the protein structure might hinder protease–protein interactions, which may also affect the divergence in the predicted and experimentally obtained results of proteolysis aimed at producing peptides [Panjaitan *et al.*, 2018]. Discrepancies concerning the release of peptides from proteins were also observed in experiments carried out *in vitro* and *in vivo/ex vivo*. For example, Darewicz *et al.* [2014] analyzed ACE-inhibiting peptides from salmon (*Salmo salar*) proteins hydrolyzed *in vitro* (with commercial enzymes) and *ex vivo* (using digestive juices from volunteers) and compared the results obtained using *in silico* analyses. They found that some *in silico* identified ACE inhibitors were common for both hydrolysates, however there were peptides identified either in the *ex vivo* or in the *in vitro* hydrolysate. According to Rawlings [2009], the likely reasons behind the differences between the results obtained in different experimental conditions include: involvement of inhibitors, and susceptibility of substrate peptide bonds to enzyme resulting from the protein-protein interactions.

To summarize, it is well-known that taste evaluation is more comprehensive than bioinformatic-assisted studies, several aspects need to be considered to get the reliable results when evaluating taste. The reliability of taste evaluation requires, *e.g.* panelist fatigue [Li-Chan, 2015], usage of an appropriate scale for sample evaluation (*e.g.* point or hedonic) [Lim, 2011], and/or limitations resulting from the usage of "machines" (*e.g.* e-tongue) [Ciosek & Wróblewski, 2011]. Thus, *in silico* studies combined with empirical methodologies enable the complete search for tasting-peptides derived from foods. However, all theoretical predictions of food taste must be confirmed [Gallego *et al.,* 2019].

CONCLUSIONS

The hybrid analysis of bovine milk protein hydrolysates confirmed that all released parent peptides contained shorter fragments with bitter taste and that some of them had the status of bitterness indicators. The presence of bitter regions in a parent peptide may determine the taste of the whole sequence. β-Casein turned out to be the richest source of peptides with potential to be bitter, as confirmed both *in silico* and *in vitro*. However, the results of *in silico* and *in vitro* hydrolysis concerning the number of released peptides as well as the effectiveness of enzyme applied differed. Twenty eight peptides with potential bitterness were identified in MPC *in vitro* hydrolysates while *in silico* hydrolysis enabled releasing 226 such sequences. The most effective enzymes in releasing peptides *in silico* were: bromelain > papain > ficin > proteinase K, whereas in the experimental conditions these were: ficin = papain > bromelain > proteinase K.

Summing up, the fragmentomic idea of research has so far been successfully employed in other scientific disciplines. We found it useful in food science when coupled with the hybrid approach. Despite the limitations of *in silico* analyses that do not fully reflect the *in vitro* results, the knowledge about the bitterness of peptide indicators, selection of enzymes for protein hydrolysis, and predictions of possible peptide products that may affect the bitter taste of a hydrolysate, contribute to providing more insights on the taste of foods, especially if unwanted. However, before introducing a food product to the market, sensory analyses are required to confirm (or not) its bitter taste.

RESEARCH FUNDING

Project financially supported by Minister of Science and Higher Education in the range of the program entitled "Regional Initiative of Excellence" for the years 2019–2022, Proj-

ect No. 010/RID/2018/19, amount of funding 12.000.000 PLN as well as the funds of the University of Warmia and Mazury in Olsztyn (Project No. 17.610.014–300).

CONFLICT OF INTEREST

The authors do not declare the conflict of interest.

REFERENCES

1. Al-Shamsi, K.A., Mudgil, P., Hassan, H.M., Maqsood, S. (2018). Camel milk protein hydrolysates with improved technofunctional properties and enhanced antioxidant potential in *in vitro* and in food model systems. *Journal of Dairy Science*, *101*(1), 47–60.
2. Arihara, K. (2006). Strategies for designing novel functional meat products. *Meat Science, 74*(1), 219–229.
3. Bucholska, J., Minkiewicz, P. (2016). The use of peptide markers of carp and herring allergens as an example of detection of sequenced and non-sequenced proteins. *Food Technology and Biotechnology, 54*(3), 266–274.
4. Bucholska, J., Minkiewicz, P., Darewicz, M., Iwaniak, A. (2018). Databases and associated bioinformatic tools in studies of food allergens, epitopes and haptens – a review. *Polish Journal of Food and Nutrition Sciences, 68*(2), 103–113.
5. Bumberger, E., Belitz, H.D. (1993). Bitter taste of enzymic hydrolysates of casein. I. Isolation, structural and sensorial analysis of peptides from tryptic hydrolysates of β-casein. *Zeitschrift für Lebensmittel-Untersuchung und Forschung, 197*(1), 14–19.
6. Cheung, L.K.Y., Aluko, R.E., Cliff, M.A., Li-Chan, E.C.Y. (2015). Effects of exopeptidase treatment on antihypertensive activity and taste attributes of enzymatic whey protein hydrolysates. *Journal of Functional Foods, 13*, 262–275.
7. Choopinham, S., Jaturasitha, S., Rakariyatham, N., Suree, N., Hataichanoke, N. (2015). Antioxidant and antihypertensive activity of gelatin hydrolysate from Nile tilapia skin. *Journal of Food Science and Technology, 52*(5), 3134–3139.
8. Ciosek, P., Wróblewski, R. (2011). Potentiometric electronic tongues for foodstuff and biosample recognition – an overview. *Sensors, 11*(5), 4688–4701.
9. Daskaya-Dikmen, C., Yucetepe, A., Karbancioglu-Guler, F., Daskaya, H., Ozcelik, B. (2017). Angiotensin-I-converting enzyme (ACE)-inhibitory peptides from plants. *Nutrients*, *9*(4), art. no. 316.
10. Darewicz, M., Borawska, J., Vegarud, G.E., Minkiewicz, P., Iwaniak, A. (2014). Angiotensin I-converting enzyme (ACE) inhibitory activity and ACE inhibitory peptides of salmon (*Salmo salar*) protein hydrolysates obtained by human and porcine gastrointestinal enzymes. *International Journal of Molecular Sciences, 15*(8), 14077–14101.
11. Deng, S., Lutema, P.C., Gwekwe, B., Li, Y., Akida, J.S, Pang, Z., Huang, Y., Dang, Y., Wang, S., Chen, M., Miao, W., Lin, H., Wang, L., Cheng, L. (2019). Bitter peptides increase engulf of phagocytes *in vitro* and inhibit oxidation of myofibrillar protein in peeled shrimp (*Litopenaeus vannamei*) during chilled storage. *Aquaculture Reports, 15,* art. no. e100234.
12. Ding, Y., Li, X., Kan, J. (2017). Isolation and identification of flavor peptides identified in douchi (traditional Chinese soybean food). *International Journal of Food Properties, 20*(suppl. 2), 1982–1994.
13. Dziuba, J., Minkiewicz, P., Mogut, D. (2011). Determination of theoretical retention times for peptides analyzed by reversed-phase high-performance liquid chromatography. *Acta Scientiarium Polonorum Technologia Alimentaria, 10*(2), 209–221.
14. Esmaeilpour, M., Ehsani, M.R., Aminlari, M., Shekarforoush, S., Hoseini, E. (2016). Antimicrobial activity of peptides derived from enzymatic hydrolysis of goat milk caseins. *Comparative Clinical Pathology, 25*(3), 599–605.
15. Gallego, M., Mora, L., Toldrá, F. (2019). The relevance of dipeptides and tripeptides in the bioactivity of dry-cured ham. *Food Production, Processing, and Nutrition*, *1*, art. no. 2.
16. Guetouache, M., Bettache, G., Medjekal, S. (2014). Composition and nutritional value of raw milk. *Issues in Biological Sciences and Pharmaceutical Research, 2*(10), 115–122.
17. Iwaniak, A., Dziuba, J. (2009). Animal and plant origin proteins as the precursors of peptides with ACE inhibitory activity. Proteins evaluation by means of *in silico* methods. *Food Technology and Biotechnology, 47*(4), 441–449.
18. Iwaniak, A., Dziuba, J. (2011). BIOPEP-PBIL tool for the analysis of the structure of biologically active motifs derived from food proteins. *Food Technology and Biotechnology, 49*(1), 118–127.
19. Iwaniak, A., Minkiewicz, P., Darewicz, M., Protasiewicz, M., Mogut, D. (2015). Chemometrics and cheminformatics in the analysis of biologically active peptides from food sources. *Journal of Functional Foods, 16*, 334–351.
20. Iwaniak, A., Minkiewicz, P., Darewicz, M., Hrynkiewicz, M. (2016a). Food protein-originating peptides as tastants – Physiological, technological, sensory, and bioinformatic approaches. *Food Research International, 89*(1), 27–38.
21. Iwaniak, A., Minkiewicz, P., Darewicz, M., Sieniawski, K., Starowicz, P. (2016b). BIOPEP database of sensory peptides and amino acids. *Food Research International, 85,* 155–161.
22. Iwaniak, A., Hrynkiewicz, M., Bucholska, J., Darewicz, M., Minkiewicz, P. (2018). Structural characteristics of food protein-originating di- and tripeptides using principal component analysis. *European Food Research and Technology, 244*(10), 1751–1758.
23. Iwaniak, A., Minkiewicz, P., Mogut, D., Darewicz, M. (2019a). Elucidation of the role of *in silico* methodologies in approaches to studying bioactive peptides derived from foods. *Journal of Functional Foods, 61*, art. no. 103486.
24. Iwaniak, A., Hrynkiewicz, M., Bucholska, J., Minkiewicz, P., Darewicz, M. (2019b). Understanding the nature of bitter-taste di- and tripeptides derived from food proteins based on chemometric analysis. *Journal of Food Biochemistry, 43*, art. no. e12500.
25. Khaldi, N. (2012). Bioinformatic approaches for identifying new therapeutic bioactive peptides in food. *Functional Foods in Health and Disease, 2*(10), 325–338.
26. Kilara, A., Panyam, D. (2003). Peptides from milk proteins and their properties. *Critical Reviews in Food Science and Nutrition, 43*(6), 607–633.
27. Kim, H., Li-Chan, E.C.Y. (2006). Application of Fourier transform Raman spectroscopy for prediction of bitterness of peptides. *Applied Spectroscopy, 60*(11), 1297–1306.
28. Krokhin, O.V. (2006). Sequence Specific Retention Calculator – a novel algorithm for peptide retention prediction in ion-pair RP-HPLC: application to 300 Å and 100 Å pore size C18 sorbents. *Analytical Chemistry, 78*(22), 7785–7795.
29. Krokhin, O.V., Craig, R., Spicer, V., Ens, W., Standing, K.G., Beavis, R.C., Wilkins, J.A. (2004). An improved model for prediction

of retention times of tryptic peptides in ion pair reversed-phase HPLC. *Molecular & Cellular Proteomics, 3*(9), 908–919.
30. Lacroix, I.M.E., Meng, G., Cheung, I.W.Y., Li-Chan, E.C.Y. (2016). Do whey protein-derived peptides have dual dipeptidyl--peptidase IV and angiotensin I-converting enzyme inhibitory activities? *Journal of Functional Foods, 21*, 87–96.
31. Lafarga, T., Hayes, M. (2017). Bioactive protein hydrolysates in the functional food ingredient industry: overcoming current challenges. *Food Reviews International, 33*(3), 217–246.
32. Li, S.S., Bu, T.T., Zheng, J.X., Liu, L., He, G.Q., Wu, J.P. (2019). Preparation, bioavailability, and mechanism of emerging activities of Ile-Pro-Pro and Val-Pro-Pro. *Comprehensive Reviews in Food Science and Food Safety, 18*(4), 10–97–1110.
33. Li-Chan, E.C.Y. (2015). Bioactive peptides and protein hydrolysates: research and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science, 1*, 28–37.
34. Lim, J. (2011). Hedonic scaling: A review of methods and theory. *Food Quality and Preference, 22*(8), 733–747.
35. Mallick, P., Schirle, M., Chen, S.S., Flory, M.R., Lee, H., Martin, D., Ranisz, J., Raught, B., Schmitt, R., Werner, P., Kuster, B., Aebersold, R. (2007). Computational prediction of proteotypic peptides for quantitative proteomics. *Nature Biotechnology, 25*(1), 125–131.
36. Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., Darewicz, M. (2008). BIOPEP and other programs for processing bioactive peptide sequences. *Journal AOAC International, 91(4)*, 965–980.
37. Minkiewicz, P., Miciński, J., Darewicz, M., Bucholska, J. (2013). Biological and chemical databases for research into the composition of animal source foods. *Food Reviews International, 29*(4), 321–351.
38. Mooney, C., Haslam, N.J., Pollastri, G., Shields, D.C. (2012). Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PLoS ONE, 7*(10), art. no. e45012.
39. Otagiri, K., Miyake, I., Ishibashi, N., Fukui, H., Kanehisa, H., Okai, H. (1983). Studies of bitter peptides from casein hydrolyzate. II. Syntheses of bitter peptide fragments and analogs of BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val) from casein hydrolysate. *Bulletin of the Chemical Society of Japan*, *56*, 1116–1119.
40. Paizs, B., Suhai, S. (2005). Fragmentation pathways of protonated peptides. *Mass Spectrometry Reviews, 24*(4), 508–548.
41. Panjaitan, F.C.A., Gomez, H.L.R., Chang, Y.-W. (2018). *In silico* analysis of bioactive peptides released from giant grouper (*Epinephelus lanceolatus*) roe proteins identified by proteomics approach. *Molecules, 23*(11), art. no. e2910.
42. Pripp, A.H., Ardö, S. (2007). Modelling relationship between angiotensin-(I)-converting enzyme inhibition and the bitter taste of peptides. *Food Chemistry, 102*(3), 880–888.
43. Rawlings, N.D. (2009). A large and accurate collection of peptidase cleavages in the MEROPS database. *Database (Oxf.), bap015*.
44. Roepstorff, P., Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomedical Mass Spectrometry, 11*(11), 601.
45. Ryan, J.T., Ross, P.R., Bolton, D., FitzGerald, G.F., Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. *Nutrients, 3*(9), 765–791.
46. Sánchez, A., Vázquez, A. (2017). Bioactive peptides: a review. *Food Quality and Safety, 1*(1), 29–46.
47. Savitzky, A., Golay, M.J.E. (1964). Smoothing and differentiation of data by simplified least squares procedures. *Analytical Chemistry, 36*(8), 1627–1638.
48. Spicer, V., Yamchuk, A., Cortens, J., Sousa, S., Ens, W., Standing, K.G., Wilkins, J. A., Krokhin, O.V. (2007). Sequence-specific retention calculator. A family of peptide retention time prediction algorithms in reversed-phase HPLC: applicability to various chromatographic conditions and columns. *Analytical Chemistry, 79*(22), 8762–8768.
49. Temussi, P.A. (2012). The good taste of peptides. *Journal of Peptide Science, 18*(2), 73–82.
50. The UniProt Consortium, 2019. UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Research, 47*(D1), D506–D515.
51. Udenigwe, C.C. (2014). Bioinformatic approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science and Technology, 36*(2), 137–143.
52. Vermeirssen, V., van der Bent, A., Van Camp, J., van Amerongen, A., Verstraete, W. (2004). A quantitative *in silico* analysis calculates angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests. *Biochimie, 86*(3), 231–239.
53. Visser, S., Slangen, C.J., Rollema, H.S. (1991). Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 548*(12), 361–370.
54. Zambrowicz, A., Timmer, M., Polanowski, A., Lubec, G., Trziszka, T. (2013). Manufacturing of peptides exhibiting biological activity. *Amino Acids, 44*(2), 315–320.
55. Zamyatnin, A.A. (2009). Fragmentomics of natural peptide structures. *Biochemistry (Moscow), 74*(13), 1575–1585.

Submitted: 6 September 2019. Revised: 24 October 2019. Accepted: 28 October 2019. Published on-line: 2 January 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 151–157
DOI: 10.31883/pjfns/116395
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Co-Cultivation Growth of *Escherichia coli* and *Staphylococcus aureus* as Two Common Dairy Contaminants

Alžbeta Medveďová, Romana Györiová, Veronika Lehotová, Ľubomír Valík*

Department of Nutrition and Food Quality Assessment, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Radlinského 9, 812 37 Bratislava, Slovakia

Key words: predictive microbiology, growth parameters, microbial interactions, *E. coli*, *S. aureus*

The overgrowth of spoilage and pathogenic bacteria may pose risk to consumers health and cause technological and economic losses. Hence, interactions among *S. aureus* 2064 and *E. coli* BR in dependence of incubation temperature and different initial counts of both microorganisms were quantitatively described based on cultivation experiments and predictive models. Statistically insignificant differences ($p \geq 0.05$) between growth rates of *E. coli* at different initial concentrations suggest that the growth rates of *E. coli* in co-cultivation with *S. aureus* were affected only by incubation temperature. The growth of *E. coli* can be reliably predicted (R^2=0.968; A_f=1.160) based on the equation $\sqrt{\mu} = 0.0283\ (T - T_{min}) + 0.1038$. The growth of *S. aureus* during its co-cultivation with *E. coli* was influenced by incubation temperature and the presence of *E. coli* as well. It was documented by relatively high discrepancies indexes (D_f 23.9–43.9%) and also by differences between growth rates at different initial microbial concentrations. These results may help in better understanding of interrelationships during sensitive foods production *(e.g.* without temperature treatment or those with intensive manual handling).

INTRODUCTION

The trend for the consumption of raw milk and raw milk products, in the context of "consuming natural" and "purchasing locally" is becoming more and more popular. Taking into account the high nutritious potential of raw milk demonstrated by the presence of various macronutrients (carbohydrates, fats and proteins with easy digestibility), micronutrients (vitamins and minerals), regulators of physiological processes (amino acids), almost neutral pH (6.4–6.7), and high water activity [Claeys *et al.*, 2013; Hahne *et al*., 2019], it is a valuable food not only for human and animals, but also for microorganisms. The sources of microbial contaminants are versatile, including the animal udder surface, feed, feces, milking equipment [Quigley *et al.*, 2013] or contact with milk handling personnel [Valík, 2013]. Besides technologically important bacteria (*e.g.* lactic acid bacteria LAB), accompanying species (*e.g. Micrococcus*) also spoilage and pathogenic bacteria may occur. The presence of spoilage bacteria can have considerable negative effects on the quality of milk and dairy products, the presence of pathogens can have more severe repercussions [Quigley *et al*., 2011; Valík, 2013]. Hence, the risk posed by consuming raw milk, whether due to the rare presence of such pathogenic bacteria that cause severe disease with high mortality rates (*e.g. Listeria monocytogenes*) or due to frequently occurring bacteria with low severity (*e.g. Campylobacter* spp., *Staphylococcus aureus* or *Escherichia coli*) is still considered high [Valík, 2013].

In our work, we focused on two bacteria: *E. coli* and *S. aureus*, considering their frequent presence in milk and in the environment (raw materials of animal and plant origin, feed, handling personnel, but also wastewater or sludge that can be reused as fertilizer in agricultural soil), their good growth in different nutrient media, genetic properties, and severity of causing diseases. Their growth, survival, and formation of harmful metabolites are dependent not only on the quantity they achieve in the media, but also on conditions of the surrounding environment and also on their mutual interactions. If the environmental conditions create a favorable growth environment, these hygienically and technologically undesirable microorganisms can multiply, produce toxic metabolites, and ultimately endanger the health of consumers [Medveďová & Valík, 2012]. Therefore it is necessary to determine relationships between microorganisms themselves and also between microorganisms and external and internal environmental factors that determine microbial growth, or lead to their inhibition. This is also the main role and contribution of predictive microbiology. Thus, if we obtain a mathematical equation describing the impact of environmental conditions on the behavior of microorganisms in food, it is natural to use such knowledge and quantify (predict) the extent and degree of their impact on microbiological quality and food safety [Ross & McMeekin, 1994]. Additionally, in specific cases,

* Corresponding Author: Tel.: 00421 2 59325524;
E-mail: alzbeta.medvedova@stuba.sk (A. Medveďová)

mathematical modelling is also required to predict the interaction of antagonist or competitively behaving populations [Vereecken *et al*., 2000; Brocklehurst *et al*., 2004; Giménez & Dalgaard, 2004; Valík & Ačai, 2016].

In this context, the aim of our work was to quantitatively describe the mutual relationships between these potentially pathogenic bacteria in dependence on incubation temperature and proportion of their initial counts that can have impact on which population will be dominating in a given environment, even if the initial counts of one of the population will be unusually high or above the legislative limits.

MATERIAL AND METHODS

Bacterial isolates

The isolate of *E. coli* BR was isolated from a Slovakian traditional "Bryndza" cheese. The isolate of *S. aureus* 2064 was isolated from a Slovakian ewes' lump cheese. Both isolates were maintained in Brain Hearth Infusion (BHI) broth (Sigma-Aldrich, St. Louis, Missouri, USA) at 5 ±0.5°C prior to analysis. Their identity was confirmed and described in our previous work [Medveďová *et al.*, 2009, 2018].

Inoculation, cultivation conditions, and bacterial counts enumeration

A standard suspension of each isolate was prepared from a 24-h old culture grown in BHI broth at 37°C. These cultures were then diluted in saline-peptone solution and from 10^{-3} dilution 0.3 mL were inoculated into 300 mL of pre-tempered ultra/high temperature treated (UHT) milk (Rajo, Bratislava, Slovakia). The nutritional composition of 100 mL of UHT milk was as follows: 1.5 g of fat, from which 0.9 g were saturated fatty acids, 3.4 g of proteins, and 4.9 g of sugars from which 4.9 g was lactose. The above inoculation procedure was aimed to reach initial concentration of *E. coli* at approximately 10^3 CFU/mL and of *S. aureus* at approximately 10^6 CFU/mL (3EC+6STA); of *E. coli* at approximately 10^6 CFU/mL and of *S. aureus* at approximately 10^3 CFU/mL (6EC+3STA); of *E. coli* at approximately 10^3 CFU/mL and of *S. aureus* at approximately 10^3 CFU/mL (3EC+3STA). The static incubation of three parallel and three replicates experiments was performed at 6, 12, 15, 18, 21, 25, 30, and 37 ±0.5°C.

Sampling was performed at predefined time intervals taking into account the incubation temperature, to reach the best possible fit of model in curvature between lag phase and exponential phase and then between exponential phase and stationary phase. We took into account data obtained during monocultural growth of the isolates. Actual density of the studied isolates was determined by 10-fold dilution in a saline-peptone solution and by using selective nutrient media, Baird-Parker agar (Merck, Darmstadt, Germany) for determination of *S. aureus* 2064 counts according to EN ISO 6888–1 and Chromocult Coliform agar (Merck, Darmstadt, Germany) for determination of *E. coli* BR counts according to National Standard Method F23. At the same time as microbial density was determined, also a pH value of the media was examined by using a WTW Inolab 720 pH meter (Weilheim, Germany).

Modelling the microbial growth

Growth parameters from each individual growth curves for both microbial isolates were determined by using a primary model of Baranyi & Roberts [1994] using an in-house Excel Add-in package 'DMFit' version 3.5 (ComBase managed by United States Department of Agriculture-Agricultural Research Service, Washington, USA; and University of Tasmania Food Safety Centre, Hobart, Australia). Subsequently, the effect of temperature on the growth rates was described by the model of Ratkowsky *et al.* [1982]:

$$\sqrt{\mu_{max}} = b\,(T - T_{min}) \quad (1)$$

where: μ_{max} is specific growth rate (1/h), T is actual incubation temperature, T_{min} is theoretical minimum temperature at which growth was observed (°C), and b is a Ratkowsky parameter [Ross & McMeekin, 1994; Valík & Ačai, 2016].

Validation and statistical analysis

To validate mathematical equations describing the temperature effect on the growth rate of the isolates, the accuracy (*Af*), bias (*Bf*), and discrepancy (*% Df*) factors were used as defined by Baranyi *et al.* [1999]. Also the ordinary least-squares criterion and regression coefficient (R^2) were used. Finally, the statistical analysis (independent Student *t*-test and ANOVA test with statistical significance of $p<0.05$) was performed using Microsoft Excel tools 2007 (Microsoft, Redmond, USA).

RESULTS AND DISCUSSION

Microbial interactions have been described intensively and also several predictive models have been introduced by *e.g.* Giménez & Dalgaard [2004], Vereecken *et al.* [2000] or Brocklehurst *et al.* [2004]. However, all these models focused on the competition or antagonistic behavior of LAB on the growth of spoilage or pathogenic bacteria, as we described also earlier in Valík *et al.* [2018] for *S. aureus* in dependence of LAB and in Ačai *et al.* [2016] for *E. coli* mutual growth with LAB. However, in this paper we would like to draw the attention to the interactions between spoilage/pathogenic bacteria themselves, since *S. aureus* and *E. coli* are frequently present in raw materials [Quigley *et al*., 2013], in which they can multiply until concentrations resulting in disease outbreaks [Medveďová & Valík, 2012]. Their growth is affected not only by environmental conditions but also by the present microbiota, the pathogenic one included.

The initial concentration of *E. coli* in experiments with 6 EC was $N_{0EC}=6.11\pm0.19$ log CFU/mL (n=24; cv=3.1%), and in experiments with 3 EC it was $N_{0EC}=3.27\pm0.32$ log CFU/mL (n=48; cv=9.9%). On the other hand, initial counts of *S. aureus* in experiments with 6 STA was $N_{0STA}=5.98\pm0.21$ log CFU/mL (n=24; cv=3.5%) and in experiments with 3 STA it was $N_{0STA}=3.40\pm0.61$ log CFU/mL (n=48; cv=17.9%).

The growth of both microbial partners was positively affected by increased incubation temperature as it is also shown by growth parameters calculated by the model of Baranyi & Roberts [1994] summarized in Table 1. Exemplary

TABLE 1. Growth parameters of *E. coli* (EC) BR and *S. aureus* (STA) 2064 during their co-cultivation in UHT milk in dependence of temperature and parameters of pH value change.

Experimental variant	μ		*lag*		N_{max}		*pH*	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*k*	lag_{pH}
Temperature 6°C								
3STA+3EC	0.02±0.00	-0.01±0.00	138.3±3.9	0±0.0	5.11±0.06	1.25±0.07	-0.01±0.00	0±0.0
3STA+6EC	0.12*[a]±0.02	-0.01±0.00	226.6*[a]±27.3	0±0.0	3.50*[a]±0.14	3.91[a]±0.09	-0.01±0.00	0±0.0
6STA+3EC	0.01[c]±0.00	-0.01±0.00	0[b,c]±0.0	35.9[b,c]±14.6	6.03[b,c]±0.17	0.97[b,c]±0.05	-0.01±0.00	300.4±5.4
Temperature 12°C								
3STA+3EC	0.10±0.01	0.14±0.00	23.9±0.7	21.0±0.6	8.08±0.06	8.65±0.04	-0.22±0.02	128.8±2.4
3STA+6EC	0.51[a]±0.02	0.13±0.00	121.7[a]±0.3	18.7[a]±0.2	8.07±0.31	8.72±0.02	-0.03±0.01	130.4±3.5
6STA+3EC	0.12[c]±0.02	0.14±0.00	75.2[b,c]±7.7	19.9±1.9	7.99[b,c]±0.02	8.70±0.06	-0.01±0.00	0±0.0
Temperature 15°C								
3STA+3EC	0.30±0.00	0.31±0.01	15.0±0.2	5.5±1.6	8.54±0.01	8.85±0.04	-0.26±0.02	44.9±2.4
3STA+6EC	0.01[a]±0.00	0.21[a]±0.00	64.1[a]±27.9	6.6 ±1.2	3.67[a]±0.07	8.62[a]±0.03	-0.07±0.01	24.2±1.6
6STA+3EC	0.30[c]±0.01	0.21[b]±0.00	16.5[b,c]±1.5	3.8[b,c]±1.1	8.69[b,c]±0.06	8.55[b]±0.00	-0.06±0.01	21.9±2.1
Temperature 18°C								
3STA+3EC	0.31±0.00	0.35±0.02	2.5±0.8	5.2±1.1	7.88±0.03	8.26±0.14	-0.05±0.01	25.1±2.5
3STA+6EC	0.10[a]±0.00	0.29[a]±0.01	17.9[a]±0.2	4.6±0.6	4.85[a]±0.00	8.62[a]±0.06	-0.44±0.08	19.3±1.8
6STA+3EC	0.26[b,c]±0.00	0.41[c]±0.01	2.8[c]±0.3	7.5[c]±0.0	8.38[b,c]±0.08	6.71[b,c]±0.00	-0.14±0.04	15.9±1.9
Temperature 21°C								
3STA+3EC	0.47±0.01	0.64±0.01	1.8±0.0	3.7±2.3	8.47±0.01	8.81±0.03	-0.49±0.12	27.2±5.4
3STA+6EC	1.40[a]±0.39	0.65±0.00	7.7±4.4	3.9 ±0.1	4.76[a]±0.05	8.99[a]±0.00	-0.10±0.01	23.7±4.8
6STA+3EC	0.69[b,c]±0.01	0.55[b,c]±0.02	2.9±0.1	2.2±1.7	8.82[b,c]±0.01	8.58[b,c]±0.03	-0.22±0.04	49.2±6.9
Temperature 25°C								
3STA+3EC	0.78±0.00	1.09±0.00	1.3±0.2	2.0±0.1	8.73±0.02	9.03±0.04	-0.61±0.15	5.1±0.4
3STA+6EC	0.05[a]±0.00	0.41[a]±0.00	0[a]±0.0	0[a]±0.0	4.89[a]±0.00	8.81[a]±0.03	-0.24±0.08	9.8±1.1
6STA+3EC	1.55[b,c]±1.26	0.89[b,c]±0.01	2.7[b,c]±0.0	1.2[b,c]±0.3	8.95[b,c]±0.04	8.69[b,c]±0.02	-0.11±0.01	4.2±0.7
Temperature 30°C								
3STA+3EC	1.03±0.01	1.09±0.01	1.4±0.3	0.4±0.2	7.47±0.06	8.32±0.01	-0.12±0.01	8.3±1.2
3STA+6EC	0.28[a]±0.00	1.08 ±0.07	0[a]±0.0	1.2 ±0.8	4.94[a]±0.00	8.91[a]±0.06	-0.10±0.01	3.6±0.5
6STA+3EC	0.86[b,c]±0.04	1.21[b]±0.01	0[b]±0.0	0±0.0	8.95[b,c]±0.02	8.20[b,c]±0.01	-0.11±0.01	4.3±0.2
Temperature 37°C								
3STA+3EC	1.43±0.01	1.82±0.01	0±0.0	0.7±0.0	8.28±0.03	8.89±0.08	-0.26±0.17	6.9±1.5
3STA+6EC	0.34[a]±0.09	2.06 ±0.12	0±0.0	1.1±0.0	4.78[a]±0.12	8.78±0.01	-0.42±0.21	4.7±1.1
6STA+3EC	0.88[b,c]±0.01	2.19±0.11	1.1[b,c]±0.3	1.5 ±0.3	8.95[b,c]±0.15	8.79±0.03	-0.34±0.12	9.1±2.4

μ – specific growth rate (1/h), *lag* – duration of lag phase (h), N_{max} – counts of isolates in the stationary phase (log CFU/mL), k – rate of pH value decrease (1/h), lag_{pH} – duration of pH value lag phase (h); * – results only for the first growth phase; statistical significant differences ($p<0.05$) within parameters [a] – in experiments 3STA+3EC and 3STA+6EC, [b] – in experiments 3STA+3EC and 6STA+3EC, [c] – in experiments 3EC+6STA and 6STA+3EC.

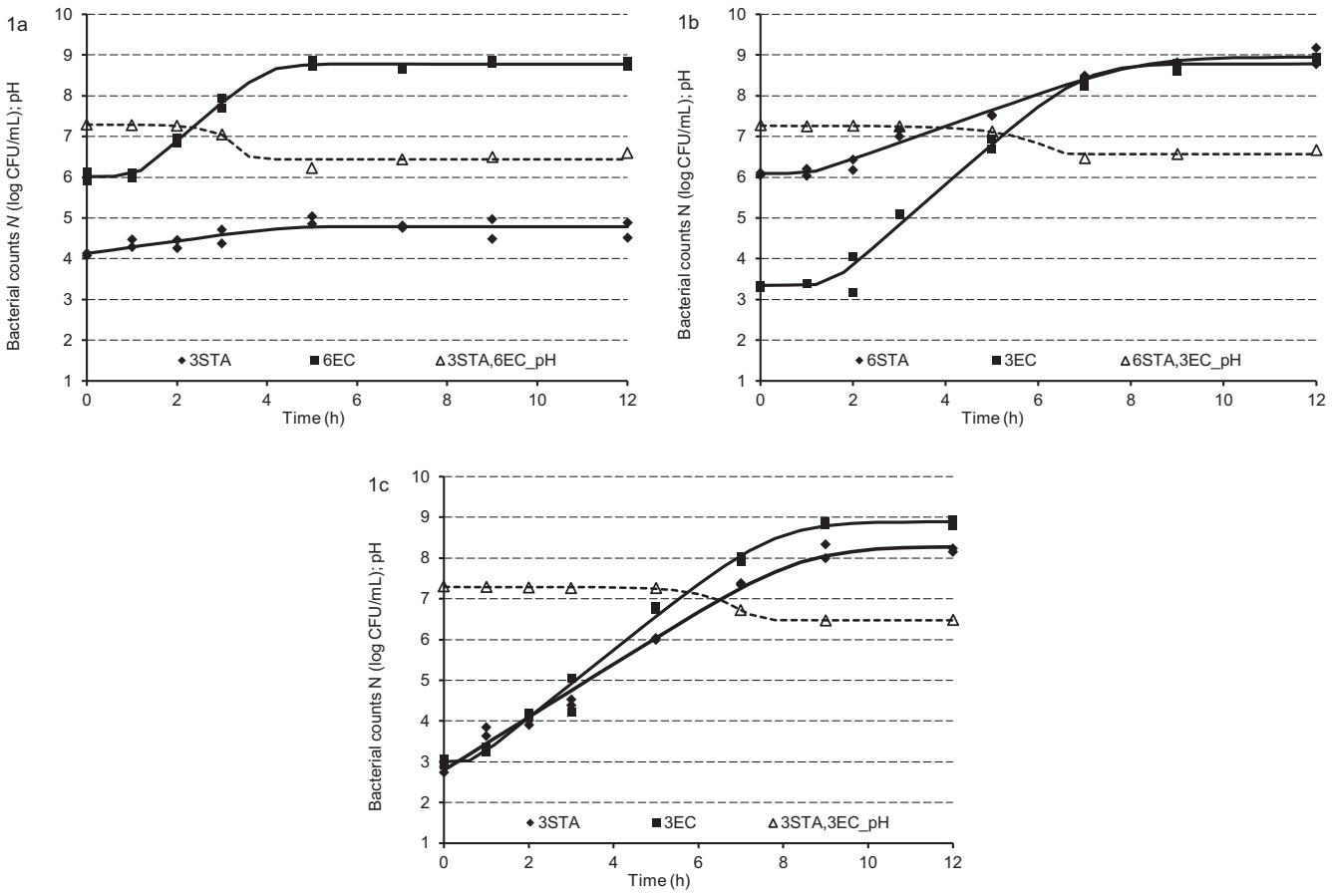


FIGURE 1. Growth of *S. aureus* 2064 and *E. coli* BR during co-cultivation in UHT milk in dependence on their initial counts (1a – 3STA+6EC; 1b – 6STA+3EC, 1c – 3STA+3EC) and subsistent change of pH value at 37°C.

growth curves at 37°C are depicted in Figure 1a-c, where one can see that if the initial concentration of *E. coli* and *S. aureus* was almost equal (3STA+3EC), both microorganisms grew very similarly to their typical final densities of about 8–9 log CFU/mL. Also in the case of dominating *S. aureus* counts (6STA+3EC), the growth of *E. coli* was affected only by the incubation temperature and not by the presence of *S. aureus*, as it is expressed by statistically insignificant differences ($p \geq 0.05$) at 6, 12, 18, and 37°C between growth rates of *E. coli* during co-culture with *S. aureus* in experiments 3EC+3STA and 3EC+6STA (missing superscript b in Table 1). Also in these experiments, *E. coli* was able to reach final densities in the stationary phase of at least 7 log CFU/mL. However, when there was an excess of *E. coli* in the media (3STA+6EC), the growth of *S. aureus* was inhibited and resulted in significantly lower final counts of *S. aureus*. The maximal *S. aureus* counts in the stationary phase in the case of *E. coli* prevalence in the media were at densities lower than 5 log CFU/mL.

Based on co-cultivation growth of *S. aureus* and *E. coli* and based on comparing their mono-cultural growth [Medveďová & Valík, 2012; Medveďová *et al.*, 2018], it can be concluded that the *S. aureus* 2064 growth is inhibited in the presence of *E. coli* BR abundance. It can probably be due to faster nutrient consumption by *E. coli* and also to the production of metabolites with antistaphylococcal properties, *e.g.* lactic acid, citric acid, and other organic acids, lower fatty acids. All these substances were identified in *E. coli* by Kim & Kim [2017] who reported about 1000 intracellular metabolites in *E. coli*. Even so, the actual produced substances are dependent on completeness of the media and other factors affecting *E. coli* biosynthesis processes [Tokuyama *et al*., 2019], in the case of *E. coli* cultivation in milk, the completeness of media is unchallenged [Claeys *et al*., 2013; Hahne *et al*., 2019].

The intensiveness and dominance of *E. coli* growth is obvious also in Figure 2. Values of its specific growth rates were in all experiments and at all temperatures higher than the specific growth rates of *S. aureus*, as the linear curve of *E. coli* specific growth rates affected by temperature was above linear curve of *S. aureus* in experiments 3STA+3EC. We may suppose that *E. coli* started to grow earlier than *S. aureus* (majority of *E. coli* lag phases are shorter than those of *S. aureus*), subsequently it started to consume nutrients and produced metabolites. Taking together, we may assume that *E. coli* was more effective in nutrient competition and together with probable antistaphylococcal metabolites it led to *S. aureus* 2064 growth inhibition during their co-cultural growth.

The only exceptions of this trend were experiments at 6 and 12°C. At 12°C, and in the case of 3STA+6EC experiment, *S. aureus* reached in the stationary phase counts of 8.07 log CFU/mL. It may be a result of slower pH value decrease (k=-0.03 1/h) compared to its decrease at other temperatures, where the slowest decrease (k=-0.07 1/h) was observed at 15°C; even it was 2.3-times faster.

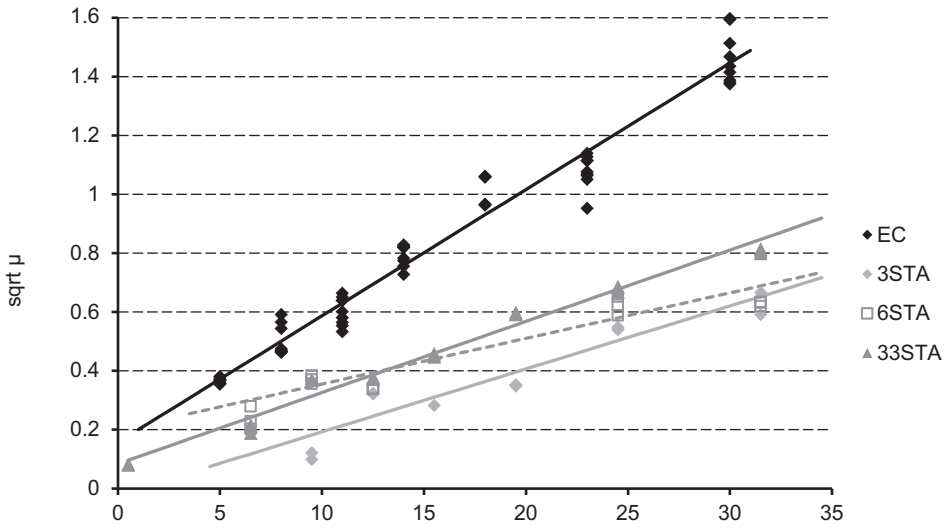


FIGURE 2. Effect of incubation temperature (expressed as $T - T_{min}$) on the specific growth rate (expressed as square root of specific growth rate μ) of *E. coli* (EC) BR in mono-culture (♦) and during its co-cultivation with *S. aureus* (STA) 2064 in 3STA+3EC (●), in 6STA+3EC (□) and 3STA+6EC (▲) in UHT milk according to Ratkowsky model. 3STA or 3EC stands for initial bacterial density of 10^3 CFU/mL, 6STA or 6EC stands for initial bacterial density of 10^6 CFU/mL.

A specific trend of bacterial behavior was observed also at 6°C, since only rotting of *E. coli* was observed at all mutual initial counts combinations. Contrary, *S. aureus* was able either to maintain its counts (6STA+3EC) or even increase its counts by about 2.59 log CFU/mL (3STA+3EC) or increase them by about 0.9 log CFU/mL in the first phase (marked with * in Table 1) and after the second lag phase lasting for 242 h, the decrease of its counts was noticed with the rate of -0.01 1/h (growth parameters from the second phase are not shown in Table 1). The ability of *S. aureus* to grow at 6°C in experiment 3STA+3EC with the specific growth rate of μ=0.02 1/h is remarkable, since in ultra-high temperature treated milk the same isolate was unable to grow at 6°C and it started to grow only at 7°C (with μ=0.006 1/h) [Medveďová & Valík, 2012]. It is also worthy of mention that at 6°C the *S. aureus* isolate was able to maintain its counts in experiment 6STA+3EC at density of 6 log CFU/mL for 36 days. It is undesirable in the case of enterotoxigenic strains as 5 log CFU/mL are the minimal counts needed for staphylococcal enterotoxins production [Delbes *et al*., 2006; Charlier *et al*., 2008]. Therefore, it seems that the presence of *E. coli* helped *S. aureus* to withstand such a low temperature better that during its mono-cultural growth.

The effect of temperature on the microbial growth in sub-optimal temperature range can be described by Ratkowsky model. As it is shown in Figure 2 and by equation 1 (Table 2), there was a linear trend in *E. coli* growth rate increase in dependence on temperature and it was not influenced by the presence of *S. aureus*. On the other hand, the growth rate of *S. aureus* was influenced not only by the incubation temperature but also by the presence of *E. coli* and mutual ratio between them (Figure 2). It is also obvious in Table 2, where 3 different equations describing the growth rate of *S. aureus* in dependence of *E. coli* initial counts are presented. an inevitable part of predictive modelling is also validation. Subsistent validation indices are also mentioned in Table 2. Since the discrepancy coefficient for the Ratkowsky model describing the growth of *E. coli* is only 16%, it is also clear that its growth was influenced only by the incubation temperature. It is also emphasized by statistically insignificant discrepancies ($p \geq 0.05$) in almost all experiments between *E. coli* growth rates at different *S. aureus* initial counts and their combinations. Moreover, also in the case of *E. coli* lag phase there were only few cases when its values were significantly ($p \geq 0.05$) influenced by *S. aureus* initial density. It was also the case of *E. coli* final densities in the stationary phase. On the other hand, discrepancy indices of 23.9–43.9% are referring to a significant effect of *E. coli* presence in the cultivation media, mutual ratio between *E. coli* and *S. aureus*, and also to the effect

TABLE 2. Validation of Ratkowsky model describing the dependence of temperature on specific growth rate of *E. coli* BR and *S. aureus* 2064 during their mutual co-cultivation.

Experimental variant	Equation describing dependence	A_f	B_f	$\%D_f$	R^2
1: EC in all	$\sqrt{\mu} = 0.028\ (T - T_{min}) + 0.104$	1.160	0.994	16.0	0.968
2: STA in 3STA+3EC	$\sqrt{\mu} = 0.036\ (T - T_{min}) + 0.133$	1.239	0.953	23.9	0.980
3: STA in 3STA+6EC	$\sqrt{\mu} = 0.021\ (T - T_{min}) - 0.021$	1.439	1.015	43.9	0.883
4: STA in 6STA+3EC	$\sqrt{\mu} = 0.024\ (T - T_{min}) + 0.259$	1.411	0.960	41.1	0.904

of incubation temperature. Besides *S. aureus* specific growth rate, also its lag phase duration and also counts in the stationary phase were significantly influenced by the presence of the second bacterial population.

Regarding the pH value of the growth media, its value in all performed experiments started to decline from an average value of 7.40±0.11 (n=96; cv=1.5%) after a given time (pH lag phase) that was as shorter as the incubation temperature was higher. The pH value decrease was observed during the growth phase of *E. coli* to an average value of pH 6.31±0.31 (n=96; cv=5.0%). The lowest pH value observed in time of reaching the stationary phase by *E. coli* indicates that its growth was accompanied by the production of acid with subsequent pH value decrease. Contrary, in the case of slower *E. coli* growth (experiments with lower values of *E. coli* specific growth rates) also its metabolic activity was slower that indicates lower pH value decrease rates *k* (Table 1). Finally, during the stationary phase of *E. coli*, the pH value started to increase again to a final value of about 6.62±0.33 (n=96; cv=5.0%). As the pH value was determined only in one of parallels, the statistical analysis of pH value parameters was not performed.

CONCLUSION

To conclude, the interactions between two frequent contaminants of raw materials, *E. coli* and *S. aureus*, as affected by temperature and their mutual ratios were described. The growth of *E. coli* isolate was affected and positively stimulated by the incubation temperature. During its growth, pH value decrease was observed as a result of *E. coli* metabolic activity. pH decrease together with competition for nutrient led to *S. aureus* growth inhibition, even its growth was positively stimulated by increasing incubation temperature. Presented results may help during production of mainly foods with minimal heat treatment with the aim to increase their safety and harmlessness. However, as many lactic acid bacteria cultures are used in the food industry, their role in spoilage / pathogenic bacteria growth is indisputable; the problem of microbial behavior and interactions is more complex and need to be described deeply. Furthermore, mathematical modelling cannot be a compromise or alternative to the established hygiene norms. Using high quality raw material is crucial in dairy technology especially in “traditional ways of production using raw milk” to increase safety of those products.

RESEARCH FUNDINGS

This work was supported by grant VEGA No. 1/0532/18.

CONFLICT OF INTEREST

None of the authors of this manuscript has a financial or personal relationship with other people or organizations that could inappropriately influence the content of this work.

REFERENCES

1. Ačai, P., Valík, Ľ., Medveďová, A., Rosskopf, F. (2016). Modelling and predicting the simultaneous growth of *Escherichia coli* and lactic acid bacteria in milk. *Food Science and Technology International*, *22*(6), 475–484.
2. Baranyi, J., Roberts, T.A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, *23*(3–4), 277–294.
3. Baranyi, J., Pin, C., Ross, T. (1999). Validating and comparing predictive models. *International Journal of Food Microbiology*, *48*(3), 159–166.
4. Brocklehurst, T.F. (2004). Challenge of the food environment. In R.C., McKellar, X., Lu (Eds.), *Modelling Microbial Responses in Food*, CRC Press Inc., Boca Raton, Florida, USA, pp. 197–232.
5. Charlier, C., Even, S., Gautier, M., LeLoir, Y. (2008). Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk. *International Dairy Journal*, *18*(2), 197–203.
6. Claeys, L.W., Cardoen, S., Daube, G., De Block, J., Dewettinck, K., Dierick, K., De Zutter, L., Huyghebaert, A., Imberechts, H., Thiange, P., Vandenplas, Y., Herman, L. (2013). Raw or heated cow milk consumption: Review of risks and benefits. *Food Control*, *31*, 251–262.
7. Delbes, C., Alomar, J., Chougui, N., Martin, J.-F., Montel, M.-Ch. (2006). *Staphylococcus aureus* growth and enterotoxin production during manufacture of cooked, semihard cheese from cows’ milk. *Journal of Food Protection*, *69*(9), 2161–2167.
8. Giménez, B., Dalgaard, P. (2004). Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage microorganisms in cold-smoked salmon. *Journal of Applied Microbiology*, *96*(1), 96–109.
9. Hahne, J., Isele, D., Berning, J., Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow’s milk with high bacterial counts and their food spoilage potential. *Food Microbiology*, *79*, 11–19.
10. ISO 4833–1:2013. Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 degrees C by the pour plate technique. ICS: 07.100.30 (2013), p. 9.
11. Kim, J., Kim, K.H. (2017). Effects of minimal media *vs*. complex media on the metabolite profiles of *Escherichia coli* and *Saccharomyces cerevisiae*. *Process Biochemistry*, *57*, 64–71.
12. Medveďová, A., Rosskopf, F., Liptáková, D., Valík, Ľ. (2018). Prediction of temperature effect on growth of two raw milk cheese isolates of *Escherichia coli* in milk. *Journal of Food Nutrition and Research*, *57*(2), 141–150.
13. Medveďová, A., Valík, Ľ. (2012). *Staphylococcus aureus*: Characterisation and quantitative growth description in milk and artisanal raw milk cheese production. In A. Eissa (Ed.), *Structure and Function of Food Engineering*, InTech, Rijeka, Croatia, pp. 71–102.
14. Medveďová, A., Valík, Ľ., Studeničová, A. (2009). The effect of temperature and a_w on the growth of *Staphylococcus aureus*. *Czech Journal of Food Sciences*, *27*, SI2, 28–35.
15. National Standard Method F23 (2005). Enumeration of *Enterobacteriaceae* by the Colony Count Technique, Standards Unit,

Evaluations and Standards Laboratory, Health Protection Agency, p. 23.
16. Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.B., Ross, R.P., Fitzgerald, G.F., Cotter, P.D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Letters*, *37*(5), 664–698.
17. Quigley, L., O'Sullivan, O., Beresford, T.B., Ross, R.P., Fitzgerald, G.F., Cotter, P.D. (2011). Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology*, *150*(2–3), 81–94.
18. Ratkowsky, D.A., Olley, J., McMeekin, T.A., Ball, A. (1982). Relationship between temperature and growth rates of bacterial cultures. *Journal of Bacteriology*, *149*(1), 1–5.
19. Ross, T., McMeekin, T.A. (1994). Predictive microbiology, review paper. *International Journal of Applied Microbiology*, *81*, 501–508.
20. Tokuyama, K., Toya, Y., Matsuda, F., Cress, B.F., Koffas, M.A.G., Shimizu, H. (2019). Magnesium starvation improves production of malonyl-CoA-derived metabolites in *Escherichia coli*. *Metbabolic Engineering*, *52*, 215–223.
21. Valík, Ľ. (2013). Risk assessment of pathogenic bacteria presence in raw milk. [http://www.mpsr.sk/sk/index.php?navID=525&navID2=525&sID=111&id=8134&start]. Accessed 10 May, 2019.
22. Valík, Ľ., Ačai, P. (2016). *Predictive microbiology and microbiological risk assessment (in Slovak)*. Publisher STU, Bratislava, Slovakia, p. 160.
23. Valík, Ľ., Ačai, P., Medveďová, A. (2018). Application of competitive models in predicting the simultaneous growth of *Staphylococcus aureus* and lactic acid bacteria in milk. *Food Control*, *87*, 145–152.
24. Vereecken, K.M., Dens, E.J., Van Impe, J.F. (2000). Predictive modelling of mixed microbial populations in food products: evaluation of two-species models. *Journal of Theoretical Biology*, *205*(1), 53–72.

Submitted: 17 July 2019. Revised: 9 December 2020. Accepted: 7 January 2020. Published on-line: 02 March 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 159–168
DOI: 10.31883/pjfns/118008
http://journal.pan.olsztyn.pl

Original article
Section: Food Technology

Technological Properties of Model System Beef Emulsions with Encapsulated Pumpkin Seed Oil and Shell Powder

Slaviša Stajić[1], Ana Kalušević[2,3], Igor Tomasevic[1], Biljana Rabrenović[2], Anđelija Božić[1], Petar Radović[1], Viktor Nedović[2], Dušan Živković[1]*

[1]*Department of Animal Source Food Technology, Faculty of Agriculture, University of Belgrade, Nemanjina 6, Belgrade 11080, Serbia*
[2]*Department of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade, Nemanjina 6, Belgrade, Serbia*
[3]*Department of Chemical and Physico-Chemical Research, Institute of Meat Hygiene and Technology, Kaćanskog 13, 11040 Belgrade, Serbia*

Key words: beef emulsions, model system, phosphate reduction, encapsulated pumpkin seed oil, instrumental colour, texture properties

The aim of this research was to examine the technological properties of beef emulsions in which fatty tissue was partially substituted with pumpkin seed oil (PSO) encapsulated in alginate or pectin matrix, and where phosphates (F treatments) were simultaneously substituted with shell powder (C treatments).

Fat replacement (in the amount of 25%) mostly had no significant influence on pH, cooking loss, purge loss, fluid release under pressure, residual nitrite level, and texture properties. On the other hand, higher yellowness and hue angle were observed when backfat was replaced with encapsulated PSO, but only in treatments with phosphates. The use of shell powder as a phosphate replacer led to significantly higher pH values and thus to significantly higher residual nitrite level: 70.87–74.64 mg/kg (C treatments) *vs*. 56.79–62.16 mg/kg (F treatments). The nitrite depletion rate during the seven-week storage was lower in C treatments. Moreover, higher lightness, yellowness and hue angle could be expected, as well as lower hardness, springiness, cohesiveness and chewiness.

For the most part, seven-week storage had no influence on the observed technological properties, except on colour properties in which an opposite trend was observed in terms of yellowness – increase in treatments with phosphates and decrease in treatments with shell powder.

Further research, which would include sensory analysis, should be conducted to determine how these altered colour and textural properties will be perceived by consumers.

INTRODUCTION

For centuries the purpose of meat products was to extend the viability of meat, which is why they were an important source of proteins and energy. Over the past fifty years, the availability of fresh meat has increased, therefore the specific sensory characteristics of meat products have become more important.

Emulsion-type meat products (frankfurters, wieners, bologna, mortadella) are worldwide popular comminuted meat products. These ready-to-serve meat products are made by comminuting and mixing chopped meat, fatty tissue and water/ice. Therefore, their specific technological properties (*e.g.* emulsion formulation and stability, water binding properties, instrumental colour and texture...) which correlate to sensory characteristics, are attributable to the protein/fat/moisture ratio. Furthermore, ingredients such as salt, phosphates, nitrites *etc*. are of significant importance for the formation of these technological (and in turn sensory) properties and their stability.

Salt and phosphates promote the extraction of myofibrillar proteins and contribute to the emulsifying process and emulsion stability. Phosphates exhibit a synergistic effect with salt, improve the water holding capacity of emulsion-type meat products and thus increase the processing yield, reduce storage loss and improve product texture, tenderness, and juiciness [Sebranek, 2009]. However, research indicates that acutely high phosphorus intake affects bone metabolism by decreasing bone formation and increasing bone resorption [Kemi *et al.,* 2007]. Furthermore, nowadays people are showing a greater interest in foods that contain bioactive or functional components [Hygreeva *et al.,* 2014], and the demand for natural, organic and/or clean label meat products has also increased. In that sense, sporadic research studies were conducted with natural calcium powders as phosphate substitutes [Cho *et al.,* 2017].

Fats play an important role in the formation and stabilization of meat emulsions (therefore technological properties)

* Corresponding Author: Tel.: +381114413272;
E-mail: stajic@agrif.bg.ac.rs (S. Stajić)

and sensory properties of emulsion-type meat products – flavour, juiciness, hardness, and mouthfeel [Choi *et al.,* 2009; Hygreeva *et al.,* 2014]. Pork backfat is the most interesting of fatty tissues due to its technical properties that have a beneficial influence on technological properties of meat products (emulsion formulation and stability, instrumental colour and texture...), which correlates to some sensory characteristics [Ospina-E *et al.,* 2010]. Also, due to its technological properties, beef is slightly preferable to pork [Mittal, 2005]. However, high SFA content and low PUFA content (especially *n*-3 PUFA) are a characteristic of fatty tissue and meat lipids that has been associated with a higher risk of some chronic disorders (*e.g.* cardiovascular diseases).

In general, technological strategies aimed at improving the fatty acid profile entail the replacement of animal fat with a different lipid which would be more in line with health recommendations [Jimenez-Colmenero *et al.,* 2015]. In that sense, a partial replacement of fatty tissue with oils (olive, flaxseed, grape seed, canola, cotton seed, hazelnut, soy, rapeseed, fish) was used to improve the FA profile of different types of meat products [Hygreeva *et al.,* 2014; Jimenez-Colmenero *et al.,* 2015]. Pumpkin seed oil is not used so often as a fatty tissue replacer in meat products as other oils, though it has good nutritional and technical properties. It is rich in oleic and linoleic acids, Δ5- and Δ7-sterols, tocopherols and other bioactive compounds [Montesano *et al.,* 2018; Rezig *et al.,* 2012]. The most beneficial health effect of pumpkin seed oil is that it prevents the growth and reduces the size of the prostate, and it was also associated with reducing cancer and heart disease risk [Montesano *et al.,* 2018]. Due to its chemical composition, it is oxidative-stable at temperatures used in the processing of emulsified-type meat products.

The application of the above-mentioned oils in the modern food preservation industry is quite limited due to difficulties caused by their unstable nature, volatility, rapid evaporation, and degradation under regular conditions. Hence, in order to obtain more stable products, oils were added as an emulsion system, gelled emulsion system or encapsulated. Encapsulation technology could be used as an efficient way of entrapping oils and preventing their degradation and undesirable interactions with the food matrix [Nedović *et al.,* 2013]. Therefore, high PUFA oils can be protected from oxidation in different food systems by different encapsulation techniques, and alginate and pectin are acceptable encapsulating/coating materials commonly used for this purpose [Nedović *et al.,* 2013].

Since the amount of fatty tissue and the presence of phosphates are of significant importance for the formation of meat emulsions and their (storage) stability, and thus for the sensory properties of emulsion-type meat products, the substitution of fatty tissue with high PUFA containing oils in meat emulsions without phosphates poses quite a challenge.

The aim of this research was to examine the technological properties of beef emulsions in which fatty tissue was partially substituted with encapsulated pumpkin seed oil and where phosphates were simultaneously substituted with shell powder. The stability of these emulsions during 7-week cold storage and the extent of changes were also observed.

MATERIAL AND METHODS

Emulsions preparation

Six different meat emulsions were prepared in order to examine the influence of phosphate substitution with shell powder and fat reduction by pumpkin seed oil (PSO) encapsulated in the calcium alginate and pectin matrices. Two groups of meat emulsions were prepared, each comprising three different meat emulsions. The first was emulsified using a commercial polyphosphate mixture (F emulsions) and the second using shell powder (C emulsions). The pH value of 0.1% (w/w) shell powder water solution (t=20.5°C) was 12.04; whereas the pH value of 0.5% (*w/w*) phosphates mixture water solution (t=20.5°C) was 7.60. Since amounts higher than 1 g/kg of shell powder increased the pH of raw emulsions to around 7 and higher, which will almost suspend colour formation and increase the possibility of spoilage [Feiner, 2006; Shahidi *et al.,* 2014], these emulsions were excluded from measurements. In both groups, ¼ of fatty tissue in two meat emulsions was substituted by PSO encapsulated in the calcium alginate (ALG emulsions) and pectin matrices (PEC emulsions). The full formulations of all meat emulsions are presented in Table 1.

Model system meat emulsions were prepared as follows: fresh beef (round muscles) and pork backfat were bought at a local store, the visible fat and connective tissue were trimmed off the meat and cut into small pieces kept frozen at -20°C until use. Before use, meat and backfat were tempered for 16 h in a cooling chamber (at 0–2°C) to a temperature between -2 and 0°C. Then, they were separately grounded through a 5 mm plate (82H, Laska, Traun, Austria) and weighed. Meat emulsions were prepared according to the following procedure. Ground and weighed meat and backfat were put in a Thermomix TM31 (Vorwerk Elektrowerke GmbH & Co. KG, Wuppertal, Germany). Ice cooled water (0–3°C) with previously dissolved ingredients was added and mixed with meat and backfat using a spoon. Then, the mixture was emulsified for 15 s at a low blender speed, followed by 45 s at the highest speed (10,200 rpm). After that, the encapsulated pumpkin seed oil was added (ALG or PEC treatments) and stirred for 15 s. Only batters with the temperature below 12°C were taken for further processing.

Emulsions were then stuffed in pre-weighted plastic tubes (50 mL, d=27 mm; approximately 50 g each), sealed and centrifuged at 3,000×*g*, 4°C, 90 s (Eppendorf, Centrifuge 5430R, Eppendorf AG, Hamburg, Germany) to eliminate air bubbles. The tubes were heated in a water bath at 80°C until 70°C in the centre was reached (15 min), then cooled and stored in the dark at 3±1°C overnight. Afterwards, all tubes were tempered at room temperature for 1 h, then the content was taken out and wiped with paper towels.

One third from each treatment was examined (day 0) while the remaining ⅔ were vacuum-packed (two in each package) in coextrusive, barrier bags (PA/PE/PE; 85 lm thick, dimensions 200 mm × 350 mm) using a tabletop vacuum machine (MVS 35x; Minipack-Torre SpA, Dalmine, Italy), and stored at 3±1°C for 42 days. During storage, analyses were conducted on day 21 and day 42. Two replications of the experiment were conducted on different days.

TABLE 1. Formulation of model system beef emulsions.

	Phosphate			Shell powder		
	CONF	ALGF	PECF	CONC	ALGC	PECC
Beef meat	500	500	500	500	500	500
Backfat	200	150	150	200	150	150
Water	300	300	300	300	300	300
Encapsulated pumpkin seed oil calcium alginate matrix	/	50		/	50	/
Encapsulated pumpkin seed oil pectin matrix	/	/	50	/	/	50
Nitric salt*	18	18	18	18	18	18
Phosphate mixture**	5	5	5	/	/	/
Shell powder***	/	/	/	1	1	1
Na-isoascorbate	0.5	0.5	0.5	0.5	0.5	0.5

*with 0.6% of $NaNO_2$; ** polyphosphate commercial mixture (sodium tripolyphosphate and disodium pyrophosphate, P_2O_5 content *ca*. 60%); *** Purifact-XTEND 15, Purifact B.V., Roosendaal, NL (CaO content >91%).
CON – treatments with all pork backfat; ALG – treatments with 25% of encapsulated pumpkin seed oil in alginate matrix; PEC – treatments with 25% of encapsulated pumpkin seed oil in pectin matrix; F – treatments with phosphates; and C – treatments with shell powder.

The encapsulation of PSO in the calcium alginate and pectin matrices using electrostatic extrusion was realized according to the procedure described by Stajić *et al.* [2014], with some modifications. The first step in the encapsulation process was the preparation of a shell solution by dissolving sodium alginate (Sigma-Aldrich, St. Louis, MO, USA) or pectin powder (Cargill, Wayzata, MI, USA) in distilled water (0.02 g/mL). Alginate (Pectin)/Oil emulsion (20%, *w/w*) was prepared using Ultra-Turrax T25 (T25 digital ULTRA-TURRAX®, IKA, Germany) at a speed of 10,000 rpm for 5 min. The emulsion was extruded through blunt stainless still needle (22 gauge) using a syringe pump under a constant flow rate of 50 mL/h. Electrostatic potential (5.0 kV) was formed by a high voltage dc unit (Model 30R, Bertan Associates, Inc., New York). The collecting solution was calcium chloride (0.02 g/mL, Analytika, Czech Republic). The distance between the needle tip and the collecting solution was 2.5 cm. After extrusion, the beads were left in the collecting solution for 30 min. After the gelling period, microbeads (Figure 1) were rinsed with distilled water.

Methods

For each treatment, six samples were weighed after stuffing and after wiping the cooked and cooled emulsions, and the weight loss was calculated by the difference between these measurements and expressed as a percentage.

During storage (days 21 and 42), six vacuum packages were used for determining purge loss. The package content (two cooked tube contents) was taken out, wiped with paper towels, and weighed. Purge loss was reported as a percentage of the initial weight (day 0). The samples were then used for further analysis.

On days 0, 21, and 42, pH values, fluid release under pressure (FRP), nitrite content [ISO2918:1975], instrumental colour on cross section, and texture profile were determined. Samples were held for equilibration to room temperature for 1 h before measurements were taken.

Eight samples (one per tube content) from each treatment were used for pH value measurements with an HI 83141 pH-meter (Hanna Instruments, Sarmeola di Rubano, Italy) equipped with a penetration probe, which was calibrated with standard buffer solutions (pH=4 and pH=7) before each measurement.

Regarding FRP, on days 0, 21, and 42, one sample height 10±0.5 mm (original diameter) was taken from six cooked tube contents of each treatment, weighed, and compressed between two filter papers (dried at 103°C, 30 min and cooled at room temperature in exicator), using the weight of 200±2 g for 5 min. After that, the sample was removed and both papers were measured. The amount of the released fluid relative to initial sample weight represents fluid release under pressure (expressed as %).

Sixteen samples (eight cooked tube contents with two cross-sections samples) were used for instrumental colour measurement using the Computer vision system (CVS) as described by Tomasevic *et al.* [2019]. Three readings were taken from each cross-section from RAW photographs and 5 × 5 pixels measuring area, using a Photoshop Average Colour Sampler Tool. Average values of these measurements were calculated and used as one iteration for statistical analysis. C* (chroma) and h (hue angle) were calculated using the following equations:

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2} \text{ and } h = \arctan b^*/a^*$$

Total colour difference (TCD), relative to CONF, was determined on days 0, 21, and 42 using the standard equation:

$$TCD = \sqrt{(L^*_X - L^*_{CONF})^2 + (a^*_X - a^*_{CONF})^2 + (b^*_X - b^*_{CONF})^2}$$

where: X – treatments (ALGF, PECF, CONC, ALGC, PECC).

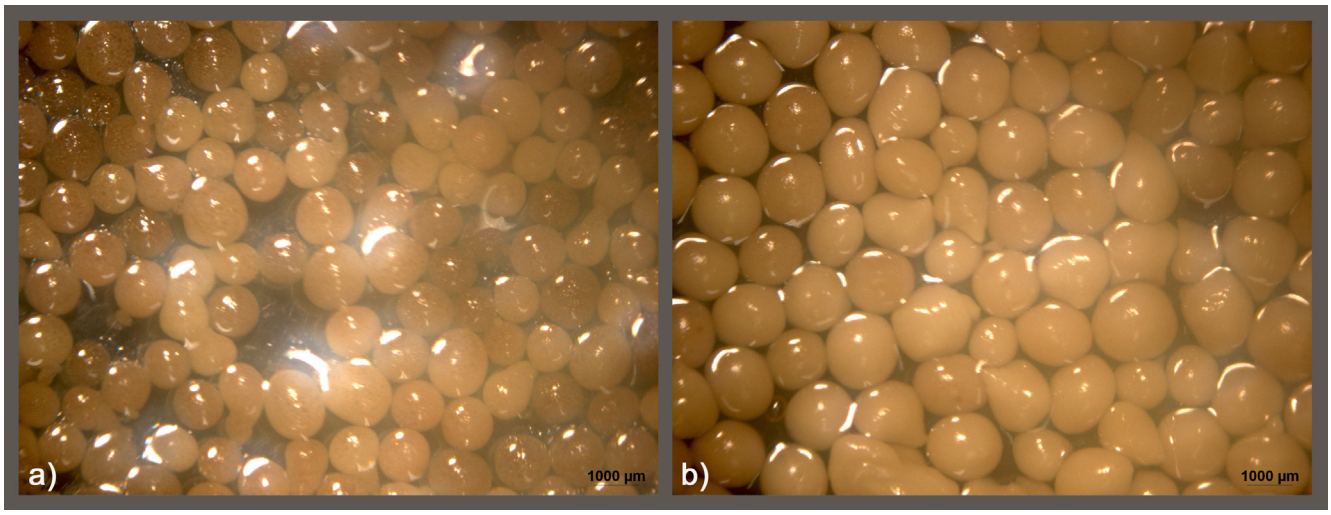


FIGURE 1. Visual appearance of encapsulated pumpkin seed oil: a) in alginate matrix; b) in pectin matrix.

Texture profile analysis was performed in the same manner as described by Stajić *et al.* [2018]. Using the available computer software, the fallowing texture parameters were obtained [Bourne, 2002]: hardness (g): the height of the force peak on the first compression cycle; adhesiveness (g × s): the work necessary to pull the compressing probe away from the sample (the negative force area of the first bite); cohesiveness (dimensionless): the ratio of the positive force areas under the first and second compressions; springiness: the ratio that the sample recovered its height after first and before second compression cycle; and chewiness (g): calculate as hardness × cohesiveness × springiness and represents work required to masticate a sample before swallowing. Twelve samples (six cooked tube contents with two sample cores, d=16 mm, h=12 mm) were used.

Statistical analysis

Two-way ANOVA was used to evaluate the effect of additive replacement, fat replacement, and its interaction. Statistica 12.5 (StatSoft, Inc., Tulsa, OK, USA) software was used to perform statistical analysis. Differences between means were determined using Tukey's HSD test at the significance level of $p<0.05$, while during the storage period they were determined using Repeated measures ANOVA.

RESULTS AND DISCUSSION

Changes of pH values

The use of shell powder as a phosphate replacer had a significant influence on pH values of meat emulsions (Table 2). Before cooking, all treatments with phosphates (F treatments) had significantly lower pH values relative to equivalent treatments with shell powder (C treatments). After cooking, pH values increased in all F treatments, while the opposite effect was observed in C treatments. When meat is subjected to heat treatments at 70–80°C, pH increases because of the reduction of free acidic groups of proteins and because of liberation cations (Ca^{2+} and Mg^{2+}) [Medyński *et al.*, 2000]. In sausage batters with phosphates, heating can increase pH value by 0.0 to 0.4 pH units [Puolanne *et al.*, 2001]. Medyński *et al.* [2000] stated that due to limitations in the reactivity of meat proteins with increased amount of lactic acid, pH value after cooking can decrease when lactic acid was added in meat. It is possible that a similar limitation can occur when a higher amount of calcium ions was added into the meat batter, especially assuming that the lower hardness of C treatments can contribute to poorer solubilisation (activation) of myofibrillar proteins when shell powder was added. The texture section covers this in more detail.

The results indicated a significant impact of additive replacement on pH values on day 0 and throughout storage, while fat replacement (and encapsulating agents as well) and interaction of factors had a sporadic no significant impact. During storage, a slight increment of pH values was observed (up to 0.09), significantly only in CONF and ALGF, but without an influence on the relations within treatments. Though significant differences were found, the pH values of all model system emulsions, on day 0 and throughout storage, were within the range for the emulsified-type of sausages made of beef [Vural & Javidipour, 2002], pork/beef [Yotsuyanagi *et al.,* 2016], and goat/beef [Stajić *et al.,* 2020].

Lee *et al.* [2011] reported that different levels (0.15–0.5%) of oyster shell powder as phosphate substituent, increased pH values in emulsified pork sausages up to the values slightly above 7. Similarly, Cho *et al.* [2017] reported significantly higher pH values in ground pork meat products when replacing 0.3% of phosphates with 0.5% of oyster shell powder. Choi *et al.* [2014] also reported a significant increase of pH values (0.5–0.7) when replacing 0.3% of phosphates with different levels (0.15–0.5%) of oyster shell powder and 0.5% of whey protein in restructured pork hams. In contrast to them, Bae *et al.* [2017] found no effect of phosphate (0.3%) replacement with 0.5% oyster shell powder on the pH of ground pork meat products before and after cooking, which differs from the results presented in this research study.

Research data indicate that partial (or even total) fat replacement with different preparations of plant oils could not lead to pH changes in emulsified-type sausages [Pintado *et al.,* 2016a; Salcedo-Sandoval *et al.,* 2015].

TABLE 2. Technological properties of uncooked* and cooked model system beef emulsions.

Technological properties	Storage time	Additive 1			Additive 2			Significance (p)		
		Fat 1	Fat 2	Fat 3	Fat 1	Fat 2	Fat 3			
		CONF	ALGF	PECF	CONC	ALGC	PECC	Additive	Fat	Additive* Fat
pH	Day 0*	5.93±0.02^{b}	5.92±0.02^{b}	5.91±0.04^{b}	6.69±0.06^{ab}	6.64±0.03^{a}	6.74±0.04^{a}	0.0000	0.0028	0.0007
	Day 0	6.06±0.02^{bB}	6.06±0.02^{bB}	6.06±0.02^{b}	6.49±0.03^{a}	6.47±0.05^{a}	6.51±0.06^{a}	0.0000	NS	NS
	Day 21	6.09±0.04^{bB}	6.09±0.02^{bB}	6.09±0.04^{b}	6.51±0.02^{a}	6.47±0.05^{a}	6.52±0.05^{a}	0.0000	NS	0.0476
	Day 42	6.13±0.01^{bA}	6.15±0.02^{bA}	6.12±0.07^{b}	6.52±0.07^{a}	6.51±0.05^{a}	6.52±0.06^{a}	0.0000	NS	NS
Cooking loss (%)	Day 0	3.77±0.30^{ab}	4.12±0.45^{ab}	3.26±0.40^{b}	4.14±0.80^{ab}	4.31±0.57^{a}	4.39±0.53^{a}	0.0034	NS	NS
Purge loss (%)	Day 21	5.04±0.50^{B}	5.48±0.55^{B}	5.54±0.57^{B}	4.67±0.51^{B}	5.15±0.37^{B}	5.13±0.47^{B}	0.0349	0.0385	NS
	Day 42	6.61±1.03^{A}	7.13±0.96^{A}	7.20±0.77^{A}	5.97±0.88^{A}	6.49±0.86^{A}	6.36±0.78^{A}	0.0235	NS	NS
Fluid release under pressure (%)	Day 0	4.14±0.46^{abA}	4.25±0.61^{abA}	3.64±0.38^{b}	4.14±0.69^{ab}	4.89±0.54^{a}	5.09±0.61^{aA}	0.0007	NS	0.0128
	Day 21	3.29±0.39^{bB}	3.40±0.54^{bB}	3.42±0.67^{b}	4.63±0.46^{a}	4.75±0.20^{a}	4.48±0.35^{aAB}	0.0000	NS	NS
	Day 42	3.05±0.31^{bB}	3.13±0.23^{bB}	3.03±0.37^{b}	4.03±0.33^{a}	4.05±0.60^{a}	3.70±0.43^{abB}	0.0000	NS	NS
Nitrite (mg/kg)	Day 0	56.79±2.95^{b}	62.16±2.16^{ab}	61.27±7.09^{ab}	72.98±7.52^{a}	70.87±6.09^{a}	74.64±7.99^{a}	0.0000	NS	NS
	Day 21	44.39±1.69^{b}	50.15±2.74^{b}	50.23±3.85^{b}	66.02±6.20^{a}	67.25±8.47^{a}	70.73±8.30^{a}	0.0000	NS	NS
	Day 42	28.39±1.42^{bc}	27.07±2.09^{c}	30.09±6.77^{abc}	42.76±9.59^{a}	42.55±7.33^{a}	41.12±3.68^{ab}	0.0000	NS	NS

Additive 1 – phosphates (F treatments); Additive 2 – shell powder (C treatments); Fat 1 – all pork backfat (CON treatments); Fat 2 – with 25% of encapsulated pumpkin seed oil in alginate matrix (ALG treatments); Fat 3 – with 25% of encapsulated pumpkin seed oil in pectin matrix (PEC treatments); NS – not significant. $^{a–c}$ Values (mean±SD) in the same row with different superscripts are significantly different ($p<0.05$). $^{A, B}$ Uppercase letters are used for comparing the samples considering the effect of storage. Values in the same column for the same property, with different superscripts are significantly different ($p<0.05$).

Emulsions stability

As mentioned before, phosphates are very important in the production of emulsified meat products. Alkaline phosphates, mostly used in meat processing [Mills, 2014], increase the pH value and facilitate the extraction of myofibrillar proteins, which enhances water binding properties and emulsification process (thus improves processing yield), gel formation during thermal processing (textural properties), and product stability during retail storage. Water binding properties are of great importance for the quality of meat systems, hence cooking loss, purge loss, and fluid release under pressure (FRP) were useful parameters to evaluate this. In general, pH values higher than the isoelectric point of meat proteins increase the water binding properties of meat systems [Mills, 2014]. Moreover, Puolanne *et al.* [2001] reported a maximum water-holding capacity for beef cooked sausages with phosphates (0.25% P_2O_5) for 2% of NaCl added and pH 6.5.

Regarding cooking loss, phosphate replacement had a significant impact. However, despite significantly lower pH values, all F treatments had lower cooking loss relative to the equivalent C treatments, significantly so only between PECF and PECC (and ALGC). Somewhat similarly to these results are the results of Cho *et al.* [2017] who reported significantly higher cooking loss in ground pork products when replacing 0.3% of phosphates with 0.5% of oyster shell powder. Furthermore, Choi *et al.* [2014] reported significantly higher cooking loss in restructured pork hams (despite higher pH values) when replacing 0.3% of phosphates with different levels (0.15–0.50%) of oyster shell powder and 0.5% of whey protein. However, Lee *et al.* [2011] found no significant differences in cooking loss of emulsified meat products when replacing 0.3% of phosphates with different levels (0.15–0.50%) of oyster shell powder and 0.5% of whey protein. Similar relations to those of cooking loss were found for FRP on day 0 – higher values in C treatments relative to equivalent F treatments, but significantly so only between PECC (and ALGC) and PECF.

After 21 days of storage, the amount of purged liquid was higher in F treatments relative to the equivalent C treatments, however no significant differences were found between any of the treatments. The purge loss increased at the end of storage (day 42), significantly in all treatments, however without an influence on the relations between treatments. On the other hand, FRP decreased during storage, significantly so in CONF, ALGF and PECF, which can be correlated with the increase of purge loss – the higher purge loss, the lower (free) water content which can be released by sample compression. Decreases of FRP during storage influenced the relations between treatments – on days 21 and 42 all F treatments (except PEC on day 42) had significantly lower FRP relative to the equivalent C treatments.

Residual nitrite

As shown in Table 2, the residual nitrite level was significantly influenced by phosphate replacement, while fat replacement and interaction of factors had no significant influ-

TABLE 3. Instrumental colour of cooked model system beef emulsions.

Instrumental colour properties	Storage time	Additive 1			Additive 2			Significance (p)		
		Fat 1	Fat 2	Fat 3	Fat 1	Fat 2	Fat 3			
		CONF	ALGF	PECF	CONC	ALGC	PECC	Additive	Fat	Additive* Fat
L*	Day 0	67.65±2.32^{ab}	66.81±1.60^{b}	67.88±1.02^{abA}	69.00±1.36^{aA}	68.69±1.34^{aA}	68.60±1.85^{aA}	0.0002	NS	NS
	Day 21	67.33±2.09	66.21±0.61	65.54±1.56^{B}	67.00±1.94^{B}	65.60±2.57^{B}	66.33±1.31^{B}	NS	0.0076	NS
	Day 42	67.69±0.98^{a}	66.60±1.72^{ab}	67.38±1.26^{abAB}	67.38±1.0^{abB}	66.06±1.66^{bB}	66.75±0.88^{abB}	NS	0.0017	NS
a*	Day 0	10.27±0.68^{C}	9.52±0.38^{B}	9.35±0.99^{B}	10.17±1.01^{B}	9.67±1.26^{C}	9.77±0.94^{C}	NS	0.0076	NS
	Day 21	12.92±0.76^{aB}	11.56±0.48^{bcdA}	12.52±0.60^{bcAB}	10.94±1.18^{dB}	12.63±1.65^{abB}	11.52±1.26^{cdB}	0.0043	NS	0.0000
	Day 42	13.83±1.37^{abA}	11.81±1.53^{cA}	12.85±1.56^{bcA}	12.85±1.77^{bcA}	15.29±1.92^{aA}	14.31±2.59^{abA}	0.0007	NS	0.0000
b*	Day 0	5.90±1.03^{bA}	8.17±1.15^{aC}	8.04±1.17^{aB}	8.85±1.05^{aA}	7.83±1.00^{aA}	8.56±0.66^{aA}	0.0000	0.0017	0.0000
	Day 21	5.02±0.51^{cB}	9.81±1.25^{aB}	10.23±1.78^{aA}	7.79±0.94^{bB}	7.21±1.23^{bA}	7.81±1.25^{bAB}	0.0034	0.0000	0.0000
	Day 42	6.00±1.03^{cA}	11.33±1.19^{aA}	10.48±1.72^{aA}	7.92±0.77^{bB}	7.77±0.70^{bA}	7.35±0.63^{bB}	0.0000	0.0000	0.0000
C*	Day 0	11.88±0.80^{cC}	12.58±0.75^{abC}	12.37±1.17^{abB}	13.49±1.37^{aB}	12.45±1.51^{abC}	13.00±0.98^{abB}	0.0029	NS	0.0112
	Day 21	13.87±0.79^{bcB}	15.19±0.96^{abB}	16.21±1.43^{aA}	13.45±1.27^{cB}	14.55±2.00^{bcB}	13.96±1.43^{bcB}	0.0002	0.0001	0.0167
	Day 42	15.10±1.50^{cA}	16.46±0.68^{abA}	16.69±1.33^{abA}	15.13±1.64^{cA}	17.17±1.86^{aA}	16.12±2.44^{abA}	NS	0.0002	NS
h	Day 0	29.75±4.41^{bA}	40.43±4.39^{aAB}	40.60±4.52^{aA}	41.01±2.06^{aA}	39.03±2.62^{aA}	41.28±2.66^{aA}	0.0000	0.0000	0.0000
	Day 21	21.24±1.92^{dB}	40.17±3.49^{aB}	38.95±4.25^{aA}	35.48±3.48^{bB}	29.62±1.93^{cB}	34.08±4.30^{bB}	NS	0.0000	0.0000
	Day 42	23.41±3.19^{cB}	43.91±6.38^{aA}	39.14±6.55^{aA}	31.84±3.92^{bC}	27.11±2.78^{bcC}	27.64±3.80^{bcC}	0.0000	0.0000	0.0000

Additive 1 – phosphates (F treatments); Additive 2 – shell powder (C treatments); Fat 1 – all pork backfat (CON treatments); Fat 2 – with 25% of encapsulated pumpkin seed oil in alginate matrix (ALG treatments); Fat 3 – with 25% of encapsulated pumpkin seed oil in pectin matrix (PEC treatments); NS – not significant. $^{a–d}$ Values (mean±SD) in the same row with different superscripts are significantly different ($p<0.05$). $^{A–C}$ Uppercase letters are used for comparing the samples considering the effect of storage. Values in the same column for the same property, with different superscripts are significantly different ($p<0.05$).

ence. Regarding partial fat replacement with different plant oil preparations, literature data varied from the reduced residual nitrite content to no effect [Salcedo-Sandoval *et al.,* 2015] as was the case in this research. Since the decrease of pH values increases the reactivity of nitrite [Skibsted, 2011], the significant influence of phosphate replacement on residual nitrite level can be attributed to the higher pH values of treatments with shell powder. The higher pH values could be the reason why nitrite depletion rate (at the end of storage) was lower [Honikel, 2008a] in C treatments [39.96% (ALGC)–44.91% (PECC)] compared to F treatments [50.01% (CONF)–56.45% (ALGF)].

Colour properties

Colour parameters were significantly influenced by phosphate and fat replacement, as well as their interaction (Table 3).

On day 0, C treatments were lighter than F treatments, however significantly only to ALGF. Regarding redness, no significant differences were found between treatments. The pH values are of significant importance for colour formation in meat systems with nitrite. The nitric oxide (NO) formulation (from added sodium nitrite), which reacts with myoglobin and forms nitrosylmyoglobin (NOMb, bright red colour), is pH dependable – lower pH values accelerate NO formation [Sebranek, 2009; Skibsted, 2011]. In meat batters with the usual pH values of 5.5–6, the nitric oxide production is low and is even lower in meat systems with higher pH values [Honikel, 2014]. At pH values above 6.5, NO formulation is almost suppressed [Feiner, 2006]. However, the addition of ascorbic acid / ascorbate accelerates nitric oxide formation and in turn colour formation as well. Furthermore, the reactivity of ascorbic acid / ascorbate increases with increasing pH [Skibsted, 2011] which could be sufficient for the NOMb formulation in meat systems with pH values slightly higher than 6.5, as was the case in this research. This is in line with the research of Glorieux *et al.* [2017] who used different types of phosphates and did not find significant differences in a* values (also in L* and b*) in emulsified-type pork sausages (with nitrite added) with pH values within the interval of 5.70–6.53. Furthermore, Bae *et al.* [2017] found no effect of phosphate (0.3%) replacement with 0.5% oyster shell powder on redness of ground pork meat products.

Phosphate and partial fat replacement resulted in higher b* and h values in all treatments compared to CONF, while significantly higher chroma values were found only in CONC relative to CONF. Bae *et al.* [2017] found no effect of phosphate (0.3%) replacement with 0.5% oyster shell powder on b* values while Cho *et al.* [2017] reported significantly lower yellowness. Though research data indicate that partial replacement of backfat in frankfurters with different oils (stabilised

in different systems) can increase b* and reduce a* values [Jiménez-Colmenero *et al.,* 2010; Pintado *et al.,* 2016b], the results of this research show no significant differences within C treatments with in any of the observed colour parameters on day 0. The significantly higher b* and h values in CONC relative to CONF could be attributed to the Maillard browning reactions which are promoted by higher pH values in cooking temperatures. Regarding F treatments, colour parameters indicating yellow tones (b* and h values) were significantly higher in treatments with PSO compared to CONF. The significantly higher b* and h values in ALGF and PECF treatments relative to CONF could be attributed to the better stability of PSO microbeads in the presence of Ca^{2+} ions [LeRoux *et al.,* 1999] from shell powder which could be also the reason for the increase of these values during storage.

During storage, all C treatments became less light, significantly so on day 42 compared to the beginning of storage, while lightness of F treatments was not changed. Redness increased significantly during storage in all treatments, and on day 21 it was significantly higher in CONF relative to all modified treatments except ALGC, and PECC at the end of storage. At the end of storage, yellowness values in C treatments were lower (significantly in CONC and PECC) compared to day 0, while in F treatments with pumpkin seed oil they were significantly higher, and unchanged in CONF. This changed the relations between treatments so that all C treatments had significantly lower b* values compared to F treatments with pumpkin seed oil, but still significantly higher than CONF. Calcium alginate lipid containing microbeads are stable under acid and neutral conditions [Zeeb *et al.,* 2015], hence lower pH values in F treatments should not influence the PSO release during storage and increase of b* values. However, research data [LeRoux *et al.,* 1999] indicate that the presence of Na^{+} can reduce the strength of alginate gels due to ion exchange (Ca^{2+} with Na^{+}) in the gel network. The increase of b* values could be a result of the release of the PSO into the meat system (lower stability of PSO microbeads) due to the higher sodium content (due to presence of sodium phosphate) in F treatments, while PSO microbeads were more stable in systems with lower sodium and with the presence of Ca^{2+} ions from shell powder. The PSO was characterised as vegetable oil with very high b* values [Rezig *et al.,* 2012]. A significant decrease of hue angle values in C treatments during storage was also observed, which indicates less yellow product.

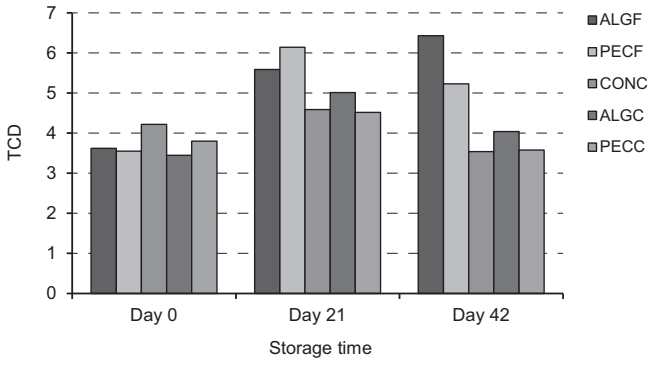


FIGURE 2. Total colour difference (TCD) compared to CONF and during the storage period; F treatments – with phosphates added; C treatments – with shell powder added; CON – all pork backfat; ALG treatments – with 25% of encapsulated pumpkin seed oil in alginate matrix; PEC treatments – with 25% of encapsulated pumpkin seed oil in pectin matrix.

TCD was calculated by a comparison to CONFs' L*a*b* values because CONF represents the model system which is usually prepared (with phosphates added and all fatty tissue). The results (Figure 2) on day 0 were very similar between modified treatments 3.5 (ALGC) – 4.2 (CONC). These values indicate that colour differences were probably perceptible by consumers [Brainard, 2003; Ramírez-Navas & Rodríguez De Stouvenel, 2012]. During storage, TCD values were higher in F treatments compared to all C treatments, reaching values higher than 6, which [Ramírez-Navas & Rodríguez De Stouvenel, 2012] marked as significant values.

Texture properties

The replacement of phosphates with shell powder had a significant influence on the observed textural properties, while fat substitution and interaction of factors had no influence or sporadic influence (Table 4). In meat systems, phosphates exhibit several actions which affect the stability of meat emulsions [Feiner, 2006; Honikel, 2008b]: alkaline phosphates (mostly used in emulsified-type meat products) increase pH, influence actomyosin complex dissociation, enhance protein solubilisation (activation) and increase the ionic strength, hence their substitution poses quite a challenge.

All F treatments had significantly higher hardness, springiness, cohesiveness and chewiness relative to equivalent C treatments on day 0 and throughout storage. On day 0, phosphate replacement with shell powder increased adhesiveness, significantly in all C treatments relative to CONF (a commonly used emulsion system – with phosphates added and all fatty tissue). Backfat replacement with encapsulated PSO led to increased adhesiveness in F and C treatments without any observed effect of encapsulating agents (alginate *vs*. pectin). During storage, the changes of the observed textural properties within the same treatment were mostly not significant except cohesiveness, where a significant increase was observed after 21 days of storage, without further significant changes until the end of storage. However, this did not lead to different relations between treatments. An increase in cohesiveness during storage was observed in emulsified-type meat products with different formulations [Pintado *et al.,* 2016b; Salcedo-Sandoval *et al.,* 2013; Stajić *et al.,* 2020].

Lee *et al.* [2011] replaced 0.3% of phosphates with 0.15, 0.30, and 0.50% of oyster shell powder (+0.50% whey protein in each) in emulsified-type pork sausages and reported a significant increase in hardness, cohesiveness, springiness and chewiness. However, Cho *et al.* [2017] reported significantly lower hardness, cohesiveness, springiness and chewiness when 0.3% of phosphates are replaced with 0.5% of oyster shell calcium powder in ground pork meat products. Similar results were also reported by Bae *et al.* [2017].

There were no differences in the values of cooking loss and purge loss between treatments, which implies similar moisture contents. Thus, differences in textural properties were probably not the result of different moisture/protein

TABLE 4. Texture profile analysis of cooked model system beef emulsions.

Instrumental texture properties	Storage time	Additive 1			Additive 2			Significance (p)		
		Fat 1	Fat 2	Fat 3	Fat 1	Fat 2	Fat 3			
		CONF	ALGF	PECF	CONC	ALGC	PECC	Adv	Fat	Additive* Fat
Hardness (g)	Day 0	1225.28±229.82^{a}	1198.17±160.77^{aAB}	1222.81±245.60^{a}	768.55±219.49^{b}	781.27±120.95^{b}	724.68±70.90^{b}	0.0000	NS	NS
	Day 21	1215.77±130.14^{a}	1074.27±108.32^{aB}	1065.05±225.98^{a}	765.40±101.11^{b}	708.46±88.49^{b}	706.61±95.47^{b}	0.0000	0.0123	NS
	Day 42	1341.55±218.34^{a}	1239.99±123.99^{aA}	1227.07±184.52^{a}	698.07±82.54^{b}	750.17±98.17^{b}	781.96±109.11^{b}	0.0000	NS	NS
Adhesiveness (g × s)	Day 0	-29.09±17.77^{b}	-16.95±4.67^{abAB}	-20.29±16.57^{ab}	-13.07±7.85^{aB}	-11.84±7.86^{a}	-7.29±2.03^{a}	0.0000	0.0478	NS
	Day 21	-20.63±10.62^{c}	-13.41±6.72^{bcA}	-11.17±6.78^{ab}	-5.86±2.86^{aA}	-6.07±2.70^{ab}	-6.11±3.81^{ab}	0.0000	0.0339	0.0236
	Day 42	-26.09±10.51^{c}	-22.20±8.65^{bcB}	-19.68±6.55^{abc}	-10.98±6.52^{aAB}	-12.79±9.28^{ab}	-10.76±7.32^{a}	0.0000	NS	NS
Springiness	Day 0	0.93±0.02^{ab}	0.94±0.02^{aA}	0.92±0.04^{ab}	0.83±0.06^{c}	0.86±0.05^{c}	0.87±0.08^{bc}	0.0000	NS	NS
	Day 21	0.92±0.03^{a}	0.91±0.02^{aB}	0.91±0.02^{a}	0.83±0.05^{b}	0.85±0.04^{b}	0.82±0.05^{b}	0.0000	NS	NS
	Day 42	0.92±0.02^{a}	0.89±0.03^{aB}	0.91±0.04^{a}	0.87±0.05^{b}	0.82±0.06^{b}	0.84±0.04^{b}	0.0000	0.0143	NS
Cohesiveness	Day 0	0.77±0.01^{aB}	0.76±0.02^{aB}	0.75±0.02^{aB}	0.61±0.09^{bB}	0.60±0.08^{bB}	0.63±0.08^{bB}	0.0000	NS	NS
	Day 21	0.81±0.02^{aA}	0.79±0.01^{aA}	0.79±0.02^{aA}	0.71±0.03^{bA}	0.70±0.04^{bA}	0.69±0.04^{bA}	0.0000	NS	NS
	Day 42	0.79±0.01^{aA}	0.79±0.01^{aA}	0.79±0.01^{aA}	0.70±0.06^{bA}	0.68±0.03^{bA}	0.70±0.03^{b}	0.0000	NS	NS
Chewiness (g)	Day 0	871.74±144.95^{a}	851.18±113.27^{a}	844.29±153.05^{a}	403.14±159.96^{b}	407.96±107.80^{b}	402.41±92.54^{b}	0.0000	NS	NS
	Day 21	898.03±111.48^{a}	778.42±80.62^{ab}	768.22±172.31^{b}	451.90±74.22^{c}	422.08±68.59^{c}	401.34±66.31^{c}	0.0000	0.0074	NS
	Day 42	973.74±149.71^{a}	874.07±109.60^{a}	877.54±123.76^{a}	429.35±83.73^{b}	419.46±72.36^{b}	459.53±82.19^{b}	0.0000	NS	NS

Additive 1 – phosphates (F treatments); Additive 2 – shell powder (C treatments); Fat 1 – all pork backfat (CON treatments); Fat 2 – with 25% of encapsulated pumpkin seed oil in alginate matrix (ALG treatments); Fat 3 – with 25% of encapsulated pumpkin seed oil in pectin matrix (PEC treatments); NS – not significant. $^{a–c}$ Values (mean±SD) in the same row with different superscripts are significantly different ($p<0.05$). $^{A–B}$ Uppercase letters are used for comparing the samples considering the effect of storage. Values in the same column for the same property, with different superscripts are significantly different ($p<0.05$).

content. Moreover, the results indicate that fat replacement had no significant influence on the observed textural properties (except adhesiveness). Phosphates promote dissociation of actomyosin complex and myofibrillar proteins extraction, enhance gelation, and in turn increase the hardness of meat products [Glorieux *et al.,* 2017]. The type of phosphates probably had little impact on textural properties [Glorieux *et al.,* 2017]. Moreover, the optimum gelling capacity of myofibrillar proteins at the temperature of 65°C occurs at pH around 6.0, while Ca^{2+} in small quantities enhances gelation [Xiong, 2014]. The possible explanation for lower hardness, springiness, cohesiveness, and chewiness could be better solubilisation (activation)/gelation of myofibrillar proteins in the presence of phosphates.

Partial replacement of backfat with different plant oil preparations does not necessary lead to significantly different textural properties [Pintado *et al.,* 2016a, b], as was observed in this research.

CONCLUSIONS

The substitution of phosphates with shell powder in model system beef emulsions significantly increased pH values (from around 0.8 in raw to about 0.4 in cooked emulsions). However, this did not alter cooking loss, purge loss, and fluid release under pressure. Nitrite content was significantly higher in the treatments with higher pH values, but no significantly lower redness values were measured. Phosphates substitution with shell power will probably lead to higher parameters indicating yellow tones (b* and h values). All the observed textural parameters were significantly altered by the phosphates substitution with shell powder – significantly lower hardness, springiness, cohesiveness, and chewiness were observed, probably due to better solubilisation (activation) of myofibrillar proteins in the presence of phosphates.

Backfat substitution with encapsulated pumpkin seed oil to the level of 25% as well as encapsulating agents altered mostly colour parameters, especially these indicating yellow tones (b* and h values) in the treatments with phosphates. Furthermore, yellowness and hue angle significantly increased during storage in these treatments, while the decrease in the treatments with shell powder was possibly due to the better stability of PSO microbeads in systems with lower sodium and with presence of Ca^{2+} ions from shell powder.

Further research should involve the application of the obtained (positive) results with the aim to develop products and perform examinations which will include microbiological and oxidative stability, and nutritional and sensory quality of meat products obtained by the optimization of a model system into a production system.

RESEARCH FUNDING

The research was financed by the Ministry of Education, Science and Technological Development, Republic of Serbia, projects III–46009 and III–46010.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs. Marija Stajić, MA, for English proofreading.

CONFLICT OF INTERESTS

Authors declare no conflict of interests.

REFERENCES

1. Bae, S.M., Cho, M.G., Jeong, J.Y. (2017). Effects of various calcium powders as replacers for synthetic phosphate on the quality properties of ground pork meat products. *Korean Journal for Food Science of Animal Resources, 37*(3), 456–463.
2. Bourne, M.C. (2002). *Food Texture and Viscosity: Concept and Measurement*, 2nd edition. Academic Press, London, UK, pp. 182–186.
3. Brainard, D.H. (2003). Color appearance and color difference specification. In S.K. Shevell (Ed.), *The Science of Color,* 2nd edition*,* Elsevier Science Ltd., Amsterdam, NL, pp. 191–216.
4. Cho, M.G., Bae, S.M., Jeong, J.Y. (2017). Egg shell and oyster shell powder as alternatives for synthetic phosphate: effects on the quality of cooked ground pork products. *Korean Journal for Food Science of Animal Resources, 37*(4), 571–578.
5. Choi, J.-S., Lee, H.-J., Jin, S.-K., Lee, H.-J., Choi, Y.-I. (2014). Effect of oyster shell calcium powder on the quality of restructured pork ham. *Korean Journal for Food Science of Animal Resources, 34*(3), 372–377.
6. Choi, Y.-S., Choi, J.-H., Han, D.-J., Kim, H.-Y., Lee, M.-A., Kim, H.-W., Jeong, J.-Y., Kim, C.-J. (2009). Characteristics of low-fat meat emulsion systems with pork fat replaced by vegetable oils and rice bran fiber. *Meat Science, 82*(2), 266–271.
7. Feiner, G. (2006). *Meat Products Handbook: Practical Science and Technology*. Woodhead Pub, Cambridge, UK, pp. 74, 151.
8. Glorieux, S., Goemaere, O., Steen, L., Fraeye, I. (2017). Phosphate reduction in emulsified meat products: impact of phosphate type and dosage on quality characteristics. *Food Technology and Biotechnology, 55*(3), 390–397.
9. Honikel, K.O. (2008a). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science, 78*(1–2), 68–76.
10. Honikel, K.O. (2008b). Composition and calories. In L.M.L. Nollet, F. Toldra (Eds.), *Handbook of Processed Meats and Poultry Analysis,* CRC Press, Boca Raton, Florida, USA, pp. 195–213.
11. Honikel, K.O. (2014). Chemical analysis for specific components / Curing agents. In M. Dikeman, C. Devine (Eds.), *Encyclopedia of Meat Sciences,* 2nd edition, Academic Press, Oxford, UK, pp. 200–205.
12. Hygreeva, D., Pandey, M.C., Radhakrishna, K. (2014). Potential applications of plant based derivatives as fat replacers, antioxidants and antimicrobials in fresh and processed meat products. *Meat Science, 98*(1), 47–57.
13. Jiménez-Colmenero, F., Herrero, A., Pintado, T., Solas, M.T., Ruiz-Capillas, C. (2010). Influence of emulsified olive oil stabilizing system used for pork backfat replacement in frankfurters. *Food Research International, 43*(8), 2068–2076.
14. Jimenez-Colmenero, F., Salcedo-Sandoval, L., Bou, R., Cofrades, S., Herrero, A.M., Ruiz-Capillas, C. (2015). Novel applications of oil-structuring methods as a strategy to improve the fat content of meat products. *Trends in Food Science & Technology, 44*(2), 177–188.
15. Kemi, V.E., Kärkkäinen, M.U.M., Lamberg-Allardt, C.J.E. (2007). High phosphorus intakes acutely and negatively affect Ca and bone metabolism in a dose-dependent manner in healthy young females. *British Journal of Nutrition, 96*(3), 545–552.
16. Lee, J.-J., Park, S.-H., Choi, J.-S., Kim, J.-H., Lee, S.-H., Choi, S.-H., Choi, Y.-I., Jung, D.-S. (2011). Effect of oyster shell powder on quality properties and storage stability of emulsion-type pork sausages. *Korean Journal for Food Science of Animal Resources, 31*(3), 469–476.
17. LeRoux, M.A., Guilak, F., Setton, L.A. (1999). Compressive and shear properties of alginate gel: Effects of sodium ions and alginate concentration. *Journal of Biomedical Materials Research, 47*(1), 46–53.
18. Medyński, A., Pospiech, E., Kniat, R. (2000). Effect of various concentrations of lactic acid and sodium chloride on selected physico-chemical meat traits. *Meat Science, 55*(3), 285–290.
19. Mills, E. (2014). Additives/Functional. In M. Dikeman, C. Devine (Eds.), *Encyclopedia of Meat Sciences,* 2nd edition, Academic Press, Oxford, UK, pp. 7–11.
20. Mittal, G.S. (2005). Meat in emulsion type sausages – An overview. *Journal of Food, Agriculture & Environment, 3*(2), 101–108.
21. Montesano, D., Rocchetti, G., Putnik, P., Lucini, L. (2018). Bioactive profile of pumpkin: an overview on terpenoids and their health-promoting properties. *Current Opinion in Food Science, 22*, SI, 81–87.
22. Nedović, V., Kalušević, A., Manojlović, V., Petrović, T., Bugarski, B. (2013). Encapsulation systems in the food industry. In S. Yanniotis, P. Taoukis, N. Stoforos, V.T. Karathanos, (Eds.), *Advances in Food Process Engineering Research and Applications*, Springer, Boston, MA, USA, pp. 229–253.
23. Ospina-E, J.C., Cruz-S, A., Pérez-Álvarez, J.A., Fernández-López, J. (2010). Development of combinations of chemically modified vegetable oils as pork backfat substitutes in sausages formulation. *Meat Science*, *84*(3), 491–497.
24. Pintado, T., Herrero, A., Ruiz-Capillas, C., Triki, M., Carmona, P., Jiménez-Colmenero, F. (2016a). Effects of emulsion gels containing bioactive compounds on sensorial, technological, and structural properties of frankfurters. *Food Science and Technology International, 22*(2), 132–145.
25. Pintado, T., Herrero, A.M., Jiménez-Colmenero, F., Ruiz-Capillas, C. (2016b). Strategies for incorporation of chia (*Salvia hispanica* L.) in frankfurters as a health-promoting ingredient. *Meat Science, 114*, 75–84.
26. Puolanne, E.J., Ruusunen, M.H., Vainionpää, J.I. (2001). Combined effects of NaCl and raw meat pH on water-holding in cooked sausage with and without added phosphate. *Meat Science, 58*(1), 1–7.
27. Ramírez-Navas, J.S., Rodríguez De Stouvenel, A. (2012). Characterization of Colombian quesillo cheese by spectrocolorimetry. *Vitae, 19*(2), 178–185.

28. Rezig, L., Chouaibi, M., Msaada, K., Hamdi, S. (2012). Chemical composition and profile characterisation of pumpkin (*Cucurbita maxima*) seed oil. *Industrial Crops and Products, 37*(1), 82–87.
29. Salcedo-Sandoval, L., Ruiz-Capillas, C., Cofrades, S., Triki, M., Jiménez-Colmenero, F. (2015). Shelf-life of n-3 PUFA enriched frankfurters formulated with a konjac-based oil bulking agent. *LWT – Food Science and Technology, 62*(1), SI, Part 2, 711–717.
30. Sebranek, J.G. (2009). Basic curing ingredients. In R. Tarté (Ed.), *Ingredients in Meat Products: Properties, Functionality and Applications,* Springer, New York, NY, USA, pp. 1–23.
31. Shahidi, F., Samaranayaka, A.G.P., Pegg, R.B. (2014). Curing / Brine curing of meat. In M. Dikeman, C. Devine (Eds.), *Encyclopedia of Meat Sciences,* 2nd edition, Academic Press, Oxford, UK, pp. 416–424.
32. Skibsted, L.H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide, 24*(4), 176–183.
33. Stajić, S., Pisinov, B., Tomasevic, I., Djekic, I., Čolović, D., Ivanović, S., Živković, D. (2020). Use of culled goat meat in frankfurter production – Effect on sensory quality and technological properties. *International Journal of Food Science & Technology, Accepted Author Manuscript*. [http://doi.org/10.1111/ijfs.14346].
34. Stajić, S., Stanišić, N., Tomasevic, I., Djekic, I., Ivanović, N., Živković, D. (2018). Use of linseed oil in improving the quality of chicken frankfurters. *Journal of Food Processing and Preservation, 42*(2), art. no. e13529.
35. Stajić, S., Živković, D., Tomović, V., Nedović, V., Perunović, M., Kovjanić, N., Lević, S., Stanišić, N. (2014). The utilisation of grapeseed oil in improving the quality of dry fermented sausages. *International Journal of Food Science & Technology, 49*(11), 2356–2363.
36. Tomasevic, I., Tomovic, V., Milovanovic, B., Lorenzo, J., Đorđević, V., Karabasil, N., Djekic, I. (2019). Comparison of a computer vision system vs. traditional colorimeter for color evaluation of meat products with various physical properties. *Meat Science, 148*, 5–12.
37. Vural, H., Javidipour, I. (2002). Replacement of beef fat in Frankfurters by interesterified palm, cottonseed and olive oils. *European Food Research and Technology, 214*(6), 465–468.
38. Xiong, Y.L. (2014). Chemical and physical characteristics of meat / Protein functionality. In M. Dikeman and C. Devine (Eds.), *Encyclopedia of Meat Sciences*, 2nd edition, Academic Press, Oxford, UK, pp. 267–273.
39. Yotsuyanagi, S.E., Contreras-Castillo, C.J., Haguiwara, M.M.H., Cipolli, K.M.V.A.B., Lemos, A.L.S.C., Morgano, M.A., Yamada, E.A. (2016). Technological, sensory and microbiological impacts of sodium reduction in frankfurters. *Meat Science, 115*, 50–59.
40. Zeeb, B., Saberi, A.H., Weiss, J., McClements, D.J. (2015). Retention and release of oil-in-water emulsions from filled hydrogel beads composed of calcium alginate: impact of emulsifier type and pH. *Soft Matter, 11*(11), 2228–2236.

Submitted: 13 December 2019. Revised: 29 January and 5 February 2020. Accepted: 7 February 2020. Published on-line: 02 March 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 169–178
DOI: 10.31883/pjfns/118528
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Altitude Effect on the Properties of Honeys from the Region of Jijel (Algeria)

Aissam Bouhala[1], Salim Ouchemoukh[2], Abdelhamid Moussi[3], Said Beldjoudi[1]*

[1]*Faculty of Nature and Life Sciences, University of Mohammed Seddik Benyahia, Jijel, BP 98, Ouled Aissa, Jijel 18000, Algeria*
[2]*Faculty of Nature and Life Sciences, Applied Biochemistry Laboratory, A. Mira University, Bejaia 06000, Algeria*
[3]*Laboratory of Genetic, Biotechnology and Valorization of Bio-Resources, University of Biskra, 07000 Biskra, Algeria*

Key words: Jijel, coastal and mountain honeys, physicochemical properties, antioxidants, antioxidant activity, antibacterial activity

Coastal and mountainous honey samples were collected from different regions in Jijel (Algeria) to evaluate their moisture content, electrical conductivity, ash content, pH, acidity, protein content, color parameters, antioxidants content, radical scavenging activity, reducing power, and antibacterial activity, to reveal the differences between coastal and mountain honeys and to determine the correlation between altitude and different parameters. The results indicate that Jijelian honeys were dark with acceptable physicochemical properties and a good bioactive potential. *Escherichia coli* was sensitive to Jijelian honeys while *Staphylococcus aureus* and *Pseudomonas aeruginosa* were more resistant. Coastal honeys had statistically significantly higher pH, electrical conductivity, ash content, color intensity, hydroxymethylfurfural (HMF) content, and reducing power than the mountainous samples ($p<0.05$), while the total acidity was higher in the mountain honeys ($p<0.05$). The altitude was significantly negatively correlated with HMF content, electrical conductivity, ash content, and pH. The correlation coefficients were -0.510, -0.405, -0.360, and -0.355, respectively.

INTRODUCTION

Honey is a natural product that honeybees produce from some plant parts or excretions of some insects that feed on plant sap [Karabagias *et al.*, 2014]. More than two hundred components have been found in honey; it is an important source of energy due to its high sugar content, mainly fructose (38%) and glucose (31%) [Alvarez-Suarez *et al.*, 2010; Bueno-Costa *et al.,* 2016]. Moreover, it has small amounts of amino acids, proteins, phenolic compounds, carotenoids, organic acids, ascorbic acid, enzymes, α-tocopherol, and oligosaccharides [Alvarez-Suarez *et al.*, 2010]. The composition and characteristics of honey are primarily determined by the food source (plants); however, environmental factors, processing, and storage affect its composition as well [Saxena *et al.*, 2010].

Phenolic compounds content and antioxidant activity have been widely used as indicators to evaluate the characteristics and bioactive properties of honey [Tahir *et al.*, 2017]. Honey contains a variety of phenolics, and is rich in antioxidants, which increases its usability potential for therapeutic purposes [Küçük *et al.*, 2007]. In addition, several other authors have mentioned the antimicrobial potential of honey [Alvarez-Suarez *et al,* 2010; Bueno-Costa *et al.*, 2016; Küçük *et al.,* 2007; Liu *et al.*, 2013]. The concentration of hydrogen peroxide, which is determined according to the level of glucose oxidase (from bees) and catalase (pollen source), in honey mainly predicts its antimicrobial potential, however, lysozyme, phenolic acids, and flavonoids are the major non-peroxide contributing factors [Tenore *et al.*, 2012]. On the other hand, the correlation of the color with bioactive compounds and antioxidant and antibacterial activities has been revealed in other studies [Bueno-Costa *et al.*, 2016]. In recent years, many authors have studied the physicochemical and bioactive properties of honeys from different regions in the world including Algeria [Bueno-Costa *et al.*, 2016; Mouhoubi-Tafinine *et al.*, 2016; Ouchemoukh *et al.*, 2007; Tahir *et al.*, 2017; Tenore *et al.*, 2012], using different analytical methods.

Many scientists have studied the characteristics and the properties of mono-floral honeys produced from different plants by honeybees [Alvarez-Suarez *et al.*, 2010; Karabagias *et al.*, 2014; Küçük *et al.*, 2007; Tenore *et al.*, 2012]. However, the aim of this study was to evaluate the characteristics (physicochemical properties, protein content, color parameters, contents of total phenolics and total flavonoids, DPPH radical scavenging activity, reducing power, and antibacterial activity) of honeys from the greenest region of Algeria (Jijel) from different altitudes, to determine the differences between coastal and mountain honeys and to reveal the correlation between the altitude and different parameters.

* Corresponding Author: Phone: +213774876893; Fax: +21334454489; E-mail: issambouhala@yahoo.fr (A. Bouhala)

MATERIALS AND METHODS

Samples

Twenty-two honey samples were collected from different regions in Jijel (Algeria). Half of these samples were collected from regions close to the Mediterranean Sea and the other half from mountain regions. All these samples were produced from hives placed in areas with diverse vegetation in order to get poly-floral honeys that are more representable of this region. In addition, honeys from hives placed in vast monoculture fields were avoided. All samples were stored at 4–5°C in airtight glass containers until analyses.

Physicochemical analyses

The physicochemical analyses were determined according to the International Honey Commission [2009]. Moisture and ash contents were expressed in g/100g. Acidity, electrical conductivity, and hydroxymethylfurfural (HMF) content were expressed in milliequivalents of sodium hydroxide required to neutralize 1 kg of honey (meq/kg), mS/cm, and mg/kg, respectively.

Protein content

The protein content was analyzed according to the Bradford method reported by Azeredo *et al.* [2003]. The absorbance was measured at 595 nm (UV-1800 UV-Vis Spectrophotometer from Shimadzu, Kyoto, Japan), against a standard solution of bovine serum albumin (0.1–1.4 mg/mL).

Color analysis

Color analysis was reviewed according to Ferreira *et al.* [2009]. Honeys in distilled water solutions of 50% (*w/v*) were centrifuged at 3000×*g* for 10 min (centrifuge Model 3–16P, Sigma Laborzentrifugen GmbH, Osterode, Germany). The color was measured spectrophotometrically at 635 nm. The Pfund scale was used to classify the honeys as follows: mm Pfund = -38.70 + 371.39 ×Abs.

Total phenolics content

The following method described by Bueno-Costa *et al.* [2016] was used to determine the total phenolics content (TPC): a honey solution of (0.1 g/mL) was centrifuged at 3000×*g* for 10 min. Then, 0.5 mL of supernatant and 2.5 mL of 0.2 N Folin–Ciocalteu reagent were mixed for 5 min. Afterwards, 2 mL of a sodium carbonate solution (75 g/L) was added and the mixture was incubated for 2 h in dark. The absorbance was measured using a spectrophotometer at 765 nm. The TPC was expressed as mg gallic acid equivalent per 100 g of sample (mg GAE/100 g).

Total flavonoids content

The total flavonoids content (TFC) was determined according to the method described by Chaikham *et al.* [2016]. A solution of honey in ddH_2O (1 mL; 0.5 g/mL) was mixed with 300 μL $NaNO_2$ (5.0%). A volume of 300 μL of $AlCl_3$ (10%) was added to the mixture, and after 6 min, 2 mL of 1M NaOH was added. A spectrophotometer was used at 510 nm to measure the absorbance. A standard curve was defined by the known concentrations of quercetin (0–40 mg/L), and the results were expressed as mg quercetin equivalent per 100 g of sample (mg QE/100 g).

DPPH radical scavenging activity

Assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (RSA) was performed according to Meda *et al.* [2005] procedure. The 0.75 mL of each honey solution in methanol (2.5–160 mg/mL) was mixed with 1.5 mL of DPPH in methanol (0.02 mg/mL). The mixture was left in the dark for 15 min and then its absorbance was measured at 517 nm. The DPPH radical solution without the sample served as the blank sample. The results were calculated based on the following formula: %Inhibition = [(blank absorbance – sample absorbance)/blank absorbance] ×100. The half maximal inhibitory concentration (IC_{50}) value of each honey sample was estimated from the plot of % inhibition *vs.* honey concentration.

Reducing power

The following method of reducing power (RP) determination was used [Küçük *et al.*, 2017]: 1 mL of a honey solution (5.0%) was added to 2.5 mL of a phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferric cyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50°C for 20 min. Afterwards, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000×*g* for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Ascorbic acid (1.0 mg/mL) was used as a reference standard.

Antibacterial activity

Agar disc diffusion assay of 100% honey concentration was used against three strains of bacteria, which were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 (Pasteur Institute of Algeria, Algeria), according to Alderman & Smith [2001]. The results were expressed in zone of growth inhibition (mm).

Statistical analysis

All tests were performed in triplicate and the results were expressed as mean ± standard deviation. The parameters of the descriptive statistics were calculated using the Microsoft Excel 2007 program. A one-way analysis of variance (ANOVA) was carried out with the STATISTICA 7.1 software to highlight the presence or absence of a significant difference between the samples of honey, which was considered statistically significant at the level of 0.05. LSD test was used as post-hoc ANOVA test ($p<0.05$). The relationships between the parameters were determined by the correlation matrix ($p<0.01$), while the comparison between means obtained for the coastal and mountain honeys was determined by Student's *t*-test using XLSTAT 2014.

RESULTS AND DISCUSSION

Physicochemical parameters of honeys

Moisture content

The Jijelian honeys had a moisture content varying between 16.7 and 19.8 g/100 g (Table 1). The results reported differed significantly ($p<0.05$) among the samples, and all

TABLE 1. Physicochemical parameters of honeys from coastal (C1-C11) and mountain (M1-M11) of the Jijel region of Algeria.

Samples locations	Altitude (m)	Moisture (g/100 g)	pH	Free acidity (meq/kg)	Lactonic acidity (meq/kg)	Total acidity (meq/kg)	EC (mS/cm)	Ash (g/100 g)
Coastal honeys								
Beni Belaid (C1)	6	17.2±0.05^{jk}	4.59±0.07^{a}	18.8±0.65^{ef}	9.2±0.51^{fgh}	28.0±1.11^{fgh}	1.13±0.01^{a}	0.81±0.02^{b}
Oued Zhour (C2)	9	18.5±0.05^{c}	4.14±0.04^{ef}	24.1±2.00^{bc}	8.5±1.41^{ghi}	32.6±3.41^{cde}	0.67±0.01^{g}	0.19±0.02^{mn}
El Janah (C3)	10	18.3±0.05^{d}	4.26±0.05^{bcd}	10.8±0.55^{j}	9.6±0.30^{fg}	20.4±0.98^{kl}	0.89±0.01^{d}	0.66±0.01^{d}
Achouat (C4)	12	18.1±0.05^{e}	4.01±0.02^{ef}	22.1±0.75^{cd}	9.2±0.45^{fgh}	31.3±1.2^{def}	0.65±0.00^{h}	0.38±0.02^{g}
El Balouta (C5)	30	19.8±0.10^{a}	4.34±0.03^{b}	12.5±0.36^{hij}	11.6±0.96^{de}	24.1±1.27^{ijk}	0.51±0.01^{l}	0.13±0.01^{o}
Boukartoum (C6)	40	18.9±0.05^{b}	4.58±0.01^{a}	14.3±2.42^{gh}	8.2±0.55^{hi}	22.5±2.94^{jkl}	1.06±0.01^{b}	0.83±0.01^{a}
Jijel (C7)	60	17.5±0.05^{g}	4.07±0.02^{fgh}	14.4±0.91^{gh}	11.2±1.25^{de}	25.6±2.16^{hij}	0.58±0.01^{j}	0.27±0.00^{j}
El Kennar (C8)	60	18.8±0.10^{b}	3.81±0.04^{i}	20.8±0.30^{de}	8.4±0.36^{ghi}	29.2±0.65^{efgh}	0.62±0.01^{i}	0.21±0.01^{lm}
El Aouana (C9)	65	17.1±0.05^{k}	4.25±0.02^{bcd}	26.2±1.40^{ab}	8.4±0.34^{ghi}	34.6±1.65^{bcd}	0.91±0.01^{c}	0.62±0.01^{e}
Timizer (C10)	70	17.3±0.05^{ij}	4.27±0.01^{bcd}	22.2±1.00^{cd}	10.4±0.65^{ef}	32.6±1.65^{cde}	0.84±0.01e	0.25±0.01^{k}
Ziama Mansouriah (C11)	120	17.1±0.05^{k}	4.19±0.01^{cde}	14.7±0.45^{gh}	12.5±0.36^{d}	27.2±0.80^{ghi}	0.43±0.01^{n}	0.17±0.01^{n}
Mean C	**44**	**18.1±1.36^{A}**	**4.23±0.23^{A}**	**18.3±5.16^{A}**	**9.7±1.47^{A}**	**28.0±4.56^{B}**	**0.75±0.23^{A}**	**0.46±0.28^{A}**
Mountain honeys								
El Milia (M1)	300	17.4±0.05^{hi}	3.64±0.03^{j}	18.8±4.45^{ef}	9.2±1.63^{fgh}	35.5±6.05^{fgh}	0.56±0.01^{k}	0.24±0.01^{k}
Ouled Yahia (M2)	310	18.5±0.05^{c}	3.80±0.01^{i}	28.3±2.45^{a}	7.6±0.55^{i}	35.9±2.95^{abc}	0.52±0.01^{l}	0.33±0.03^{i}
Ghebala (M3)	330	16.7±0.05^{l}	3.68±0.03^{j}	22.3±1.83^{cd}	16.5±0.95^{b}	38.8±2.71^{a}	0.48±0.01^{m}	0.35±0.00^{hi}
Bordj Thar (M4)	340	17.5±0.05^{gh}	3.89±0.06^{i}	20.7±0.80^{de}	10.4±0.72^{ef}	31.1±1.50^{def}	0.63±0.01^{i}	0.37±0.01^{gh}
Oudjana (M5)	400	18.8±0.10^{b}	4.28±0.02^{bc}	12.5±2.57^{hij}	7.2±0.43^{i}	19.7±3.00^{l}	0.62±0.00^{i}	0.41±0.01^{f}
Djimla (M6)	510	18.5±0.05^{c}	4.01±0.02^{h}	20.5±0.69^{de}	9.2±0.75^{fgh}	29.7±1.37^{efg}	0.82±0.01^{e}	0.62±0.01^{e}
Ouled Askeur (M7)	520	17.5±0.05^{gh}	4.01±0.00^{h}	13.6±1.56^{hi}	18.8±0.60^{a}	32.4±2.11^{cde}	0.75±0.01^{f}	0.33±0.00^{i}
Taksana (M8)	570	17.5±0.05^{g}	4.01±0.02^{h}	22.4±0.45^{cd}	9.6±0.75^{fg}	32±1.20^{de}	0.51±0.01^{l}	0.27±0.01^{j}
Selma (M9)	640	17.9±0.05^{f}	4.18±0.02^{de}	16.5±1.77^{fg}	11.2±1.27^{de}	27.7±2.96^{fghi}	0.29±0.01^{o}	0.14±0.02^{o}
Teyana (M10)	695	16.7± 005^{l}	4.04±0.01^{gh}	28.6±0.98^{a}	8.4±0.26^{ghi}	37.0±1.21^{ab}	0.65±0.01^{gh}	0.22±0.01^{kl}
Erraguen (M11)	700	18.8±0.05^{b}	4.12±0.03^{efg}	10.9±1.32^{ij}	14.5±0.85^{c}	25.4±2.12^{hij}	0.62±0.02^{i}	0.23±0.01^{k}
Mean M	**483**	**17.8±0.76^{A}**	**3.97±0.20^{B}**	**19.9±5.93^{A}**	**11.5±3.80^{A}**	**31.4±5.60^{A}**	**0.59±0.14^{B}**	**0.32±0.13^{B}**

EC: electrical conductivity. – Values are presented as mean ± standard deviation (n=3). Values C1-C11 and M1-M11 with lowercase superscript differ significantly (LSD test, $p<0.05$). Means for coastal and mountain honeys (C and M, respectively) marked with different capital letters in superscript are significantly different (t-test, $p<0.05$)

samples were within the limits (>20%) prescribed as per the Codex Alimentarius Commission Standard for honey [2001]. The moisture content of honey is related to different factors like the period of harvesting, ripening process, and climatic conditions [Finola *et al.*, 2007], and it is an important criterion because a higher water content could cause fermentation [Ribeiro *et al.*, 2014].

pH

Honey has an acidic nature with a pH level ranging between 3.2 and 4.5 [da Silva *et al.*, 2016]. The texture, the stability, and the shelf life of honey are affected by the pH level, and low pH usually prevents the development of microorganisms [Kumar *et al.*, 2018]. As can be seen from Table 1, the pH values ranged from 3.64 to 4.59 and were significantly different among the samples ($p<0.05$). The pH value was significantly higher in the coastal honeys than in the mountain ones with mean values of 4.23 against 3.97 ($p<0.05$). In addition, the pH value was significantly negatively correlated with the altitude (r=-0.355; $p<0.01$) (Table 3). On the other hand, Ribeiro *et al.* [2014] and Karabagias *et al.* [2014] reported different pH limits with 2.98–4.15 for Brazilian honeys and 3.40–5.31 for Greek unifloral honeys, respectively. Generally, plant source, soil, inorganic molecules, and honey ripening process can affect the pH level of honey [Ribeiro *et al.*, 2014].

Free, lactonic, and total acidities

In honey, organic acids represent less than 0.50% of the total composition. Nevertheless, they have a major impact on honey acidity, which influences honey flavor and boosts chemical reactions and bioactive activities [Cavia *et al.*, 2007]. In addition, gluconic acid is the most important acid presented in honey, and it comes originally from the activity of glucose oxidase provided by bees through the ripening process [Karabagias *et al.*, 2014]. Table 1 shows the results of measurements of free, lactonic, and total acidities of honey from the Jijel region of Algeria. The free acidity ranged from 10.8 to 28.6 meq/kg. All samples were within the allowed limits fixed by the European Honey Commission (under 50 meq/kg) [Karabagias *et al.*, 2014], showing the honey freshness and the absence of undesirable fermentations [Finola *et al.*, 2007]. On the other hand, Azonwade *et al.* [2018] reported a different range of free acidity within 35.7 and 40.5 meq /kg for Beninese honeys. The equilibrium between organic acids and their corresponding lactones and other mineral ions (*e.g.* phosphate) can be the main factor describing the level of free acidity [Finola *et al.*, 2007]. In addition, the lactonic acidity ranged from 7.2 to 18.8 meq/kg (Table 1). Fröschle *et al.* [2018] reported different lactonic acidity range (14.5±8.2 meq/kg) of Jatropha honey. Finally, the total acidity ranged from 19.7 to 38.8 meq /kg (Table 1). According to literature data, the total acidity ranged from 11.94 to 58.03 meq/kg as reported by Chakir *et al.* [2016] for Maroccan honeys and from 18 to 145.50 meq/kg as determined by Alqarni *et al.* [2016] for national and international Saudi honeys, which were higher than our results. The samples differed significantly ($p<0.05$) in free, lactonic, and total acidities. In addition, the mean value of total acidity was significantly ($p<0.05$) higher in the mountain honeys than in the coastal honeys (31.4 and 28.0 meq/kg). Moreover, free acidity correlated strongly with total acidity (r=0.856) (Table 3), although Kumar *et al.* [2018] observed even a stronger correlation between these parameters for Indian honeys (r=0.920). Finally, only lactonic acidity was negatively correlated with the altitude (r=-0.286; $p<0.05$) (Table 3).

Electrical conductivity (EC)

Electrical conductivity (EC) fell between 0.29 and 1.13 mS/cm (Table 1). The results observed for the honeys differed significantly ($p<0.05$), and EC negatively correlated with the altitude (r=-0.405; $p<0.01$) (Table 3). EC values of coastal and mountain samples were significantly different ($p<0.05$) with the mean values of 0.75 mS/cm against 0.59 mS/cm. However, Can *et al.* [2015] and Karabagias *et al.* [2014] reported higher EC with 0.3 to 1.5 mS/cm for Turkish honey and 0.31 to 2.49 mS/cm for Greek unifloral honeys, respectively. On the other hand, Flores *et al.* [2015] found the EC of honeydew honeys was higher than 0.8 mS/cm. Indeed, mineral salt, organic acid, and protein levels are the most important factors that influence the EC of honey. Moreover, it is an indicator used to distinguish floral honeys from honeydew honeys [Can *et al.*, 2015; Subbiah *et al.*, 2015]. Generally, honeydew honeys have EC greater than 0.8 mS/cm [Codex Alimentarius Commission Standard for Honey, 2001]. Hence, the tested honeys of Jijel included five coastal samples and one mountain sample with an EC value higher than 0.8 mS/cm, indicating that these samples are more likely to be honeydew honeys.

Ash content

Table 1 shows that ash content of honeys from the Jijel region of Algeria ranged from 0.13 to 0.83 g/100 g. Ouchemoukh *et al.* [2007] found a lower ash content for Algerian honey from a different region (0.06 to 0.54 g/100 g). Statistically, the results showed significant differences in ash content ($p<0.05$) and coastal honeys presented higher ash content than the mountain honeys. In addition, a negative correlation (r=-0.360; $p<0.01$) (Table 3) was noted between ash content and altitude. da Silva *et al.* [2016] stated that the ash content in honey ranged from 0.02 to 1.03 g/100 g. The ash indicates the inorganic components and it may be used to indicate environmental pollution [Karabagias *et al*., 2014] and to distinguish the floral origin of honey, which is ≤0.6 g/100 g for blossom honeys and ≤1.2 g/100 g for honeydew honeys [Kumar *et al.,* 2018]. Therefore, the five samples with ash content above 0.6 g/100 g can be determined as honeydew honeys. The correlation coefficient between electrical conductivity and ash content was 0.885 ($p<0.01$). Likewise, Ouchemoukh *et al.* [2007] and Saxena *et al.* [2010] obtained a higher correlation for some Algerian and Indian honeys (0.92 and 0.98), respectively.

Hydroxymethylfurfural content

Hydroxymethylfurfural (HMF) is a furanic compound indicating honey freshness. It is formed as a result of sugars dehydration in acidic conditions (caramelization) throughout heat treatment of food as an intermediate in the Maillard reaction [Pasias *et al.*, 2017] and its content is affected by the sugar content nature, organic acids, pH, water content, and plant source [da Silva *et al.*, 2016]. In this study, HMF content of all honeys was under the maximum limits (40 mg/kg) approved by the Codex Alimentarius Commission Standard for Honey [2001], and ranged from 2.4 to 10.8 mg/kg (Table 2), indicating the freshness of Jijelian honeys. In addition, HMF content was highly significantly negatively correlated with the altitude (r=-0.510; $p<0.01$) (Table 3). The statistical analysis shows that the honeys differed significantly in terms of HMF content ($p<0.05$), which was significantly higher in the coastal samples than in the mountain samples with the mean values at 7.3 mg/kg and 4.4 mg/kg, respectively.

Protein content

Protein represents between 0.2 and 1.6 g/100 g of honey produced by *Apis mellifera*. Both animal and vegetal sources contribute to the presence of proteins and amino acids in honey [da Silva *et al.*, 2016]. The protein content of honeys from the Jijel region of Algeria is presented in Table 2. It ranged from 35 to 900 mg/100g, which was similar to the results obtained by Ouchemoukh *et al.* [2007] for Algerian honey from a different region. On the other hand, Azeredo *et al.* [2003] and Saxena *et al.* [2010] reported lower protein contents for some Brazilian and Indian honeys, respectively. Protein content showed no significant correlation with the altitude.

TABLE 2. Hydroxymethylfurfural (HMF) content, protein content, color intensity and antibacterial activity of honey (from coastal (C1-C11) and mountain (M1-M11) of the Jijel region of Algeria.

Samples	HMF (mg/kg)	Protein (mg/100 g)	Pfund scale (mm)	Color	Zone of growth inhibition (mm)*		
					Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
Coastal honeys							
C1	6.3 ± 1.57^{cdef}	309 ± 18^{gh}	101 ± 10^{g}	amber	9±1.0	0	0
C2	10.8 ± 3.81^{a}	900 ± 25^{a}	122 ± 9^{ef}	dark amber	16±0.6	8±0.6	11±1.0
C3	7.2 ± 0.95^{bcde}	89 ± 9^{m}	141 ± 12^{cd}	dark amber	10±1.7	0	0
C4	8.4 ± 1.66^{bc}	217 ± 22^{k}	79 ± 9^{hij}	light amber	8±0.0	0	0
C5	4.9 ± 1.00^{efgh}	81 ± 9^{m}	52 ± 4^{k}	light amber	10±0.6	0	0
C6	7.6 ± 2.35^{bcd}	582 ± 36^{b}	216 ± 15^{a}	dark amber	10±1.0	0	0
C7	8.2 ± 1.81^{bcd}	469 ± 24^{c}	149 ± 18^{cd}	dark amber	0	0	0
C8	6.1 ± 0.59^{cdef}	425 ± 26^{d}	96 ± 8^{gh}	amber	13±1.7	10±1.0	10±0.0
C9	7.9 ± 1.96^{bcd}	428 ± 38^{d}	179 ± 26^{b}	dark amber	8±1.0	0	0
C10	9.1 ± 1.78^{ab}	246 ± 18^{jk}	190 ± 14^{b}	dark amber	10±0.6	0	0
C11	3.3 ± 0.83^{hi}	98 ± 10^{m}	139 ± 11^{de}	dark amber	7±0.0	0	0
Mean C	**7.3 ± 2.05^{A}**	**349 ± 248^{A}**	**134 ± 50^{A}**	**dark amber**	–	–	–
Mountain honeys							
M1	4.3 ± 0.73^{fghi}	35 ± 6^{n}	23 ± 6^{l}	white	10±0.6	9±1.7	0
M2	3.0 ± 0.22^{hi}	312 ± 20^{gh}	137 ± 11^{de}	dark amber	8±0.0	0	0
M3	9.2 ± 1.15^{ab}	339 ± 8^{fg}	16 ± 3^{l}	extra white	10±1.0	0	0
M4	3.1 ± 0.5^{hi}	157 ± 19^{i}	91 ± 8^{ghi}	amber	9±1.0	0	0
M5	3.7 ± 0.65^{ghi}	294 ± 14^{hi}	158 ± 20^{c}	dark amber	9±0.6	0	0
M6	6.0 ± 1.45^{defg}	$362\pm13_{ef}$	90 ± 2^{ghi}	amber	10±0.0	0	0
M7	2.4 ± 0.45^{i}	374 ± 16^{e}	63 ± 6^{jk}	light amber	0	0	0
M8	4.2 ± 0.63^{fghi}	267 ± 17^{ij}	148 ± 6^{cd}	dark amber	12±1.0	0	0
M9	3.7 ± 0.88^{ghi}	136 ± 18^{i}	76 ± 8^{ij}	light amber	12±1.7	8±0.0	0
M10	7.0 ± 0.61^{bcde}	567 ± 29^{b}	105 ± 7^{fg}	amber	9±0.0	0	0
M11	2.4 ± 0.17^{i}	141 ± 5^{i}	108 ± 8^{fg}	amber	11±1.7	0	0
Mean M	**4.4 ± 2.12^{B}**	**271 ± 147^{A}**	**92 ± 46^{B}**	**amber**	–	–	–

* Undiluted honeys were analyzed. Values are presented as mean ± standard deviation (n=3). Values C1-C11 and M1-M11 with lowercase superscript differ significantly (LSD test, $p<0.05$). Means for coastal and mountain honeys (C and M, respectively) marked with different capital letters in superscript are significantly different (*t*-test, $p<0.05$).

Color analysis

Honey color is a strong indicator of pigments existence, like carotenoids and flavonoids, which provide a good antioxidant activity [Kek *et al.,* 2014]. Color and flavor of honey are connected to each other; while light colored honeys are mild, the darker ones present a strong flavor [Belay *et al.,* 2015]. The color of Jijelian honeys ranged from extra white to dark amber (Table1), and was arranged as follows (Table 2): dark amber (45.45%), amber (27.27%), light amber (18.18%), white color and extra white (4.54%). On the other hand, Bueno-Costa *et al.* [2016] reported light amber (41.7%), amber (25%), and dark amber (33.3%) for Brazilian honeys, while Finola *et al.* [2007] found white (27%), extra white (30%), white (27%), extra light amber (13%), and amber (3%) colors for Argentinian honeys. In addition, 63.63% of the coastal honeys were dark amber, while only 27.27% of the mountain honeys were in dark amber color. Honey color negatively correlated with the altitude ($r=-0.268$; $p<0.05$) (Table 3). Generally, darker honeys tend to have more ash, nutrients, and antioxidants according to their higher correlation with bioactive compounds and different bioactive activities compared to the light colored honeys. In addition, honey color is very important for commercialization, as it attracts the consumers and set their preferences [da Silva *et al.*, 2016].

TABLE 3. Pearson correlation coefficients among parameters.

	Altitude	Moisture	pH	EC	Ash	Free acidity	Lactonic acidity	Total acidity	Pfund scale	HMF	Protein	TPC	TFC	RSA IC_{50}	RP
Altitude	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Moisture	-0.168	1	–	–	–	–	–	–	–	–	–	–	–	–	–
pH	-0.355**	0.255*	1	–		–	–	–	–	–	–	–	–	–	–
EC	-0.405**	0.003	0.565**	1	–	–	–	–	–	–	–	–	–	–	–
Ash	-0.360**	-0.076	0.524**	0.885**	1	–	–	–	–	–	–	–	–	–	–
Free acidity	0.045	-0.391**	-0.336**	-0.011	0.005	1	–	–	–	–	–	–	–	–	–
Lactonic acidity	0.286*	-0.228	-0.187	-0.230	-0.239	-0.307*	1	–	–	–	–	–	–	–	–
Total acidity	0.202	-0.524**	-0.445**	-0.136	-0.124	0.856**	0.230	1	–	–	–	–	–	–	–
Pfund scale	-0.268*	0.102	0.552**	0.437**	0.501**	0.038	-0.415**	-0.187	1	–	–	–	–	–	–
HMF	-0.510**	-0.118	0.176	0.341**	0.324**	0.366**	-0.135	0.301*	0.234	1	–	–	–	–	–
Protein	-0.138	-0.012	0.108	0.306*	0.104	0.415**	-0.204	0.314*	0.323**	0.544**	1	–	–	–	–
TPC	-0.150	0.015	0.314*	0.339**	0.455**	0.062	-0.270*	-0.083	0.738**	0.108	0.053	1	–	–	–
TFC	-0.010	0.019	0.371**	0.335**	0.391**	0.104	-0.278*	-0.045	0.770**	0.024	0.185	0.802**	1	–	–
RSA IC_{50}	0.093	-0.038	-0.202	-0.297*	-0.315**	0.020	0.321**	0.195	-0.691**	-0.038	-0.286*	-0.725**	-0.714**	1	–
RP	-0.265*	-0.099	0.294*	0.323**	0.360**	0.093	-0.203	-0.016	0.615**	0.067	0.058	0.665**	0.609**	-0.663**	1

* Correlation is significant at the 0.05 level. EC: electrical conductivity, HMF: Hydroxymethylfurfural, TPC: Total phenolic content, TFC: Total flavonoid content, RSA: DPPH radical scavenging activity, RP: Reducing power. ** Correlation is significant at the 0.01 level.

Total phenolics content

Phenolics are natural compounds known by their high importance in scientific and therapeutic research [Alvarez--Suarez *et al.*, 2010]. Their level in honey affects the profiling of the the antioxidant power and some sensory properties (*e.g.* color) [Tahir *et al.*, 2017]. Total phenolics content (TPC) of Jijelian honeys was obtained in the range of 48.19 to 147.50 mg GAE/100 g (Figure 1) and it differed significantly ($p<0.05$) among the samples. Whereas, it was not significantly correlated with the altitude (Table 3). Bueno-Costa *et al.* [2016] found lower values (61.16–111.37 mg GAE/100 g) for Brazilian honeys, while Flores *et al.* [2015] reported higher values (79.5–187 mg GAE/100 g) for Spanish honeydew honeys. In honey, the content of phenolics is determined by food source, geo-graphical origin, processing, handling, and storage [Flores *et al.*, 2015].

Total flavonoids content

Flavonoids have a substantial contribution to the antioxidant properties of honey, and they are described as the most important functional compounds of honey [da Silva *et al.*, 2016]. The total flavonoids content (TFC) of honeys from Jijel varied between 5.54 and 46.88 mg QE/100 g (Figure 1), and it differed significantly ($p<0.05$). Furthermore, TFC showed no significant correlation with the altitude (Table 3). Chaikham *et al.* [2016] obtained higher TFC values ranging between 31.52 and 60.73 mg QE/100 g for Thai monofloral honeys, whereas Tenore *et al.* [2012] obtained lower values ranging between 6.85 and 23.17 mg QE/100 g for Italian monofloral honeys. On the other hand, Flores *et al.* [2015] reported lower values in the range of 6.6 and 13.1 mg QE/100 g for Spanish honeydew honeys. Several researchers have already reported that the floral source affects the flavonoid content of honey, and the environmental and climatic conditions depict the nectar composition of melliferous flora [Sousa *et al.*, 2016].

DPPH radical scavenging activity

Recently, honeybees and honey products have been utilized as natural antioxidant sources. In addition, to assess the bioactive features of honey, the antioxidant activity is considered among the most valuable methods [Tahir *et al.*, 2017], which is largely evaluated as DPPH radicals scavenging activity [Liu *et al.*, 2013]. Figure 2 shows the DPPH radical scavenging activity (RSA) expressed as IC_{50} of the tested honeys, which differed significantly ($p<0.05$) between 4.20 and 17.92 mg/mL. Lower IC_{50} means better radical scavenging activity. Escuredo *et al.* [2013] reported similar values in the range from 8.6 to 17.8 mg/mL for Spanish honeys. However, Meda *et al.* [2005] and Beretta *et al.* [2005] reported higher RSA with IC_{50} in ranges from 1.63 to 29.13 mg/mL for Burkinabe honeys and from 1.63 to 45.45 mg/mL for Italian honeys, respectively. RSA was not significantly correlated with the altitude (Table 3).

Reducing power

The reducing power is widely known as a strong criterion of antioxidant capacity [Küçük *et al.*, 2007]. The absorbance

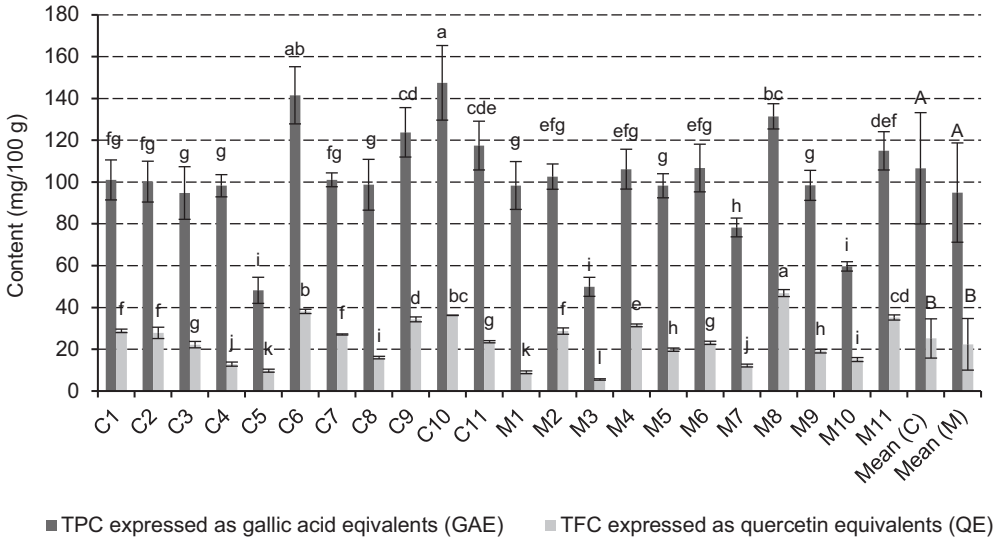


FIGURE 1. Total phenolic content (TPC) and total flavonoid content (TFC) of honeys from coastal (C1-C11) and mountain (M1-M11) Jijel region of Algeria. Bars C and M present mean values for coastal and mountain honeys, respectively. Capital letter (A or B) above these bars indicate no statistically differences between the two group of honeys (t-test, $p \geq 0.05$). Values C1-C11 and M1-M11 with different small letters (a-l) above bars differ significantly (LSD test, $p < 0.05$).

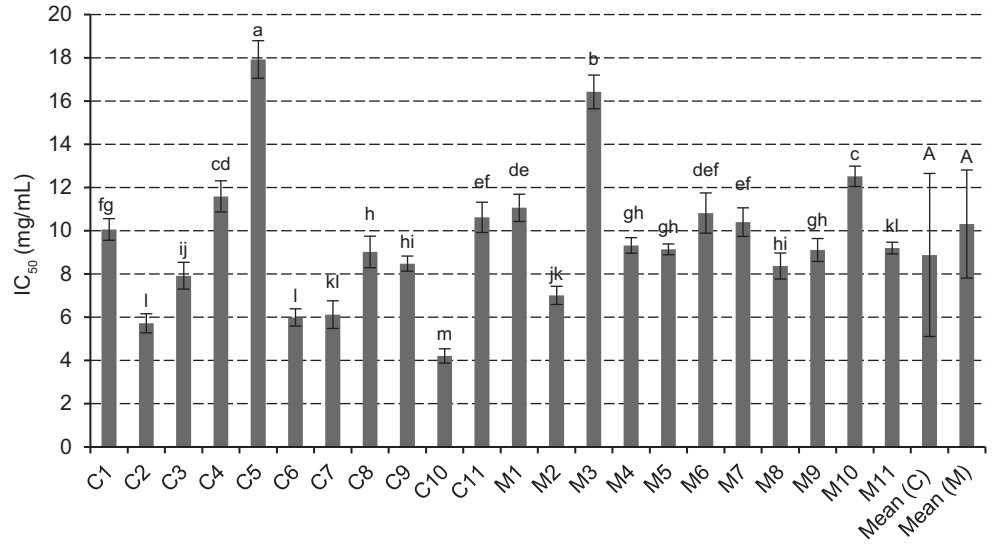


FIGURE 2. DPPH radical scavenging activity (RSA) of honeys from coastal (C1-C11) and mountain (M1-M11) of Jijel region Algeria. Bars C and M present mean values for coastal and mountain honeys, respectively. Capital letter (A) above these bars indicate no statistically significant differences between the two groups (t-test, $p \geq 0.05$). Values C1-C11 and M1-M11 with different small letters (a-m) above bars differ significantly (LSD test, $p < 0.05$).

values of RP assay differed significantly ($p < 0.05$) between 0.11 and 0.47 (Figure 3) and were negatively correlated with the altitude (r=-0.265; $p < 0.05$) (Table 3). Küçük *et al.* [2007] reported that RP varied from 0.11 to 0.78 for three concentrations (1, 5 and 10%) of Turkish honeys, while Saxena *et al.* [2010] reported that it ranged between 0.38 and 0.59 for 10% (v/v) of Indian honeys. Moreover, the coastal honeys presented better reducing power than the mountain honeys (0.35 against 0.29). The reducing power may differ due to the presence of different types of phenolic compounds, non-phenolic compounds (vitamins and amino acids) and other molecules such as enzymes (glucose oxidase and catalase) [Mouhoubi-Tafinine *et al.*, 2016].

Color intensity, TPC, TFC, RSA ($1/IC_{50}$), and RP were highly correlated with each other, with correlation coefficients ranging from r=0.609 to r=0.802 (Table 3) (TFC and RP showed the weakest correlation, while TPC and TFC showed the strongest correlation). Beretta *et al.* [2005] found that the correlation coefficients between color, phenolic content, antiradical activity against DPPH$^{\bullet}$ ($1/IC_{50}$) and ferric reducing antioxidant power (FRAP) of honey from different origin ranged between 0.884 and 0.993. In addition, Alvarez-Suarez *et al.* [2010] found strong correlations between color, TPC, TFC, Trolox equivalent antioxidant capacity (TEAC) and FRAP of monofloral Cuban honeys with r in the range of 0.83–0.97. Furthermore, Ferreira *et al.* [2009] reported

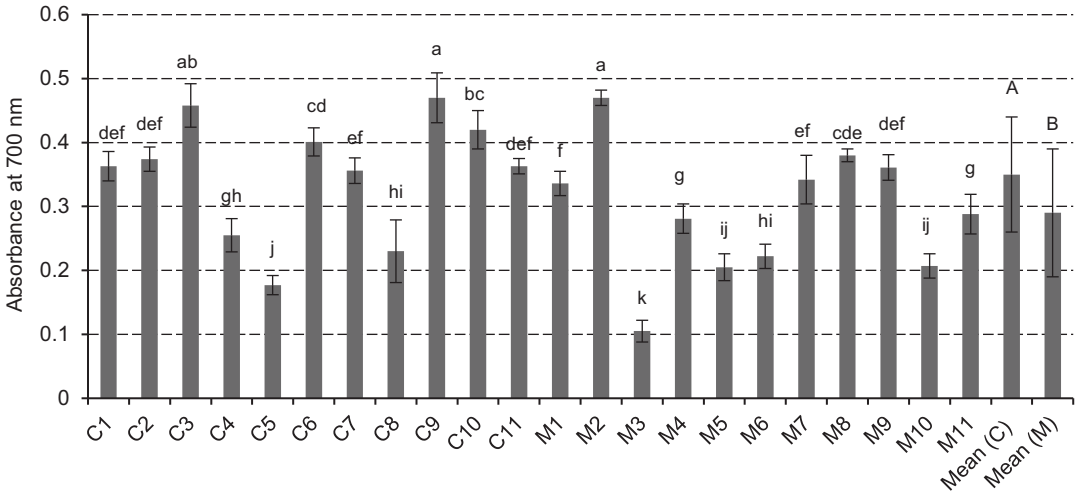


FIGURE 3. Ferric reducing power (RP) of honeys from coastal (C1-C11) and mountain (M1-M11) Jijel region of Algeria. Bars C and M present mean values for coastal and mountain honeys, respectively. Different capital letters (A-B) above these bars indicate significant differences between the two groups (*t*-test, $p<0.05$). Values C1-C11 and M1-M11 with different small letters (a-k) above bars differ significantly (LSD test, $p<0.05$). The absorbance value for 1 mg/mL of ascorbic acid (used as standard) at 700 nm was 1.487.

that dark honey had higher phenolics content, DPPH radical scavenging activity, and reducing power than amber honey and light honey.

Antibacterial activity

The antibacterial activity of honey is mostly depicted by the collective effect of acidity, osmolarity, hydrogen peroxide activity, and phenolic compound content [Molan, 1992]. In this study, 90.90% of Jijelian honeys had an antibacterial activity against *E. coli*, 18.18% against *S. aureus*, and only 9.09% against *P. aeruginosa* (Table 2). Therefore, the Jijelian honeys were more efficient against *E. coli* than *S. aureus* and *P. aeruginosa*. Ten samples of each coastal and mountain honeys presented antibacterial activity against *E. coli* while only C2, C8, M1, and M9 presented antibacterial activity against *S. aureus*. However, only two coastal honeys, C2 and C8, had antibacterial activity against *P. aeruginosa*. Hence, only two coastal samples (C2 and C8) presented antibacterial activity against the three strains of bacteria. Molan [1992] mentioned that honey has antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa*. Our results showed that *E. coli* was the most sensitive microorganism to the Jijelian honeys and *P. aeruginosa* was the most resistant one. On the other hand, Bueno-Costa *et al.* [2016] and Alvarez-Suarez *et al.* [2010] reported that *S. aureus* was the most sensitive microorganism toward Brazilian and Cuban honeys, respectively.

CONCLUSION

The tested honeys from Jijel region of Algeria had a good quality regarding physicochemical parameters, phenolic contents, and bioactive activities and they differed significantly among the samples. In addition, the antibacterial activity analysis showed Jijelian honeys were efficient against *E. coli* and not a good choice against *P. aeruginosa* and *S. aureus*. The coastal samples had higher pH, conductivity, ash and HMF contents, color intensity, and reducing power than the mountainous samples. Whereas, the total acidity was higher in the mountainous honeys. In addition, mountain honeys did not present an antibacterial activity against *P. aeruginosa.* Finally, the altitude was significantly negatively correlated with HMF content, electrical conductivity, ash content, and pH. Further research on the physiochemical properties of honey is recommended and important in order to establish the criteria of assessing the quality of honey.

ACKNOWLEDGEMENTS

The authors thank Pr. Khelili and Pr. Belghobsi for providing the Laboratory of Pharmacology and Phytochemistry (University of Jijel- Algeria) and Pr. Jamie Ellis for providing the Laboratory of Honeybee Research & Extension Lab (University of Florida- USA) to perform some analyses.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

REFERENCES

1. Alderman, D.J., Smith, P. (2001). Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture*, *196*(3–4), SI, 211–243.
2. Alqarni, A.S., Owayss, A.A., Mahmoud, A.A. (2016). Physicochemical characteristics, total phenols and pigments of national and international honeys in Saudi Arabia. *Arabian Journal of Chemistry*, *9*(1), 114–120.
3. Alvarez-Suarez, J.M., Tulipani, S., Díaz, D., Estevez, Y., Romandini, S., Giampieri, F., Elisabetta D., Paola A., Stefano B., Battino, M. (2010). Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food and Chemical Toxicology*, *48*(8–9), 2490–2499.
4. Azeredo, L.C., Azeredo, M.A.A., de Souza, S.R., Dutra, V.M.L. (2003). Protein contents and physicochemical properties in hon-

ey samples of *Apis mellifera* of different floral origins. *Food Chemistry, 80*(4), 249–254.
5. Azonwade, F.E., Paraïso, A., Agbangnan Dossa, C.P., Dougnon, V.T., N'Tcha, C., Mousse, W., Baba-Moussa, L. (2018). Physicochemical characteristics and microbiological quality of honey produced in Benin. *Journal of Food Quality*, *2018*, art. no. 1896057.
6. Belay, A., Solomon, W.K., Bultossa, G., Adgaba, N., Melaku, S. (2015). Botanical origin, color, granulation, and sensory properties of the Harenna forest honey, Bale, Ethiopia. *Food Chemistry*, *167*, 213–219.
7. Beretta, G., Granata, P., Ferrero, M., Orioli, M., Facino, R.M. (2005). Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Analytica Chimica Acta*, *533*(2), 185–191.
8. Bueno-Costa, F.M., Zambiazi, R.C., Bohmer, B.W., Fábio, C.C., Da Silva, W.P., Zanusso, J. T., Dutra, I. (2016). Antibacterial and antioxidant activity of honeys from the state of Rio Grande do Sul, Brazil. *LWT – Food Science and Technology*, *65*, 333–340.
9. Can, Z., Yildiz, O., Sahin, H., Akyuz, E., Silici, S., Kolayli, S. (2015). An investigation of Turkish honeys : Their physico-chemical properties, antioxidant capacities and phenolic profiles. *Food Chemistry*, *180*, 133–141.
10. Cavia, M.M., Fernández-Muiño, M.A., Alonso-Torre, S.R., Huidobro, J.F., Sancho, M.T. (2007). Evolution of acidity of honeys from continental climates : Influence of induced granulation. *Food Chemistry*, *100*(4), 1728–1733.
11. Chaikham, P., Kemsawasd, V., Apichartsrangkoon, A. (2016). Effects of conventional and ultrasound treatments on physico-chemical properties and antioxidant capacity of floral honeys from Northern Thailand. *Food Bioscience*, *15*, 19–26.
12. Chakir, A., Romane, A., Ferrazzi, P., Luigi, G. (2016). Physico-chemical properties of some honeys produced from different plants in Morocco. *Arabian Journal of Chemistry*, *9*, Suppl. 2, S946–S954.
13. Codex Alimentarius Commission Standards (2001). CODEX STAN 12–1981, Rev.1 (1987), Rev.2.
14. da Silva, P.M., Gauche, C., Gonzaga, L.V., Costa, A.C.O., Fett, R. (2016). Honey: Chemical composition, stability and authenticity. *Food Chemistry*, *196*, 309–323.
15. Escuredo, O., Míguez, M., Fernández-González, M., Seijo, M. C. (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chemistry*, *138*(2–3), 851–856.
16. Ferreira, I.C.FR., Aires, E., Barreira, J. C.M., Estevinho, L.M. (2009). Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. *Food Chemistry*, *114*(4), 1438–1443.
17. Finola, S., Lasagno, M.C., Marioli, J.M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry*, *100*(4), 1649–1653.
18. Flores, M.S.R., Escuredo, O., Seijo, M.C. (2015). Assessment of physicochemical and antioxidant characteristics of *Quercus pyrenaica* honeydew honeys. *Food Chemistry*, *166*, 101–106.
19. Fröschle, M., Horn, H., Spring, O. (2018). Characterization of *Jatropha curcas* honeys originating from the southern highlands of Madagascar. *LWT – Food Science and Technology*, *93*, 525–533.
20. International Honey Commission (2009). Harmonized Methods of the International Honey Commission. p. 63 [http://www.bee-hexagon.net/en/network.htm].
21. Karabagias, I.K., Badeka, A.V, Kontakos, S., Karabournioti, S., Kontominas, M.G. (2014). Botanical discrimination of Greek unifloral honeys with physico-chemical and chemometric analyses. *Food Chemistry*, *165*, 181–190.
22. Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W., Chua, L.S. (2014). Total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and *Trigona* spp. bees. *Agriculture and Agricultural Science Procedia*, *2*, 150–155.
23. Küçük, M., Kolaylı, S., Karaoğlu, Ş., Ulusoy, E., Baltacı, C., Candan, F. (2007). Biological activities and chemical composition of three honeys of different types from Anatolia. *Food Chemistry*, *100*(2), 526–534.
24. Kumar, A., Gill, J.P.S., Bedi, J.S., Manav, M., Ansari, M.J., Walia, G.S. (2018). Sensorial and physicochemical analysis of Indian honeys for assessment of quality and floral origins. *Food Research International*, *108*, 571–583.
25. Liu, J.R., Ye, Y.L., Lin, T.Y., Wang, Y.W., Peng, C.C. (2013). Effect of floral sources on the antioxidant, antimicrobial, and anti-inflammatory activities of honeys in Taiwan. *Food Chemistry*, *139*, 938–943.
26. Meda, A., Lamien, C.E., Romito, M., Millogo, J., Nacoulma, O.G. (2005). Determination of total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry*, *91*(3), 571–577.
27. Molan, P.C. (1992). The antibacterial activity of honey. 1. The nature of antibacterial activity. *Bee World*, *73*(1), 5–28.
28. Mouhoubi-Tafinine, Z., Ouchemoukh, S., Tamendjari, A. (2016). Antioxydant activity of some Algerian honey and propolis. *Industrial Crops & Products*, *88*, SI, 85–90.
29. Ouchemoukh, S., Louaileche, H., Schweitzer, P. (2007). Physicochemical characteristics and pollen spectrum of some Algerian honeys. *Food Control*, *18*(1), 52–58.
30. Pasias, I.N., Kiriakou, I.K., Proestos, C. (2017). HMF and diastase activity in honeys: a fully validated approach and a chemometric analysis for identification of honey freshness and adulteration. *Food Chemistry*, *229*, 425–431.
31. Ribeiro, R.D.O.R., Máralém, E.T., Carneiro, C. da Silva, Monteiro, M.L.G., Conte, C.A., Mano, S., de Jesus, E.F.O. (2014). Classification of Brazilian honeys by physical and chemical analytical methods and low field nuclear magnetic resonance (LF ^{1}H NMR). *LWT – Food Science and Technology*, *55*(1), 90–95.
32. Saxena, S., Gautam, S., Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, *118*(2), 391–397.
33. Sousa, J.M., de Souza, E., Marques, G., Meireles, B., Cordeiro, Â., Gullónd, B., Pintado, M.M., Magnani, M. (2016). Polyphenolic profile and antioxidant and antibacterial activities of monofloral honeys produced by *Meliponini* in the Brazilian semiarid region. *Food Research International*, *84*, 61–68
34. Subbiah, B., Stembridge, A.L., Morison, K.R. (2015). Measurement and calculation of the electrical conductivity of model honey solutions. Melbourne, Australia: *APCChE 2015 Congress*, 27 September – 1 October 2015, 2261–2269 [https://search.informit.com.au/documentSummary;dn=732764357650043;res=IELENG].

35. Tahir, H.E., Xiaobo, Z., Zhihua, L., Jiyong, S., Zhai, X., Wang, S., Mariod, A.A. (2017). Rapid prediction of phenolic compounds and antioxidant activity of Sudanese honey using Raman and Fourier transform infrared (FT-IR) spectroscopy. *Food Chemistry*, *226*, 202–211.
36. Tenore, G.C., Ritieni, A., Campiglia, P., Novellino, E. (2012). Nutraceutical potential of monofloral honeys produced by the Sicilian black honeybees (*Apis mellifera* ssp. *sicula*). *Food and Chemical Toxicology*, *50*(6), 1955–1961.

Submitted: 5 October 2019. Revised: 8 December 2019 and 9 February 2020. Accepted: 28 February 2020. Published on-line: 08 April 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 179–188
DOI: 10.31883/pjfns/119674
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Proximate Composition, Mineral Profile and Trypsin-Inhibitory Activity of West African Leafy Vegetables: Influence of Urea Micro-Dosing and Harvest Time

Modoukpè Imayath Djibril Moussa[1], Adeola Monisola Alashi[2], Carole Nadia Adjouavi Sossa-Vihotogbé[3], Pierre Bienvenu Irénikatché Akponikpè[4], Mohamed Nasser Baco[5], André Jonas Djènontin[4], Rotimi Emmanuel Aluko[2], Noël Houédougbé Akissoé[1]*

[1]*Ecole de Nutrition, des Sciences et Technologies Alimentaires, Faculté des Sciences Agronomiques, Université d'Abomey-Calavi, 03 BP 2819 Jéricho, Cotonou, République du Bénin*
[2]*Department of Food and Human Nutritional Sciences, Faculty of Agricultural and Food Sciences, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2*
[3]*Département de Nutrition et Sciences Agro-Alimentaires, Faculté d'Agronomie, Université de Parakou, République du Bénin*
[4]*Laboratoire d'Hydraulique et de Modélisation Environnementale, Faculté d'Agronomie, Université de Parakou, 03 BP 351, Parakou, République du Bénin*
[5]*Laboratoire Société-Environnement, Faculté d'Agronomie, Université de Parakou, BP 27, Parakou, République du Bénin*

Key words: green leafy vegetables, harvest time, mineral composition, urea micro-dosing, trypsin inhibition

In order to ensure the nutritional quality of leafy vegetables produced under intensive cultivation systems, the proximate composition, mineral profile, and trypsin-inhibitory activity of three priority West African vegetable species (*Amaranthus cruentus*, *Ocimum gratissimum*, and *Solanum macrocarpon*) produced with urea micro-doses (20, 40, and 60 kg/ha) were compared with control leaves raised without urea. Plants were harvested three consecutive times to determine effects on nutrient and trypsin-inhibitory activity. Proximate and mineral compositions were mainly species-dependent ($p<0.05$) though the 60 kg/ha urea dose produced the significantly ($p<0.05$) highest moisture content in leaves. Calcium, copper, iron, and zinc contents were significantly ($p<0.05$) influenced by harvest time and its interaction with vegetable species. Furthermore, trypsin inhibition was significantly ($p<0.05$) higher for the second and third leaf harvests, which suggests that initial wounding from the first harvest enhanced synthesis of the inhibitor protein molecules.

INTRODUCTION

Plant foods play a key role in the maintenance of human life as they supply the needed nutritional elements that boost the immune system [Fonge *et al.,* 2016; Das *et al.,* 2017]. Vegetables are food resources that are considered as healthy foods worldwide [Souza *et al.,* 2016; Das *et al.,* 2017]. Among all vegetables, green leafy vegetables are available all year round, at relatively low cost, and are widely used as basic ingredients in traditional dishes and nutrition therapy [Fonge *et al.,* 2016; Souza *et al.,* 2016; Das *et al.,* 2017]. In the Republic of Benin, a biodiversity inventory revealed 187 plant species used as indigenous leafy vegetables with *Amaranthus cruentus* (*Amaranthaceae*), *Ocimum gratissimum* (*Lamiaceae*), and *Solanum macrocarpon* (*Solanaceae*) being among the priority species [Dansi *et al.,* 2008]. These species are essential in West African diets, because they are used in the preparation of sauces [Dansi *et al.,* 2008; Vodouhè *et al.,* 2012]. They are also rich in fibers, minerals, vitamins, and polyphenols, which are involved in the growth and maintenance of good health for all age groups [Nana *et al.,* 2012; Adewale *et al.,* 2014; Akinwunmi & Omotayo, 2016]. The extracts obtained from these leafy vegetables have antioxidant and enzyme inhibitory activities that provide evidence for sustaining their use in traditional medicine to prevent and manage several human ailments [Nana *et al.,* 2012; Adewale *et al.,* 2014; Djibril Moussa *et al.,* 2019a, b].

However, these leafy vegetables contain anti-nutrients such as protease inhibitors, which may have a negative impact on human health, by reducing protein digestibility and limiting the availability of essential amino acids [Patel & Zaveri, 2015]. Thus, protease inhibitors can limit the caloric value of diets, which could be useful in disease conditions where excessive catabolic activity (*e.g.*, pancreatitis) is undesirable [Jedinak *et al.,* 2010]. Smaller quantities

* Corresponding Author: E-mail: dimayath@gmail.com (M.I. Djibril Moussa)

of trypsin inhibitors induced satiety by promoting the secretion of cholecystokinin, a satietogenic hormone associated with limited food, energy, and weight intakes [Carvalho *et al.,* 2016]. Hence, consumption of leafy vegetable extracts having trypsin-inhibitory activities could be beneficial for the management of metabolic abnormalities resulting from high food and energy intakes. Moreover, anti-viral effects of trypsin inhibitors [Shahwar *et al.,* 2012] could extend their health benefits, especially in rural areas where diarrhea is a common occurrence. Otherwise, trypsin inhibitors are proteinaceous in nature, which means that they are heat sensitive and can be partially or completely denatured and inactivated at elevated temperatures (>60°C) [Allen *et al.,* 2012; Pesoti *et al.,* 2015].

The nutritional profile of leafy vegetables is affected by soil fertility [Fonge *et al.,* 2016; Souza *et al.,* 2016; Das *et al.,* 2017], while efficient use of fertilizers (mineral and/or organic) is of great interest because of consumer demand for healthier food sources [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. A recent farming system known as fertilizer micro-dosing technology involves the application of small quantities of mineral fertilizer at an optimized time, depth, and distance to the target plant [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. This technology gave promising prospects in terms of crops productivity although the combined application of mineral and organic fertilizers was recommended for its sustainability [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. However, the effect of fertilizer micro-dosing on the nutritional properties of farming products, including leafy vegetables is far from being fully understood. Moreover, leaves maturity stage at harvest is an important factor that could influence their nutrient contents since plants' development stage affected nutrients uptake by plants [Sossa-Vihotogbé *et al.,* 2013; Bvenura & Afolayan, 2014a, b]. Therefore, this study was undertaken to assess proximate composition, mineral profile, and trypsin-inhibitory activity of *A. cruentus*, *O. gratissimum*, and *S. macrocarpon* leaves produced with urea micro-doses and harvested at different times.

MATERIALS AND METHODS

Materials

Plants (*A. cruentus*, *O. gratissimum*, and *S. macrocarpon*) were cultivated between January and June 2016 in an experimental farm at the Northern Center of National Agricultural Research Institute ("INRAB") in Republic of Benin (9°57' N, 2°43' E and 358 m a.s.l.; Ina village, Bembereke district). Seeds were bought from "INRAB" and grown in nurseries for four (*A. cruentus*) or six (*O. gratissimum* and *S. macrocarpon*) weeks. The experimental design was a randomized complete block, in four replicates for each vegetable species, with three rates of urea (20, 40, and 60 kg/ha) applied immediately after transplanting through micro-dosing technology [Likpètè *et al.,* 2019] while the control plots contained no urea (0 kg/ha). Cattle manure (5 t/ha) was applied a week before transplantation to all the experimental plot units (6 m × 1 m) as base fertilizer. Harvests were done, on the same plants by cutting the main stems at a height of 10 cm from soil level, excluding those of the border lines, at 4–6–8, 6–10–14, and 8–12–16 weeks after transplanting for *A. cruentus*, *S. macrocarpon*, and *O. gratissimum*, respectively. These harvest times correspond to late vegetative stage (before flower initiation) for each species. For the purpose of this study, samples of 2.5 kg of fresh leaves with stems were harvested from each experimental plot for each vegetable species at each harvest, then edible leaves were washed and oven-dried at 60°C using a Memmert UN450 drier (Memmert GmbH + Co. KG, Schwabach, Germany), for a minimum of 24 h or until a constant weight was achieved. Dried leaves were ground using a Cuisinart Grinder (Model DCG-12 BCC, Cuisinart, ON, Canada) and leaf powder was stored at -20°C.

Proximate composition analysis

Proximate analysis was carried out in duplicate using standard methods [AOAC, 2005; AOCS, 2009]. Moisture was determined by oven-drying (Heraeus T 5042 EK oven, Heraeus, Hanau, Germany) of fresh leaves at 105°C for 24 h [AOAC, 2005; method 930.15]. Protein content was determined as total nitrogen (× 6.25) using Kjeldahl method after H_2SO_4 (95%) digestion using Kjeldahl pastilles as a catalyst followed by distillation into 4% (*w/v*) H_3BO_3 and titration with 0.1 M HCl [AOAC, 2005; method 990.03]. Crude fat content was determined after petroleum ether extraction in an Ankom XT10 extraction system (ANKOM Technology, NY, USA) [AOCS, 2009; method Am 5–04]. Crude fiber content was estimated after digestion with H_2SO_4 (1.25%, *v/v*) and NaOH (0.3 M), using an Ankom 220 fibre analyzer [AOCS, 2009 method Ba6a-05]. Ash content was determined by the incineration method in a muffle furnace (Vulcan A-550, Dentsply Ceramco Inc, York, PA, USA) at 550°C overnight for 6 h [AOAC, 2005; method 923.03]. Except for moisture content, proximate composition was expressed on dry weight basis (dwb).

Mineral profile analysis

Mineral profile (dwb) was determined in duplicate by digesting white ashes in a mixture of HNO_3 and $HClO_4$ (4:1) for 24 h [AOAC, 2005; method 968.08D]. The supernatant was filtered using Whatman No. 42 filter paper and the filtrate was analyzed by atomic absorption spectroscopy (PerkinElmer AAnalyst 200, PerkinElmer, MA, USA) to assess calcium, copper, iron, magnesium, manganese, potassium, sodium, and zinc contents. Phosphorus content was determined by colorimetric method at 680 nm using a UV mini 1240 spectrophotometer (Shimadzu Corp., Kyoto, Japan) [AOAC, 2005; method 968.08D]. The nutritional interrelationships between mineral levels were evaluated by calculating the following content ratios: calcium/magnesium, calcium/phosphorus, calcium/potassium, iron/copper, sodium/magnesium, sodium/potassium, and zinc/copper [Watts, 2010].

Trypsin inhibition assay

Trypsin-inhibitory activity was assessed (in triplicate) by Patel & Zaveri [2015] method with slight modifications using aqueous extract of the leaf powder [Djibril Moussa *et al.,* 2019b]. Leaf powder (10 g) was transferred into double de-

ionized water (200 mL) and the mixture was stirred for 2 h at 60°C before centrifuging for 30 min (5000 × g at 4°C). The resultant supernatant was stored while the precipitate was redispersed in water (200 mL). The process above was repeated and both supernatants were pooled together, concentrated using a vacuum evaporator and freeze-dried. The dried extracts (0.16–250.00 μg/mL) and 4(-2-aminoethyl) benzenesulfonyl-fluoridehydrochloride (AEBSF, 0.78–25.00 μg/mL) were dissolved in 20 mM Tris-HCl buffer (pH 7.5). A 200 μL aliquot of the sample was mixed with 200 μL of trypsin (270 μg/mL) and incubated for 5 min at 37°C and 400 rpm using a thermomixer (Eppendorf AG, Hamburg, Germany). Thereafter, 500 μL of *N*-benzoyl-DL-arginine-*p*-nitroanilidehydrochloride (BAPNA, 0.9 mM) was added and agitated (400 rpm) for 10 min at 37°C. The addition of 100 μL of acetic acid (30%) stopped the reaction with continuous shaking (400 rpm) for 5 min at 37°C. The absorbance of the mixture was determined in comparison with that of a blank at 410 nm using a microplate reader Synergy H4 (Biotek Corp., Winooski, VT, USA) coupled with Gen5 2.04 data analysis software. Concentration of extracts required to inhibit 50% of trypsin activity was estimated using Prism 6 software (GraphPad Corp., La Jolla, USA).

Statistical analysis

Data were analyzed using R statistical software version 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria). Three-way analysis of variance on repeated measures was performed to investigate the main and interaction effects of vegetable species, urea micro-doses, and harvest times on proximate composition, mineral profile, and trypsin-inhibitory activity of the leafy vegetables. Significance was set at 5% and means were segregated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Variation in proximate composition of the leafy vegetables

Moisture content was the most abundant component followed by protein, fiber, and fat. The range of moisture content of *S. macrocarpon* (86.0–90.1 g/100 g) was higher than those of *A. cruentus* (76.7–87.6 g/100 g) and *O. gratissimum* (79.3–87.4 g/100 g) which are similar to those reported for *Justicia tenella* and *Sesamum radiatum* (72.0–83.3 g/100 g) by Sossa-Vihotogbé *et al.* [2013]. High moisture content is an index of freshness and fast perishability due to the activity of water-soluble enzymes involved in metabolic processes and microbial growth [Vodouhè *et al.,* 2012; Adjatin *et al.,* 2013]. The range for protein and fiber contents of *S. macrocarpon* (27.6–34.1 g/100 g and 16.7–25.5 g/100 g) were higher respectively than those of *A. cruentus* (21.2–26.3 g/100 g and 9.8–12.7 g/100 g) and *O. gratissimum* (17.1–26.9 g/100 g and 13.1–16.7 g/100 g) which are comparable to those reported earlier [Sossa-Vihotogbé *et al.,* 2013; Akinwunmi & Omotayo, 2016] for *A. cruentus* (17.0 g/100 g and 10.4 g/100 g), *J. tenella* (17.2–33.8 g/100 g and 8.7–13.1 g/100 g), *O. gratissimum* (12.2 g/100 g and 10.5 g/100 g), and *S. radiatum* (18.8–27.8 g/100 g and 6.9–10.9 g/100 g). Protein contents in leaves (>12 g/100 g, dwb) suggest their potency as good sources of proteins, which may act as anabolic agents and as alternative sources of energy through gluconeogenesis when carbohydrates metabolism is impaired [Vodouhè *et al.,* 2012; Adjatin *et al.,* 2013]. The potential preventive effects of high fiber contents against gastrointestinal disorders, absorption of excess cholesterol and colon cancer [Vodouhè *et al.,* 2012; Adjatin *et al.,* 2013] suggest that these three species are good candidates for healthy maintenance of the human body.

Fat contents in *A. cruentus* (1.0–4.0 g/100 g), *O. gratissimum* (2.7–5.4 g/100 g), and *S. macrocarpon* (2.6–4.6 g/100 g) were similar and are within the range previously reported [Sossa-Vihotogbé *et al.,* 2013; Akinwunmi & Omotayo, 2016] for *A. cruentus* (1.2 g/100 g), *S. macrocarpon* (3.0 g/100 g), *J. tenella*, and *S. radiatum* (1.9–7.3 g/100 g). Conversely, higher fat contents were reported for *O. gratissimum* (6.6 g/100 g), *T. occidentalis* (7.6 g/100 g), and *V. amygdalina* (6.0 g/100 g) by Akinwunmi & Omotayo [2016]. The range of ash recorded for *O. gratissimum* (14.6–17.8 g/100 g) were lower than those of *A. cruentus* (22.1–24.9 g/100 g) and *S. macrocarpon* (16.6–23.9 g/100 g). The ash contents recorded were higher than that reported [Sossa-Vihotogbé *et al.,* 2013; Akinwunmi & Omotayo, 2016] for *J. tenella* (10.4–17.0 g/100 g), *O. gratissimum* (5.7 g/100 g), *S. radiatum* (7.7–12.3 g/100 g), and *T. occidentalis* (11.2 g/100 g) but similar to the values reported for *A. cruentus*, *S. macrocarpon*, and *V. amygdalina* (15.5–23.1 g/100 g) by Akinwunmi & Omotayo [2016]. Ash content is an index of mineral levels in food products, and it indicates potential health benefits because of the key roles of minerals in various metabolic processes [Adjatin *et al.,* 2013; Bvenura & Afolayan, 2014a, b].

Proximate composition as linked to vegetable species, urea micro-doses, and harvest times

There was no significant effect of urea micro-doses (except on moisture content, $p=0.03$) and their interaction with vegetable species and/or harvest times on proximate composition of the leafy vegetables (Table 1). The significantly highest moisture contents were found with the application of 60 kg/ha of urea (85.1 g/100 g). Moreover, moisture ($p=0.02$) and ash ($p=0.04$) contents were significantly influenced by interaction of vegetable species and harvest times (Table 1) with the highest contents recorded at the second harvest of *S. macrocarpon* (Figure 1a) and at the first harvest of *A. cruentus* (Figure 1b). Vegetable species had a significant effect on moisture ($p<0.01$), fat ($p=0.03$), fibers ($p=0.02$), protein ($p=0.02$), and ash ($p<0.01$) contents while moisture content was significantly ($p=0.01$) affected by harvest times (Table 1). The highest fibers and protein contents were recorded with *S. macrocarpon* whereas *A. cruentus* and *O. gratissimum* had the highest ash and fat contents, respectively (Table 2). Genotype and its interaction with fertilization type and rate were reported to influence plant responsiveness to nutrient availability [Sossa-Vihotogbé *et al.,* 2013; Fonge *et al.,* 2016]. Thus, the proximate composition of the leafy vegetables was expected to vary with vegetable species, urea micro-doses, and harvest times.

The lack of significant effect of urea micro-doses on proximate composition can be explained by the fertilization method applied. Indeed, high protein contents were related

TABLE 1. Effect of vegetable species (VS), urea micro-doses (UMD), and harvest times (HT) on proximate composition and mineral profile of the vegetable leaf powders as well as on trypsin-inhibitory activity of vegetable leaf extracts.

Variables		Main factors			Interaction between factors			
		VS	UMD	HT	VSxUMD	VSxHT	UMDxHT	VSxUMDxHT
Proximate composition	Moisture	4696.2β**	487.1*	12702.9**	146.5	648.7*	53.1	61.2
	Fat	543.0*	26.3	27.4	33.8	40.9	18.6	22.4
	Fibers	1382.8*	23.6	30.9	21.7	770.3	73.5	11.3
	Protein	1371.6*	3.6	0.2	3.0	98.8	1.7	7.1
	Ash	5618.2**	2.9	122.9	30.9	303.4*	50.2	64.8
Major minerals and their ratios	Calcium	124.5	8.3	1596.7*	16.4	198.7*	2.8	7.1
	Magnesium	3089.7*	10.7	135.1	69.0	205.1	7.1	12.8
	Phosphorus	2107.9*	9.6	0.6	0.8	133.7	12.3	2.9
	Potassium	186.1*	28.6	11.0	11.4	160.9	3.3	10.5
	Sodium	710.7*	3.4	5.2	2.6	3.5	7.8	9.4
	Calcium/magnesium	217.2*	12.6	8.8	2.2	45.7	5.6	8.6
	Calcium/phosphorus	1427.1*	25.9	1584.9*	19.4	349.3*	34.9	13.9
	Calcium/potassium	504.5*	11.2	612.1*	5.0	412.9*	4.0	1.9
	Sodium/magnesium	687.2*	27.3	2.3	2.4	2.6	12.8	3.9
	Sodium/potassium	1389.6*	17.1	6.7	4.9	0.8	14.8	5.4
Trace minerals and their ratios	Copper	4675.1**	3.5	188.3*	0.9	493.3*	6.6	4.9
	Iron	6885.1**	49.5	564.9*	17.8	205.4*	8.1	17.1
	Manganese	2477.3*	28.8	382.5*	45.5	86.9	9.6	17.1
	Zinc	579.1*	179.5*	152.2*	42.7	567.5*	99.1	42.0
	Iron/copper	1609.8*	152.4	249.5*	281.8	266.3*	10.2	23.3
	Zinc/copper	2101.9*	71.3	274.0*	78.7	1535.3*	91.1	58.7
Trypsin inhibition		1692.5***	37.4*	4.2	13.4	15.9	165.2**	180.4**

VS is used for the three vegetable species (*A. cruentus*, *O. gratissimum* and *S. macrocarpon*), UMD correspond to the four urea micro-doses (0, 20, 40, and 60 kg/ha) and HT indicate the three successive harvests of *A. cruentus* (4, 6 and 8 weeks after transplanting, respectively), of *O. gratissimum* (8, 12, and 16 weeks after transplanting, respectively) and of *S. macrocarpon* (6, 10 and 14 weeks after transplanting, respectively). β: F-value. Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

to sufficient nitrogen and moisture contents in the soil, which increase nutrient uptake by plants, plant growth, and synthesis of nitrogen-based compounds [Souza *et al.,* 2016]. Moreover, urea micro-dosing optimizes nitrogen application in order to avoid any nitrogen deficiency while ensuring adequate uptake by the root system [Tovihoudji *et al.,* 2017; Likpètè *et al.,* 2019]. Likewise, higher ash contents recorded when compared to those previously reported [Sossa-Vihotogbé *et al.,* 2013; Akinwunmi & Omotayo, 2016] suggest that urea micro-dosing has promoted plant nutrients uptake, despite the lack of direct relation between urea micro-doses and ash contents. Given that the application of organic fertilizer increases synthesis of carbon-based compounds including fibers [Fonge *et al.,* 2016; Souza *et al.,* 2016; Das *et al.,* 2017], the lack of effect of urea micro-doses on fiber and protein contents indicates that the based organic fertilizer contributed to a sufficient level of organic nitrogen [Souza *et al.,* 2016]. The significant effects of harvest times on proximate composition agreed with findings of Sossa-Vihotogbé *et al.* [2013] for *J. tenella* and *S. radiatum* leaves and could be linked to the occurrence of structural changes within plants over time [Sossa-Vihotogbé *et al.,* 2013].

Mineral profile and its potential effects on human micronutrient status

Potassium and calcium were the most abundant major minerals found in *A. cruentus* (4.2–6.8 g/100 g and 2.7–5.0 g/100 g), *O. gratissimum* (3.9–4.8 g/100 g and 2.1–2.9 g/100 g), and *S. macrocarpon* (5.1–8.2 g/100 g and 2.2–3.8 g/100 g) followed by magnesium (*A. cruentus*: 1.0–1.6 g/100 g, *O. gratissimum*: 0.4–0.6 g/100 g, *S. macrocarpon*: 0.5–1.0 g/100 g), phosphorus (*A. cruentus*: 0.3–0.4 g/100 g, *O. gratissimum*: 0.4–0.6 g/100 g, *S. macrocarpon*: 0.5–0.8 g/100 g), and sodium (<0.1 g/100 g). The levels of specific minerals in the vegetables are similar to

values reported for calcium (1.2–4.0 g/100 g), magnesium (0.3–0.9 g/100 g), phosphorus (0.2–0.8 g/100 g), potassium (3.9–7.0 g/100 g), and sodium (0.02–1.5 g/100 g) in *Solanum nigrum* [Bvenura & Afolayan, 2014a] whereas lower contents (<0.1 g/100 g) were reported for *A. cruentus*, *O. gratissimum*, and *S. macrocarpon* [Akinwunmi & Omotayo, 2016]. Iron and manganese were the most abundant trace minerals determined in *A. cruentus* (271.3–971.9 mg/kg and 501.2––976.4 mg/kg, respectively), *O. gratissimum* (132.6–442.2 mg/kg and 89.8–125.6 mg/kg, respectively), and *S. macrocarpon* (153.8–606.3 mg/kg and 109.6–213.9 mg/kg, respectively) followed by zinc (*A. cruentus*: 41.7–171.1 mg/kg, *O. gratissimum*: 34.5–78.5 mg/kg, *S. macrocarpon*: 41.9–661.6 mg/kg) and copper (*A. cruentus*: 7.2–11.2 mg/kg, *O. gratissimum*: 11.8––30.3 mg/kg, *S. macrocarpon*: 13.5–27.4 mg/kg). The ranges of iron and copper in the vegetables fit with values previously reported [Bvenura & Afolayan, 2014b; Akinwunmi & Omotayo, 2016] for *S. macrocarpon* (403.1 mg/kg and 12.8 mg/kg), *S. nigrum* (178.0–766.0 mg/kg and 7.2–23.5 mg/kg), and *V. amygdalina* (467.9 mg/kg and 25.5 mg/kg). Zinc contents are similar to those reported for *O. gratissimum* (44.7 mg/kg) and *S. nigrum* (19.0–78.0 mg/kg) but lower than those of *A. cruentus* (951.5 mg/kg) and *T. occidentalis* (1023.1 mg/kg) [Bvenura & Afolayan, 2014b; Akinwunmi & Omotayo, 2016]. Manganese contents in *A. cruentus* were higher than values reported [Akinwunmi & Omotayo 2016] for *O. gratissimum* (4.5 mg/kg) and *S. macrocarpon* (49.6 mg/kg).

Mean values for calcium, copper, iron, magnesium, phosphorus, potassium, and zinc are higher than their recommended daily intakes (RDI) for children and adults [Trumbo *et al.,* 2001; Sawka, 2005; Bergman *et al.,* 2009]. Thus, consumption of these vegetables could improve human micronutrient status although high quantities (100–300 g) of dried leaf powders must be consumed to meet the RDI. Conversely, our values for sodium and manganese contents are lower than the RDI for children and adults [Trumbo *et al.,* 2001; Sawka, 2005], suggesting that these vegetables are poor-sodium and -manganese sources. Considering that sauces that accompany starchy staple foods are currently the common consumption forms of these vegetables [Dansi *et al.,* 2008; Vodouhè *et al.,* 2012], it is likely that the quantities of the vegetables (either leaf powders or fresh leaves) required to meet these RDI cannot be easily consumed in a day. Thus, a diversification of vegetables consumption forms is needed to improve individual nutritional profile.

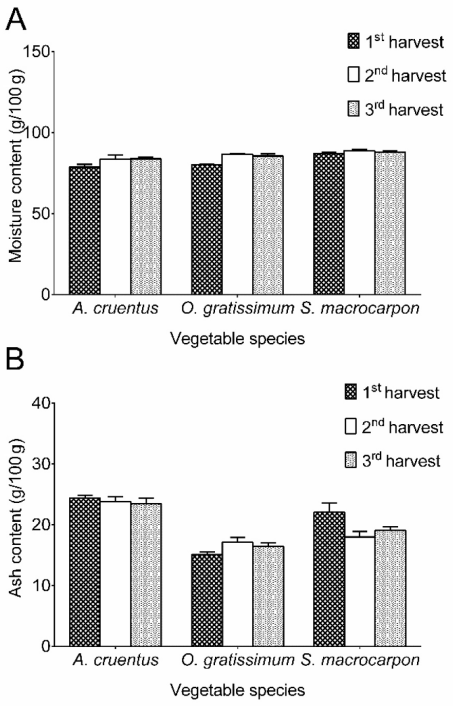


FIGURE 1. Variations in moisture (A) and ash (B) contents of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis (except for moisture content). 1st, 2nd, and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

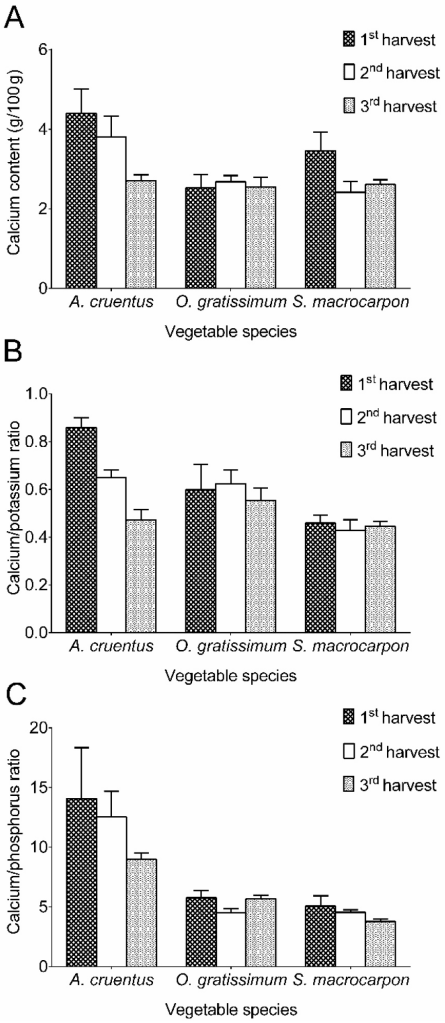


FIGURE 2. Variations in calcium content (A), calcium/potassium ratio (B) and calcium/phosphorus ratio (C) of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis. 1st, 2nd and 3rd harvests correspond to 4, 6 and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

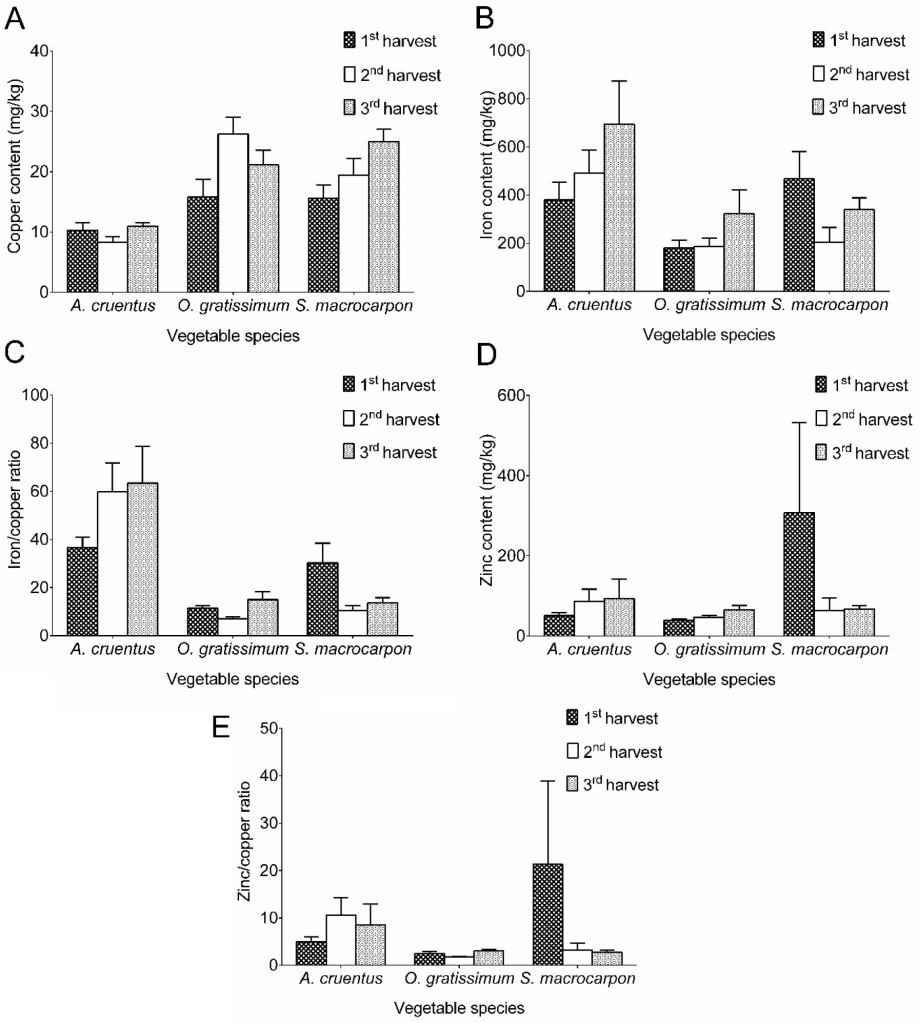


FIGURE 3. Variations in iron (A), zinc (B) and copper (C) contents as well as in iron/copper (D) and zinc/copper (E) ratios of the leafy vegetable species produced with four urea micro-doses (0, 20, 40, and 60 kg/ha) depending on harvest times.

Results are expressed on dry weight basis. 1st, 2nd and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

Mineral ratios and their potential effects on human health disorders

The calcium/magnesium and calcium/phosphorus ratios of *A. cruentus* (2.3–3.5 and 8.3–18.3, respectively), *O. gratissimum* (4.6–6.2 and 4.3–6.4, respectively), and *S. macrocarpon* (2.6–4.5 and 3.6–6.2, respectively) were higher than their calcium/potassium (*A. cruentus*: 0.5–0.9, *O. gratissimum*: 0.5––0.7 and *S. macrocarpon*: 0.4–0.5), sodium/magnesium (<0.1), and sodium/potassium (<0.1) ratios. The calcium/magnesium ratios are below (*A. cruentus*) or within (*O. gratissimum* and *S. macrocarpon*) the acceptable range of 3–11 [Watts, 2010], indicating potency for low or balanced glucose metabolism, which is inversely associated with the risk of type 2 diabetes [Watts, 2010]. Inversely, the calcium/phosphorus ratios (>1.8–3.6) of these vegetables may increase intestinal absorption of calcium, which is useful for the formation of strong bones and teeth [Watts, 2010; Adjatin *et al.,* 2013] but the calcium/potassium ratios (<2.2–6.2) could promote hyperthyroidism and its related complications [Watts, 2010]. The sodium/magnesium (<2–6) and sodium/potassium (<1.4–3.4) ratios associated with these vegetables could contribute to high blood pressure lowering and anti-inflammatory activities [Watts, 2010; Adjatin *et al.,* 2013]. The iron/copper ratio of *A. cruentus* (31.7–87.9), and *O. gratissimum* (6.3–18.5) were higher than their zinc/copper ratio (*A. cruentus*: 4.0–15.5 and *O. gratissimum*: 1.7–3.3). A wide range was found for zinc/copper ratio of *S. macrocarpon* (2.3–49.0) when compared to its iron/copper ratio (8.3–42.9). The iron/copper (>0.2–1.6) and zinc/copper (>4–12) ratios observed may increase the risk of oxidative stress-related diseases because of the key role of these ratios in the maintenance of the oxidant/antioxidant balance [Watts, 2010]. The values of iron/copper ratio may also affect iron utilization by decreasing its incorporation into hemoglobin and promoting anemia [Watts, 2010]. These findings indicate that some adjustments (preferably by food-to-food fortification) are needed to avoid the adverse effects predicted for calcium/potassium, iron/copper, and zinc/copper ratios.

Mineral composition as affected by vegetable species, urea micro-doses and harvest times

The effect of urea micro-doses (except for zinc content, $p=0.04$) and their interaction with vegetable spe-

cies and/or harvest times were not significant ($p>0.05$) for the mineral contents and ratios (Table 1). Given that ash content is an index of mineral levels, the lack of significant influence of urea micro-doses and of their interaction with other factors on the mineral profile was expected due to the lack of direct relation between urea micro-doses and ash contents in this study. Vegetable species, harvest times, and their interaction had respectively significant effects on copper ($p<0.01$, $p=0.04$ and $p=0.03$), iron ($p<0.01$, $p=0.03$ and $p=0.04$), and zinc ($p=0.03$, $p=0.04$ and $p=0.03$) contents as well as on calcium/phosphorus ($p=0.02$, $p=0.02$ and $p=0.03$), calcium/potassium ($p=0.03$), iron/copper ($p=0.02$, $p=0.04$ and $p=0.04$), and zinc/copper ($p=0.01$, $p=0.04$ and $p=0.02$) ratios (Table 1). Harvest time and its interaction with vegetable species had significant effects on calcium content ($p=0.02$ and $p=0.04$, respectively) (Table 1). The interaction of vegetable species and harvest times revealed that the first harvest of *A. cruentus* had the highest calcium content (Figure 2a), calcium/potassium ratio (Figure 2b), and calcium/phosphorus ratio (Figure 2c). The highest copper content was recorded at the second harvest of *O. gratissimum* (Figure 3a) whereas the third harvest of *A. cruentus* had the highest iron content (Figure 3b) and iron/copper ratio (Figure 3c). Conversely, the highest zinc content (Figure 3d) and zinc/copper ratio (Figure 3e) were recorded at the first harvest of *S. macrocarpon*. Significant effect of harvest time was recorded for manganese content ($p=0.03$) (Table 1), with the highest content found at the first (339.8 mg/kg) and the second (330.5 mg/kg) harvests.

Vegetable species had a significant effect on magnesium ($p=0.01$), manganese ($p=0.01$), phosphorus ($p=0.01$), potassium ($p=0.04$), and sodium ($p=0.02$) contents as well as on calcium/magnesium ($p=0.04$), calcium/phosphorus ($p=0.02$), calcium/potassium ($p=0.03$), sodium/magnesium ($p=0.02$), and sodium/potassium ($p=0.02$) ratios (Table 1). The highest magnesium, manganese, and sodium contents as well as sodium/magnesium and sodium/potassium ratios were found in *A. cruentus* (Table 2). Phosphorus content was the highest in *S. macrocarpon* while *O. gratissimum* had the highest calcium/magnesium ratio (Table 2). Higher potassium contents were found in *A. cruentus* and *S. macrocarpon* (Table 2). Plant growth depends greatly on water and nutrient availability in the soil and their uptake by the root system depending on plants' developmental stage although genotype and its interaction with fertilization type and rate influence plant responsiveness to nutrient availability [Sossa-Vihotogbé *et al.,* 2013; Fonge *et al.,* 2016]. Mineral fertilization supplies plants in readily available nutrients unlike organic fertilization, which delays nutrients release and availability due to slow mineralization [Souza *et al.,* 2016; Das *et al.,* 2017]. Thus, variation in the rate of nutrients availability into the soil and their uptake by plants may explain the significant differences observed in calcium content and trace mineral profile according to harvest time. The decrease in calcium content with increase of harvest time could be linked to the immobility of this mineral once taken up into plants tissues, which stops its translocation from older to growing parts of the plants [Bvenura & Afolayan, 2014a, b] while that of zinc contents may be linked to zinc accumulation in young plants [Bvenura & Afolayan, 2014b]. The increase in iron and copper contents with increase of harvest time could be related to high organic matter concentration into the soil that promotes a continuous release of easily absorbable minerals into the soil thereby, enhancing their uptake by plants and their contents in leaves as plants matured [Bvenura & Afolayan, 2014a, b].

TABLE 2. Effect of vegetable species on proximate and mineral compositions of the leafy vegetables produced with four urea micro-doses and harvested three successive times.

Variables		*Amaranthus cruentus*	*Ocimum gratissimum*	*Solanum macrocarpon*
Proximate composition[1]	Moisture	82.0±0.1 β[c]	84.1±0.0[b]	87.9±0.0[a]
	Fat	2.2±0.1[c]	4.2±0.0[a]	3.2±0.0[b]
	Fibers	11.4±0.2[c]	14.6±0.0[b]	20.4±0.0[a]
	Protein	23.6±0.1[b]	22.2±0.0[c]	30.3±0.2[a]
	Ash	23.9±0.0[a]	16.2±0.0[c]	19.7±0.1[b]
Major minerals[1] and their ratios	Calcium	3.6±0.0[a]	2.6±0.1[a]	2.8±0.0[a]
	Magnesium	1.2±0.0[a]	0.5±0.0[c]	0.8±0.0[b]
	Phosphorus	0.3±0.0[c]	0.5±0.0[b]	0.6±0.0[a]
	Potassium	5.6±0.0[a]	4.4±0.0[b]	6.3±0.1[a]
	Sodium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
	Calcium/ magnesium	3.0±0.0[c]	5.4±0.1[a]	3.6±0.0[b]
	Calcium/ phosphorus	11.9±0.1[a]	5.3±0.1[b]	4.5±0.0[b]
	Calcium/ potassium	0.7±0.0[a]	0.6±0.0[a]	0.4±0.0[b]
	Sodium/ magnesium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
	Sodium/ potassium	0.1±0.0[a]	0.0±0.0[b]	0.0±0.0[b]
Trace minerals[2] and their ratios	Copper	9.8±0.2[b]	21.1±0.2[a]	20.0±0.1[a]
	Iron	521.9±4.9[a]	229.7±8.4[c]	337.0±7.4[b]
	Manganese	694.7±11.5[a]	106.7±0.1[c]	154.2±0.2[b]
	Zinc	77.1±0.0[b]	50.2±0.9[c]	146.5±1.1[a]
	Iron/copper	53.3±0.7[a]	11.2±0.3[c]	18.1±0.4[b]
	Zinc/copper	8.1±0.3[b]	2.4±0.0[c]	9.1±0.2[a]

Units: [1] g/100 g; [2] mg/kg. Results are expressed on dry weight basis (except for moisture content). β: Mean values ± standard error. Mean values with different alphabets within the same row are significantly different ($p<0.05$).

Trypsin inhibition as related to vegetable species, urea micro-doses, and harvest times

The IC_{50} value of AEBSF (3.7 μg/mL) was lower than that of *A. cruentus* (0.2–0.3 mg/mL), *O. gratissimum* (<0.1 mg/mL), and *S. macrocarpon* (0.0–4.8 mg/mL). Values recorded for trypsin inhibition from *Juglans regia* (42.6 μg/mL) and *Prunus spinosa* (970.2 μg/mL) by Jedinak *et al.* [2010] and for *Justicia gendarussa* (13.4 μg/mL) by Patel & Zaveri [2015] are within the range found for *S. macrocarpon* and *O. gratissimum*. There were significant ($p<0.01$) effects of the interaction of vegeta-

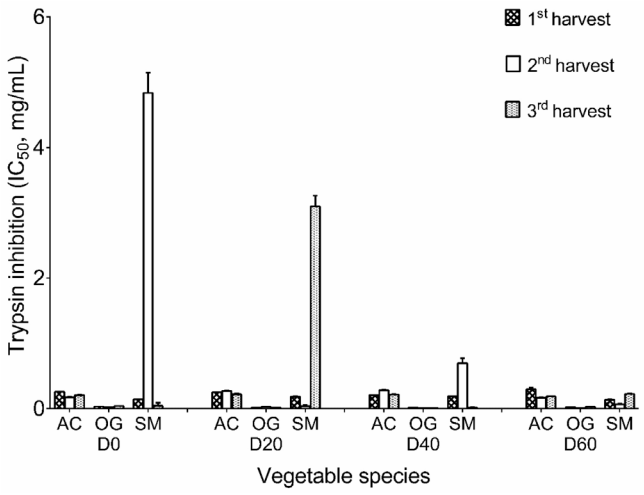


FIGURE 4. Trypsin-inhibitory activity of aqueous vegetable leaf extracts produced with four urea micro-doses and harvested at different times.

A. cruentus, O. gratissimum and *S. macrocarpon* were respectively coded AC, OG and SM; D0, D20, D40, and D60 indicate applied urea doses (0, 20, 40, and 60 kg/ha, respectively); 1st, 2nd, and 3rd harvests correspond to 4, 6, and 8 weeks after transplanting, respectively for *A. cruentus*; 8, 12, and 16 weeks after transplanting, respectively for *O. gratissimum* and 6, 10, and 14 weeks after transplanting, respectively for *S. macrocarpon*.

ble species, urea micro-doses, and harvest times on IC_{50} values for trypsin inhibition by aqueous leaf extracts (Table 1). The lowest IC_{50} for trypsin inhibition by *A. cruentus* and *O. gratissimum* extracts were obtained from the samples produced with 60 kg/ha of urea and harvested the second time while that of *S. macrocarpon* was from the sample cultivated with 40 kg/ha of urea and collected at the third harvest (Figure 4). Irrespective of vegetable species, urea micro-doses and harvest times, the lowest IC_{50} value was recorded for *O. gratissimum* (Figure 4). The interactive effect of vegetable species, urea micro-doses, and harvest times on the trypsin inhibitions was not expected considering that trypsin inhibitors are usually protein molecules and that there was no evidence of such an interactive effect on protein contents. The interactive effect confirmed the beneficial role of fertilization on the synthesis of nitrogen-based compounds [Souza *et al.,* 2016], including trypsin inhibitors. Plants synthesize protease inhibitors during their normal ontogeny and as a wound-activated response to mechanical wounding [Clemente *et al.,* 2019]. Thus, plant cutting (at harvest) activates stress of mechanical wounding and the up-regulation of genes encoding for synthesis of protease inhibitors [Clemente *et al.,* 2019], thereby leading to the lowest trypsin IC_{50} value at the third harvest (*S. macrocarpon*). The lowest IC_{50} found at the second harvest of *A. cruentus* and *O. gratissimum* could have resulted from a species-specific response to a high urea dose (60 kg/ha) and mechanical wounding at harvests. The first harvest has probably caused physiological stress that enhanced the synthesis of the trypsin-inhibitory protein molecules.

The activity of trypsin inhibitors of products from plant origin were reported to markedly increase after heat treatment at 60°C while a loss of activity occurred above 60°C and the decreasing rate depended on the type of the heat treatment applied (dry or moist) [Allen *et al.,* 2012; Pesoti *et al.,* 2015]. Indeed, a less decreasing rate was recorded for dry-heated products with an average loss of 10% of activity when temperature increase by 20°C whereas a rapid decreasing rate occurred for the moist-heated products (loss of 45% of activity from 60 to 80°C and of 20% of activity from 80 to 100°C) [Allen *et al.,* 2012]. Based on these findings, we can assert that oven-drying (at 60°C) of the studied leaves and the aqueous extraction of dry leaf powder (at 60°C) did not inactivate their trypsin inhibitors. Regardless of the applied heat treatment type, the trypsin inhibitors are rapidly inactivated by very high temperatures (>100°C) [Allen *et al.,* 2012; Pesoti *et al.,* 2015]. Given that the studied vegetables are usually consumed in sauce and that their cooking temperature is very high (>100°C), we may assume that trypsin inhibitors could be inactivated by the moist heat treatment, which has been shown to provide better inactivation of trypsin inhibitors than dry heat treatment [Allen *et al.,* 2012; Pesoti *et al.,* 2015]. However, to the best of knowledge, there is no scientific report on the effect of heat treatment on the status of the trypsin inhibitors in sauces made with the studied leafy vegetables. Considering that various ingredients are combined during cooking, there is a need for further studies that will explore the complexity of biochemical reactions occurring during cooking in order to provide clear answers regarding the inactivation of trypsin inhibitors in leafy vegetables during sauces preparation.

CONCLUSION

This study highlighted the nutritional properties of leafy vegetables produced using urea micro-doses and harvested at different times. Consumption of *A. cruentus* leaves could improve diets quality due to their high mineral contents while the richness of *S. macrocarpon* leaves in fibers could facilitate digestion and reduce the potential for colon carcinogenesis. The effects of vegetable species and harvest times were more marked than that of urea micro-doses and uphold the recommendation of early (the first two) harvests for the vegetable species in order to fully benefit from their high nutritional properties. The aqueous leaf extracts had potent trypsin inhibition; a useful nutraceutical property for the management of high energy intake diets and of disease conditions associated with excessive protein catabolism. Moreover, the trypsin-inhibitory activity may be helpful against virus-dependent diarrhea. However, the nutritional and functional properties exhibited by the studied vegetables were based on *in vitro* assays and should be confirmed by *in vivo* animal and human trials.

ACKNOWLEDGEMENTS

The authors would like to thank graduate students and other colleagues who worked on the experimental fields that produced the vegetables.

RESEARCH FUNDING

This work was supported by the International Development Research Centre (IDRC) and Global Affairs Canada, through the Canadian International Food Security Research Fund (CIFSRF) Project No. 107983 under IDRC Grant [No. 107983–003].

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Adewale, O.B., Onasanya, A., Fadaka, O.A., Iwere, H., Anadozie, S.O., Osukoya, A.O., Olayide, I.I. (2014). *In vitro* antioxidant effect of aqueous extract of *Solanum macrocarpon* leaves in rat liver and brain. *Oxidants and Antioxidants in Medical Sciences*, *3*(3), 225–229.
2. Adjatin, A., Dansi, A., Badoussi, E., Sanoussi, A.F., Dansi, M., Azokpota, P., Ahissou, H., Akouegninou, A., Akpagana, K., Sanni, A. (2013). Proximate, mineral and vitamin C composition of vegetable Gbolo [*Crassocephalum rubens* (Juss. ex Jacq.) S. Moore and *C. crepidioide*s (Benth.) S. Moore] in Benin. *International Journal of Biological and Chemical Sciences*, *7*(1), 319–331.
3. Akinwunmi, O.A., Omotayo, F.O. (2016). Proximate analysis and nutritive values of ten common vegetables in South-West (Yoruba land) Nigeria. *Communications in Applied Sciences*, *4*(2), 79–91.
4. Allen, J.C., Corbitt, A.D., Maloney, K.P., Butt, M.S., Truong, V.D. (2012). Glycemic index of sweet potato as affected by cooking methods. *The Open Nutrition Journal*, *6*(1), 1–11.
5. AOAC (2005). Official Methods of Association of Official Analytical Chemists, 18th ed., Gaithersburg, USA.
6. AOCS (2009). Official Methods and Recommended Practices of American Oil Chemists' Society, 6th ed., Denver, USA.
7. Bergman, C., Gray-Scott, D., Chen, J.J., Meacham, S. (2009). What is next for the Dietary Reference Intakes for bone metabolism related nutrients beyond calcium, phosphorus, magnesium, vitamin D, and fluoride? *Critical Reviews in Food Science and Nutrition*, *49*(2), 136–144.
8. Bvenura, C., Afolayan, A.J. (2014a). Maturity effects on mineral concentration and uptake in *Solanum nigrum* L. *Acta Agriculturae Scandinavica, Section B – Soil and Plant Science*, *64*(8), 657–665.
9. Bvenura, C., Afolayan, A.J. (2014b). Mineral uptake in *Solanum nigrum* L. cultivated on fertilizer amended soils of the Eastern Cape, South Africa. *Journal of Applied Botany and Food Quality*, *87*, 220–226.
10. Carvalho, F.M.C., Lima, V.C.O., Costa, I.S., Medeiros, A.F., Serquiz, A.C., Lima, M.C.J.S., Serquiz, R.P., Maciel, B.L.L., Uchôa, A.F., Santos, E.A., Morais, A.H.A. (2016). A trypsin inhibitor from tamarind reduces food intake and improves inflammatory status in rats with metabolic syndrome regardless of weight loss. *Nutrients*, *8*(10), 544–558.
11. Clemente, M., Corigliano, M., Pariani, S., Sanchez-Lopez, E., Sander, V., Ramos-Duarte, V. (2019). Plant serine protease inhibitors: biotechnology application in agriculture and molecular farming. *International Journal of Molecular Sciences*, *20*(6), 1345–1365.
12. Dansi, A., Adjatin, A., Adoukonou-Sagbadja, H., Falade, V., Yedomonhan, H., Odou, D., Dossou, B. (2008). Traditional leafy vegetables and their use in the Benin Republic. *Genetic Resources and Crop Evolution*, *55*(8), 1239–1256.
13. Das, S., Hussain, N., Gogoi, B., Buragohain, A.K., Bhattacharyaa, S.S. (2017). Vermicompost and farmyard manure improves food quality, antioxidant and antibacterial potential of *Cajanus cajan* (L. Mill sp.) leaves. *Journal of the Science of Food and Agriculture*, *97*(3), 956–966.
14. Djibril Moussa, M.I., Alashi, A.M., Sossa-Vihotogbe, C.N.A., Akponikpe, P.B.I., Djenontin, A.J., Baco, M.N., Akissoé, N.H., Aluko, R.E. (2019a). Inhibition of renin-angiotensin system enzymes by leafy vegetables polyphenol extracts related to fertilizer micro-dosing and harvest time. *Acta Horticulturae*, *1238*, 73–80.
15. Djibril Moussa, M.I, Alashi, A.M., Sossa-Vihotogbé, C.N.A., Akponikpè, P.B.I, Baco, N.M., Djènontin, A.J, Aluko, R.E., Akissoé, N.H. (2019b). Fertilizer micro-dosing and harvesting time of indigenous leafy vegetables affect *in vitro* antioxidant activities. *Journal of Food Bioactives*, *6*, 118–130.
16. Fonge, B.A., Bechem, E.E., Awo, E.M. (2016). Fertilizer rate on growth, yield, and nutrient concentration of leafy vegetables. *International Journal of Vegetable Science*, *22*(3), 274–288.
17. Jedinak, A., Valachova, M., Maliar, T., Sturdik, E. (2010). Antiprotease activity of selected Slovak medicinal plants. *Pharmazie*, *65*(2), 137–140.
18. Likpètè, D.D., Adjogboto, A., Akponikpe, P.B.I., Djenontin, A.J., Baco, M.N., Sossa-Vihotogbe, C.N.A., Agbossou, E.K. (2019). Water use efficiency of local amaranth as affected by timing and application methods of fertilizer micro-dosing. *Acta Horticulturae*, *1238*, 45–54.
19. Nana, F.W., Hilou, A., Millogo, J.F., Nacoulma, O.G. (2012). Phytochemical composition, antioxidant and xanthine oxidase inhibitory activities of *Amaranthus cruentus* L. and *Amaranthus hybridus* L. extracts. *Pharmaceuticals*, *5*(6), 613–628.
20. Patel, S.S., Zaveri, M.N. (2015). Trypsin and protein denaturation inhibitory activity of leaf and root of *Justicia gendarussa*. *Asian Journal of Pharmaceutical Science and Technology*, *5*(4), 217–223.
21. Pesoti, A.R., Oliveira, B.M., Oliveira, A.C., Pompeu, D.G., Gonçalves, D.B., Marangoni, S., Silva, J.A., Granjeiro, P.A. (2015). Extraction, purification and characterization of inhibitor of trypsin from *Chenopodium quinoa* seeds. *Food Science and Technology*, *35*(4), 588–597.
22. Sawka, M.N. (2005). Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate. Washington, DC, USA, The National Academies Press.
23. Shahwar, D., Raza, M.A., Shafiq-Ur-Rehman, Abbasi, M.A., Atta-Ur-Rahman (2012). An investigation of phenolic compounds from plant sources as trypsin inhibitors. *Natural Product Research*, *26*(12), 1087–1093.
24. Sossa-Vihotogbé, C.N.A., Anihouvi, B.V., Akissoe, H.N., Amadji, L.G., Hounhouigan, D.J. (2013). Effect of organic and mineral fertilization on proximate composition of three leafy vegetables harvested at different periods. *International Journal of Biological and Chemical Sciences*, *7*(1), 271–286.
25. Souza, M.R.M., Pereira, P.R.G., Magalhães, I.P.B., Sediyama, M.A.N., Vidigal, S.M., Milagres, C.S.F., Baracat-Pereira, M.C. (2016). Mineral, protein and nitrate contents in leaves of *Pereskia aculeata* subjected to nitrogen fertilization. *Pesquisa Agropecuária Tropical*, *46*(1), 43–50.
26. Tovihoudji, P.G., Akponikpè, P.B.I., Agbossou, E.K., Bertin, P., Bielders, C.L. (2017). Fertilizer microdosing enhances maize yields but may exacerbate nutrient mining in maize cropping systems in northern Benin. *Field Crops Research*, *213*, 130–142.
27. Trumbo, P., Yates, A.A., Schlicker, S., Poos, M. (2001). Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chro-

mium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association*, *101*(3), 294–301.

28. Vodouhè, S., Dovoedo, A., Anihouvi, V.B., Tossou, R.C. (2012). Influence du mode de cuisson sur la valeur nutritionnelle de *Solanum macrocarpum, Amaranthus hybridus* et *Ocimum gratissimum*, trois légumes-feuilles traditionnels acclimatés au Bénin. *International Journal of Biological and Chemical Sciences*, *6*(5), 1926–1937 (in French; English abstract).
29. Watts, D.L. (2010). HTMA mineral ratios. A brief discussion of their clinical importance. *Trace Elements Newsletter*, *21*, 1–3.

Submitted: 19 December 2019. Revised: 12 February, 16 and 20 March 2020. Accepted: 25 March 2020. Published on-line: 17 April 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 189–198
DOI: 10.31883/pjfns/119719
http://journal.pan.olsztyn.pl

Original article
Section: Food Quality and Functionality

Effect of the Growth Stage of False Flax (*Camelina sativa* L.) on the Phenolic Compound Content and Antioxidant Potential of the Aerial Part of the Plant

Magdalena Karamać[1], Francesco Gai[2], Pier Giorgio Peiretti[2]*

[1]*Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10–748 Olsztyn, Poland*
[2]*National Research Council, Institute of Sciences of Food Production, 10095 Grugliasco, Italy*

Key words: false flax, camelina, morphological stage, antioxidant activity, phenolic acids, flavonoids

The phenolic compound profile and antioxidant potential of the false flax (*Camelina sativa* L.) plant, harvested at five morphological stages, that is, from the vegetative to the ripe seed-pod stage, have been investigated. False flax extracts were prepared using 80% (*v/v*) methanol, and the total phenolic content (TPC), the contents of the individual phenolics and antioxidant activity, measured as the Trolox equivalent antioxidant capacity (TEAC), ferric-reducing antioxidant power (FRAP), DPPH$^{\bullet}$ scavenging activity and the ability to inhibit the oxidation of β-carotene-linoleic acid emulsion, were determined. The TPC of the plant, at different growth stages, ranged from 49.2 to 59.1 mg GAE/g of extract and from 1.46 to 3.10 mg GAE/g of fresh matter (FM). Four main phenolic compounds were identified (chlorogenic acid, rutin, quercetin 3-*O*-glucoside, and quercetin glycoside). The chlorogenic acid content and the sum of flavonoids increased in the extracts from the vegetative to the bud stage, reaching 35.9 and 49.5 mg/g of extract, respectively, and gradually decreased in the subsequent growth stages. The plant extracts at the bud and flowering stages generally had the highest antioxidant activity in the polar systems (TEAC, FRAP and DPPH assays). The ripe seed-pod stage showed the highest antioxidant potential in these conditions when the results were expressed on FM basis. The best antioxidant activity in the lipid emulsion system was shown for the false flax extracts at the flowering and ripe seed-pod stages. Our research has indicated the possibility of using the aerial part of *C. sativa* as a source of ingredients with protective antioxidant activity.

INTRODUCTION

False flax (*Camelina sativa* L.) is a member of the *Brassicaceae* family and is an ancient crop that is native to Europe in areas from the Mediterranean Sea to the Arctic Circle. The commercially utilized part of *C. sativa* is its seeds, due to their oil content, which accounts for up to 42% of the seed matter [Peiretti & Meineri, 2007; Belayneh *et al.*, 2017]. False flax seed oil is appreciated as a food ingredient, and interest in it as a potential low input source of biofuel has recently been growing [Berti *et al.*, 2016]. From a nutritional point of view, a high content of its polyunsaturated fatty acids (about 50% of the total fatty acids) and its favorable fatty acid profile (its α-linolenic acid content constitutes up to 40% of the total fatty acids) are important [Zubr & Matthäs, 2002; Kirkhus *et al*., 2013]. *C. sativa* oil is also a rich source of antioxidants, such as tocopherols, phytosterols and phenolic compounds, which are beneficial to human health and increase the oxidative stability of the oil [Zubr & Matthäs, 2002; Abramovič *et al*., 2007; Kirkhus *et al.*, 2013; Belayneh *et al.*, 2017]. Among the phenolic compounds, sinapic, *p*-hydroxybenzoic, salicylic and gallic acids, as well as catechin and quercetin, have been identified in false flax seed oil [Terpinc *et al.*, 2012a].

The main by-product of false flax seeds are cakes, obtained after seed oil pressing, which can be exploited as a fat- and protein-rich ingredient for animal nutrition [Berti *et al.*, 2016]. However, false flax cake has also recently been considered as a valuable source of phenolic compounds for a potential use in selected foods or food formulations [Terpinc *et al.*, 2012b; Rahman *et al.*, 2018a, b]. *C. sativa* cake contains more phenolic compounds than its oil. Terpinc *et al.* [2012a] reported that the total phenolic content (TPC) in oil and cake was 9.1 and 1666 mg chlorogenic acid eq/100 g of dry matter (DM), respectively. The phenolic compounds identified in cake and defatted meal included mainly flavonoids and hydroxycinnamic acids [Terpinc *et al.*, 2011; Rahman *et al.*, 2018a]. Sinapine, the ester of sinapic acid and choline, has often been determined as the main phenolic compound in false flax cake or meal [Salminen *et al.*, 2006; Terpinc *et al.*, 2011]. Hydroxybenzoic acids have also been identified [Terpinc *et al.*, 2012a; Rahman *et al.*, 2018a], although Salminen *et at.* [2006] did not find them in false flax seed meal. Condensed tannins are another class of phenolic compounds that have been determined in false flax cake [Matthäus & Zubr, 2000]. Rahman *et al.* [2018a] identified several procyanidin dimers among these tannins.

The phenolic compound profile of false flax cake determines its significant antioxidant activity [Matthäus, 2002; Quezada & Cherian, 2012]. Rahman *et al.* [2018b] showed

* Corresponding author: Tel.: +39 0116709232;
E-mail: francesco.gai@ispa.cnr.it (F. Gai)

that *C. sativa* meals inhibited the *in vitro* oxidation of low density lipoprotein and DNA damage and effectively inhibited pancreatic lipase and α-glucosidase activities. The bioactivity of the phenolic compounds of false flax can be enhanced by the presence of other phytochemicals. Das *et al.* [2014] reported a synergy of NAD(P)H quinone oxidoreductase 1 (NQO1, phase II detoxification enzyme) induction for a combination of quercetin and some alkyl sulfinyl glucosinolates found in *C. sativa* seeds. False flax cake has been studied not only because of its potentially health-promoting food compounds, but also as a food ingredient to increase the shelf-life of products. It has been proved, for instance, that camelina meal effectively inhibits the oxidation of lipids and protein in cooked pork meat patties [Salminen *et al*., 2006].

As shown above, false flax seeds (both oil and defatted meal) have been studied extensively for their composition and the presence of bioactive substances. However, the composition of the other parts of the *C. sativa* is not so well known. Peiretti & Meineri [2007] determined the nutritive value and fatty acid profile of the aerial part of the plant during growth, but did not analyze the phytochemicals. To the best of our knowledge, apart from the studies related to its seeds, only one study has been carried out on the phenolic compounds of *C. sativa*. Onyilagha *et al.* [2003] identified the presence of flavonoids in false flax leaves. It seems important to complete our knowledge about the phenolic compounds of the aerial part of *C. sativa*, especially because of the ever-growing interest in this plant [Faure & Tepfer, 2016]. The composition of the phenolic compounds changes during the growth cycle of plants [Gai *et al.*, 2017; Karamać *et al.*, 2019; Pavlović *et al.*, 2019], therefore, the purpose of our research has been to determine the phenolic compound profile and antioxidant potential of the aerial part of false flax at several morphological stages, that is, from the vegetative to the ripe seed-pod stage, in order to select those with the highest parameters as a potential source of phenolic antioxidants.

MATERIAL AND METHODS

Plant material collection

The false flax seeds were obtained from Ornitalia Product Service s.a.s. (Colleredo di Monte Albano (UD), Italy). The plant was cultivated in the Western Po Valley near Cuneo, Italy. The stands were seeded in the spring (15 May), and no irrigations or fertilizers were applied after sowing. The aerial parts of the plants were collected, using edging shears (0.1 m cutting width), at five growth stages from the vegetative to the ripe seed-pod stage (Table 1), on subplots of 2 m^2 randomly located in 4 × 10 m^2 plots with three replicates cut to a 1 to 2 cm stubble height. The sampling time ranged from the end of June to the end of July. Sampling was not performed on rainy days and was carried out in the morning, only after the disappearance of dew. The collected plant material was frozen immediately and then freeze-dried (LIO-5P, 5Pascal, Trezzano sul Naviglio, Italy). After grinding in a mortar (particles passing through a 1 mm screen), the samples were stored in tightly closed vessels at -20°C until analysis.

Standards and reagents

The Folin-Ciocalteu's phenol reagent (FCR), gallic acid, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH radical), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), β-carotene, linoleic acid, Tween 40, butylhydroxyanisole (BHA), and high performance liquid chromatography (HPLC) standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). The remaining chemicals (analytical grade) and HPLC solvents (gradient grade) were acquired from Avantor Performance Materials (Gliwice, Poland).

Extract preparation

The phenolic compounds were extracted from freeze-dried false flax plants using 80% (*v/v*) methanol at 65°C and a 1:10 (*v/w*) material-to-solvent ratio, according to a procedure previously described by Karamać *et al.* [2018]. The crude extracts were dried by evaporating methanol under vacuum (Rotavapor R-200, Büchi Labortechnik, Flawil, Switzerland) and freeze drying the remaining aqueous residue (FreeZone Freeze Dry System, Labconco, Kansas City, MO, USA). A mass balance was carried out to evaluate the extraction yield.

Total phenolic content determination

The TPC of the false flax extracts and plants was determined using a colorimetric assay with FCR and gallic acid as the standard [Kosińska *et al.*, 2011]. The results were expressed as mg of gallic acid equivalents (GAE) per g of extract or per g of plant fresh matter (FM).

Trolox equivalent antioxidant capacity determination

The Trolox equivalent antioxidant capacity (TEAC) was determined using the $ABTS^{\bullet+}$ decolorization assay [Re *et al.*, 1999]. First, the ABTS radical cations were activated by mixing aqueous solutions of ABTS (7 mM) and sodium persulfate (2.45 mM), and then shaking the mixture overnight in the dark. The $ABTS^{\bullet+}$ stock solution was diluted with methanol to an absorbance of 0.720 at 734 nm (DU-7500 spectrophotometer, Beckman Instruments, Fullerton, CA, USA) immediately before conducting the colorimetric reaction. A 2 mL portion of this solution was vortexed with 20 μL of the extract solution in methanol (1.5 mg/mL) and the reaction was carried out at 37°C for 6 min. The absorbance was then measured at 734 nm. Trolox was used as the standard. The results were expressed as mmol Trolox equivalents (TE) per g of extract or μmol TE per g of plant FM.

Ferric-reducing antioxidant power determination

The ferric-reducing antioxidant power (FRAP) assay was performed as previously described by Benzie & Strain [1996]. In brief, the extracts were dissolved in water (1 mg/mL), and portions of a 75 μL solution were mixed with 2.25 mL of a FRAP reagent and 225 μL of water, both warmed to 37°C. The FRAP reagent consisted of 10 volumes of 300 mM acetate buffer, pH 3.6, 1 volume of 10 mM TPTZ in 40 mM HCl, and 1 volume of 20 mM $FeCl_3 \times 6H_2O$. The absorbance of the reaction mixtures was

measured at 593 nm (DU-7500 spectrophotometer) after 30 min of incubation at 37°C. The FRAP values were calculated on the basis of the calibration curve prepared for ferrous sulfate. The results were expressed as mmol Fe^{2+} equivalents per g of extract or μmol Fe^{2+} equivalents per g of plant FM.

Scavenging the DPPH radicals

The DPPH$^{\bullet}$ scavenging activity of the false flax extracts was determined as described by Brand-Williams *et al.* [1995]. Methanolic solutions of the extract were prepared in the 1.2–6.0 mg/mL concentration range. A 0.1 mL aliquot of the extract solution was vortexed with 2 mL of methanol and 0.25 mL of methanolic solution of DPPH$^{\bullet}$ (1 mM). The reaction was conducted in the darkness at ambient temperature. After 20 min, the absorbance was measured at 517 nm. The curves of absorbance *vs*. extract concentration in the reaction mixture were plotted and the EC_{50} values were calculated. EC_{50} was defined as the concentration of extract needed to scavenge 50% of the initial DPPH$^{\bullet}$.

Oxidation of β-carotene-linoleic acid emulsion

The ability of the false flax extracts to inhibit the oxidation of the emulsion system with β-carotene and linoleic acid was determined according to Miller [1971], with some modifications, as described in a previous publication [Karamać *et al.*, 2018]. A 20 μL aliquot of a methanolic solution of extracts (1 mg/mL) or BHA (0.5 mg/mL) was pipetted into plate wells. The emulsion (250 μL) was then added. Emulsion without an antioxidant was used as a control sample. The plate was immediately placed in an Infinite M1000 microplate reader (Tecan, Männedorf, Switzerland) heated to 42°C, and the absorbance was measured at 470 nm. The plate was left in the device for 180 min and absorbance was read every 15 min. The percentage of non-oxidized β-carotene was calculated for each measurement time.

HPLC analysis

The HPLC-DAD analysis of the phenolic compounds of the false flax extracts was performed using a Shimadzu system (Kyoto, Japan) consisting of two LC-10AD_{vp} pumps, an SCL-10A_{vp} system controller and an SPD-M10A_{vp} diode array detector (DAD). The pre-packed Luna C18 (4.6×250 mm, 5 μm, Phenomenex, Torrance, CA, USA) column was connected to an HPLC system. Methanolic solutions of the extracts (6.67 mg/mL) were injected (20 μL) into the column and the compounds were eluted for 30 min with a mobile phase in a linear gradient system consisting of 5–60% acetonitrile in water (*v/v*) with trifluoroacetic acid (0.1, *v/v*). The flow rate was 1 mL/min. The DAD scanned over a wavelength range of 200 to 400 nm. The eluted compounds were identified from a comparison of their retention times and UV spectra with the corresponding standards. The calibration curves of the standards were used to quantify the compounds. Chlorogenic acid was determined at 320 nm and flavonoids at 350 nm.

Statistical analysis

Three samples of the aerial parts of false flax were collected for each growth stage and duplicate extracts were prepared separately from each sample. The chemical determinations were carried out at least in triplicate. The results were reported as means with standard deviations. An analysis of variance (ANOVA) and Fisher's LSD test were performed at a level of $p<0.05$ (GraphPad Prism; GraphPad Software, San Diego, CA, USA) to evaluate the significance of any differences among the mean values. A Principal Component Analysis (PCA) was performed to describe any variations among the growth stages. Statistica 13.1 software (StatSoft Corp., Kraków, Polska) was used for the PCA.

RESULTS AND DISCUSSION

False flax is a plant which has a short growth cycle and reaches maturity after an average cultivation of 92 days [Berti *et al.*, 2016]. In our study, the aerial part of plants was harvested between 42 and 70 days after sowing, corresponding to vegetative and ripe seed-pods stages, respectively (Table 1). The five growth stages selected in our experiment corresponded to 4 (development of the vegetative parts), 5 (inflorescence emergence), 6 (flowering), 7 (development of the fruit), and 8 (ripening) phenological growth stage of false flax, respectively, according to the Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie scale [Martinelli & Galasso, 2011]. In line with the literature, the flowering plants collected on the 56th day of the growth cycle (Table 1), showed that 50% of the flowering of spring genotypes of false flax may be obtained within 36–59 days of planting [Berti *et al.*, 2016].

TABLE 1. Extraction yield and total phenolic content (TPC) of the extract and fresh matter (FM) of the false flax plant at different growth stages.

Growth stage	Days after sowing	Extraction yield (%)	TPC	
			mg GAE/g extract	mg GAE/g FM
Vegetative	42	24.0±0.5^{a}	49.2±1.4^{b}	1.46±0.04^{c}
Bud	49	21.8±0.7^{b}	56.9±2.1^{a}	2.16±0.21^{b}
Flowering	56	18.8±0.6^{c}	59.1±6.0^{a}	2.31±0.17^{b}
Early seed-pod	64	18.2± 0.6^{c}	57.6±1.8^{a}	2.48±0.11^{b}
Ripe seed-pod	70	19.1±1.3^{c}	55.4±3.8^{ab}	3.10±0.43^{a}

GAE, gallic acid equivalents. Means with different letters in the column are significantly different ($p<0.05$).

Extraction yield and total phenolic content

Table 1 reports the extraction yield and TPC of the false flax at different plant growth stages. The highest yield was obtained from the extraction of false flax at the vegetative stage. The plants extracted at the subsequent growth states had lower yields, although the flowering, early and ripe seed-pod stage values did not differ significantly ($p \geq 0.05$). In a previous research, in which the aerial part of amaranth was extracted, we found that the budding stage of the plant also allowed a higher extraction yield to be obtained than at the early flowering or grain filling stages [Karamać *et al.*, 2019].

The TPC of the false flax extracts ranged from 49.2 to 59.1 mg GAE/g and the differences in TPC were not statistically significant ($p \geq 0.05$) for several growth stages (Table 1). Considerable variations in TPC were observed when the values were expressed on a fresh plant matter basis. The highest TPC (3.10 mg GAE/g FM) was determined in false flax at the ripe seed-pod stage and the lowest value (1.46 mg GAE/g FM) was noted for the vegetative stage. This greater variability in TPC calculated for FM than for the extract may be due to the decreasing water content in false flax during growth, which has been noted in this study and in the previous ones [Peiretti & Meineri, 2007]. The TPCs of the plant extracts at all the growth stages (Table 1) were higher than the values reported by Matthäus [2002] for defatted seed extracts obtained using several types of solvents (3.2–21.8 mg GAE/g of extract). The TPC determined in the FM of the false flax at the ripe seed-pod stage (Table 1) was similar to those found in *C. sativa* seed meal (3940 μg/g) [Salminen & Heinonen, 2008] and in seeds and defatted meal (3248.3 and 4591.8 μg GAE/g, respectively) [Quezada & Cherian, 2012]. However, Terpinc *et al.* [2012a] reported about 3–5 times higher TPC of defatted cake and seeds, which is probably the result of a different way of expressing the results (using chlorogenic

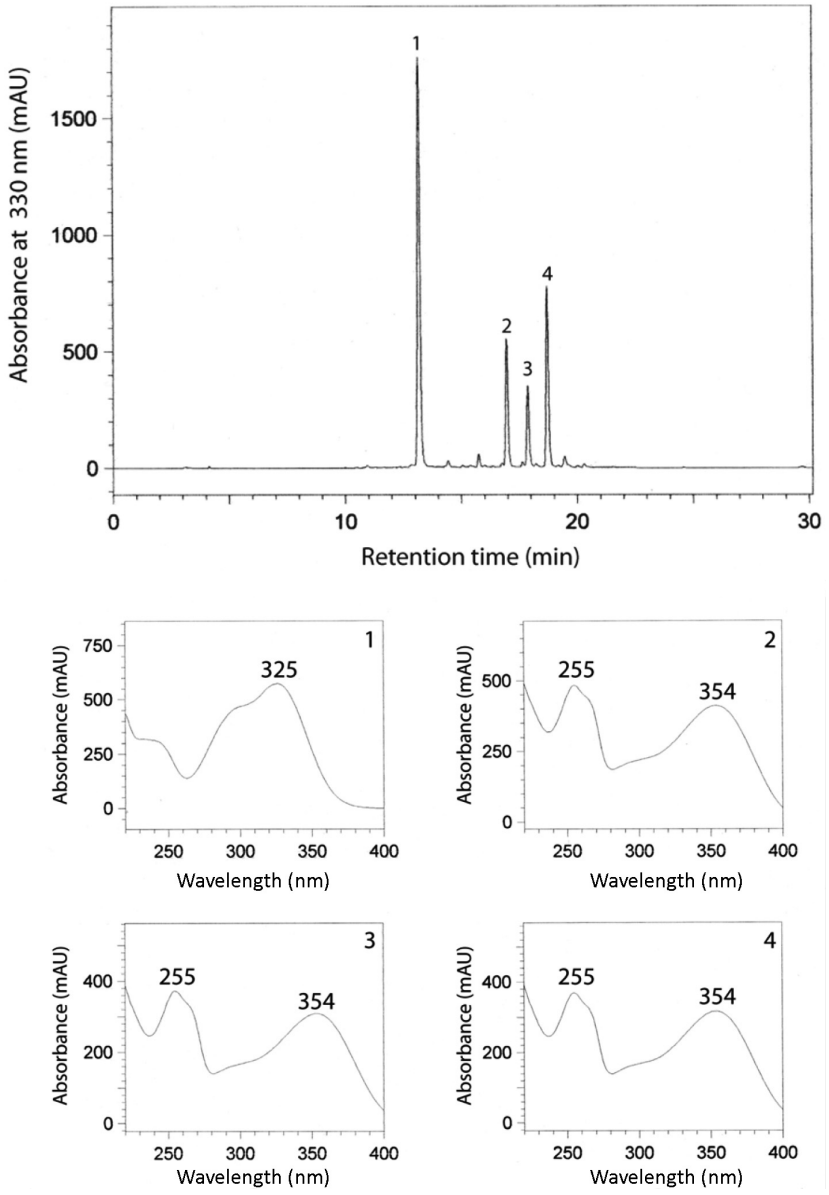


FIGURE 1. HPLC-DAD separation of the phenolic compounds of the false flax extract; 1 – chlorogenic acid; 2 – rutin; 3 – quercetin 3-*O*-glucoside; 4 – quercetin glycoside; Graphs 1–4, UV-DAD spectra of compounds 1–4, respectively.

TABLE 2. Phenolic compound content of the extracts (mg/g extract) of false flax at different growth stages.

Growth stage	Chlorogenic acid	Rutin	Quercetin 3-*O*-glucoside	Quercetin glycoside[1]
Vegetative	31.6±1.1[b]	13.3±0.7[a]	8.8±1.1[a]	21.5±1.2[ab]
Bud	35.9±1.3[a]	15.1±0.8[a]	10.0±1.3[a]	24.4±1.4[a]
Flowering	31.7±1.8[b]	13.9±1.1[a]	11.0±0.4[a]	19.3±3.1[b]
Early seed-pod	23.4±2.4[c]	10.9±2.0[b]	10.4±0.4[a]	21.4±0.9[ab]
Ripe seed-pod	19.0±0.6[d]	10.3±1.3[b]	10.3±1.0[a]	19.0±2.1[b]

[1]Expressed as quercetin equivalents. Means with different letters in the column are significantly different ($p<0.05$).

TABLE 3. Phenolic compound content of the fresh matter (mg/g FM) of false flax at different growth stages.

Growth stage	Chlorogenic acid	Rutin	Quercetin-3-*O*-glucoside	Quercetin glycoside[1]
Vegetative	0.94±0.03[d]	0.39±0.02[c]	0.26±0.03[d]	0.64±0.04[b]
Bud	1.36±0.05[a]	0.57±0.03[a]	0.38±0.05[c]	0.93±0.05[a]
Flowering	1.24±0.07[b]	0.54±0.04[ab]	0.43±0.02[b]	0.76±0.12[b]
Early seed-pod	1.01±0.10[cd]	0.47±0.09[bc]	0.45±0.02[b]	0.92±0.04[a]
Ripe seed-pod	1.06±0.03[c]	0.57±0.07[a]	0.57±0.06[a]	1.06±0.12[a]

[1]Expressed as quercetin equivalents. Means with different letters in the column are significantly different ($p<0.05$).

acid as a standard and converting the value to dry weight). In short, we found that the TPC of the aerial part of false flax, especially of older plants, was at a similar level to those of by-products obtained after seed oil pressing, which are considered a good source of phenolic antioxidants for potential food use [Rahman *et al.*, 2018a].

Distribution of the individual phenolic compounds

The HPLC-DAD separation of the false flax extracts is shown in Figure 1. Four main peaks (1–4), with retention times in the 13.2–18.9 min range, are visible on the chromatogram. These peaks correspond to compounds with the maxima absorption of UV-Vis spectra at 325 nm (compound 1) or at 255 and 354 nm (compounds 2–4). They were identified as chlorogenic acid, rutin (quercetin 3-*O*-rutinoside), quercetin 3-*O*-glucoside, and quercetin glycoside, respectively. The presence of quercetin glycosides in the aerial part of false flax was consistent with a report of Onyilagha *et al.* [2003], who characterized flavonoids in the leaves of *C. sativa* and found that quercetin derivatives were the only type of flavonoid present. These authors identified four quercetin glycosides (quercetin heptose-pentose-deoxypentose derivative, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside and -*O*-galactoside, and quercetin 3-*O*-glucuronide). Quercetin glycosides were also identified in the seeds, cake and defatted seed meal of false flax, although they were accompanied by other flavonoids, such as catechin, epicatechin, dihydrodihydroxyisorhamnetin, daidzin, and myricetin [Terpinc *et al.*, 2011; 2012a; Rahman *et al.*, 2018a]. Chlorogenic acid was previously detected in defatted false flax seeds [Rahman *et al.*, 2018a]. Surprisingly, sinapic acid and its choline ester (sinapine), which have frequently been determined in *C. sativa* seeds [Matthäus & Zubr, 2000; Salminen *et al.*, 2006; Amyot *et al.*, 2019], have not been instead identified in the aerial part of false flax at all investigated stages in our study, even at the ripe seed-pod stage.

The individual phenolic compound contents expressed in the extracts and FM of the false flax at different growth stages are reported in Tables 2 and 3, respectively. Chlorogenic acid was the most abundant phenolic constituent in the first four growth states (in both the extracts and FM), while its content was equal to the level of quercetin glycoside at the ripe seed-pod stage. The chlorogenic acid content increased from the vegetative to the bud stage, reaching 35.9 mg/g in the extract and 1.36 mg/g in the FM of the plant, and then gradually decreased. This trend has not been observed for individual flavonoids. The growth stages were divided into two groups ($p<0.05$) on the basis of the rutin level in the extracts; vegetative, bud and flowering stages with a high content, and early and ripe seed-pod stages with a low content. The rutin content values expressed in the fresh plant matter ranged from 0.39 to 0.57 mg/g FM, with the lowest level being found for the vegetative stage. No significant differences ($p\geq0.05$) were reported for the quercetin 3-*O*-glucoside content expressed as mg/g of extract, while a growth stage effect appeared when expressed on FM basis, with the lowest and the highest values (0.26 to 0.57 mg/g FM) being determined in the vegetative and ripe seed-pod stage, respectively. The quercetin glycoside content ranged from 19.0 to 24.4 mg/g of extract or from 0.64 to 1.06 mg/g of FM, and changed irregularly with the age of the false flax. Interestingly, the amount of flavonoids in the extract was the most abundant at the bud stage (49.5 mg/g of extract), as was the chlorogenic acid. In the case of fresh plant matter, the highest amounts of flavonoids were found in the bud and ripe seed-pod stages (1.88 and 2.20 mg/g of FM, respec-

TABLE 4. Trolox equivalent antioxidant capacity (TEAC) and ferric-reducing antioxidant power (FRAP) of the false flax extract and fresh matter (FM) of the plant at different growth stages.

Growth stage	TEAC		FRAP	
	mmol TE/g extract	μmol TE/g FM	mmol Fe^{2+}/g extract	μmol Fe^{2+}/g FM
Vegetative	0.28 ± 0.00^{b}	8.4 ± 0.4^{c}	0.93 ± 0.04^{ab}	27.5 ± 1.3^{b}
Bud	0.32 ± 0.01^{a}	12.2 ± 1.1^{b}	1.05 ± 0.04^{a}	40.0 ± 4.0^{a}
Flowering	0.31 ± 0.02^{a}	12.3 ± 0.6^{b}	0.96 ± 0.19^{a}	37.7 ± 7.1^{a}
Early seed-pod	0.30 ± 0.01^{ab}	13.0 ± 0.4^{b}	0.89 ± 0.16^{ab}	38.4 ± 6.9^{a}
Ripe seed-pod	0.30 ± 0.02^{ab}	16.5 ± 2.4^{a}	0.77 ± 0.09^{b}	42.9 ± 2.9^{a}

TE, Trolox equivalents. Means with different letters in the column are significantly different ($p<0.05$).

tively). The higher flavonoid content than that of hydroxycinnamic acids noted for all the growth stages was consistent with literature data regarding *C. sativa* seed meal [Salminen *et al.*, 2006]. Onyilagha *et al.* [2003] determined the content of quercetin glycosides in false flax leaves and found a very low value (50 μg/g FM) compared to that reported in our study, even at the vegetative stage of the plant (1.29 mg/g FM), in which the proportion of leaves to other botanical parts was high. However, it should be taken into account that the aforementioned authors did not analyze the compounds directly in the crude extract, but re-extracted them in butanol and then used semi-preparative TPC, which could have caused a "loss" of a part of the flavonoids. However, the important contribution of chlorogenic acid to the phenolic compound pool determined in our study was not found for false flax seeds or for the by-products from oil pressing [Rahman *et al.* 2018a]. According to the authors, who analyzed free and esterified forms of extractable phenolics of defatted meal, the free chlorogenic acid content was only 17.02 μg/g and the content of caffeic acid liberated from esters was 16.36 μg/g [Rahman *et al*., 2018a]. In the same study, rutin was the major extractable flavonoid. Its defatted meal content (277.5 μg/g) was lower than the lowest content quantified in the present study at the vegetative growth stage (Table 3). These differences could be ascribed to the different distributions of phenolics in the examined plant part (seed *vs*. leaf), as reported by several authors for other plants belonging to the *Brassicaceae* family [Ferreres *et al.*, 2005; 2006].

Antioxidant potential

The TEAC and FRAP of the false flax extracts and of the fresh plant matter are shown for different growth stages in Table 4. The results (both assays) obtained for the extracts were similar for the different growth states, although significant statistical differences ($p<0.05$) were found for some of them. These limited TEAC and FRAP variations between the growth stages were consistent with those found for TPC. Moreover, the trend of the TEAC changes in the fresh plant matter during the growth cycle was similar to that of TPC, with the highest value for the ripe seed-pod stage (16.5 mmol TE/g extract) and the lowest for the vegetative stage (8.4 mmol TE/g of extract). The FRAP determined for the FM of the plant at the vegetative stage was lower

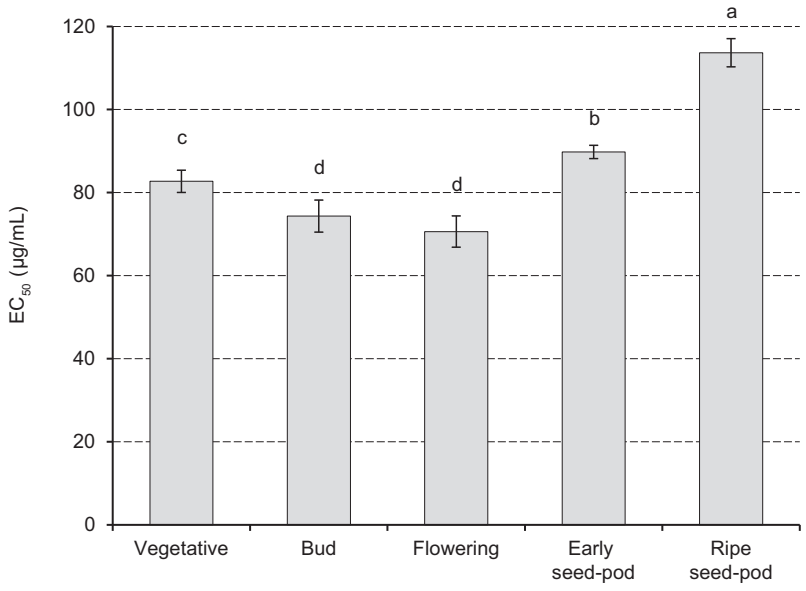


FIGURE 2. DPPH radical scavenging activity of false flax extracts obtained at different growth stages of the plant.

(27.5 μmol Fe^{2+}/g of FM) than those of the other growth stages (38.4–42.9 μmol Fe^{2+}/g of FM), which did not differ significantly ($p \geq 0.05$). The TEACs of the aerial part of the false flax were generally lower than the values found in the literature for *C. sativa* seeds and defatted meal [Quezada & Cherian, 2012; Rahman *et al*., 2018a]. However, comparable TEAC and FRAP values to those obtained in our experiments were reported in a study carried out on another oilseed plant, perilla (*Perilla frutescens* L.), considering changes in its antioxidant activity during the growth cycle [Gai *et al*., 2017]. The TEAC and FRAP of the aerial part of perilla ranged from 7 to 15 μmol TE/g of FM and from 23 to 57 μmol Fe^{2+}/g of FM, respectively, with the lowest and the highest values being determined in the medium vegetative and full flowering growth stages, respectively. On the contrary, lower TEAC and FRAP values were determined at the vegetative and reproductive stages in the aerial part of soybean plants, as reported by Peiretti *et al*. [2019], who found a range from 6.3 to 7.9 μmol TE/g of FM and from 21 to 29 μmol Fe^{2+}/g of FM, respectively.

The DPPH radical scavenging activity of the false flax extracts, obtained at different false flax growth stages, expressed as EC_{50}, is shown in Figure 2. Significant differences ($p<0.05$) can be observed between the growth stages, with the highest antiradical activity against DPPH$^{\bullet}$ for the plant extracts at the bud and flowering stages (with the lowest EC_{50} value of 70.6–74.3 μg/mL). The highest EC_{50} value (113.7 μg/mL) was found for the ripe seed-pod extract stage. The DPPH$^{\bullet}$ scavenging activity of the aerial part of false flax was lower than the antiradical activity against DPPH$^{\bullet}$ of the seeds and cake [Terpinc *et al.*, 2012a, b]. These differences (which were also mentioned earlier for TEAC) may be due to the different phenolic compound profiles of the aerial part and seeds of false flax. Mainly flavonoids, in the form of glycosides, which are known to have less ability to scavenge free radicals than their aglycons [Rice-Evans *et al.*, 1996], were found in the aerial parts (Table 3). On the other hand, chlorogenic acid is a powerful antioxidant [Shahidi & Chandrasekara, 2010]. Its DPPH$^{\bullet}$ scavenging activity was found to be slightly higher than that of sinapic acid and sinapine [Shahidi & Chandrasekara, 2010] – major phenolic compounds of *C. sativa* seeds belonging to phenolic acids and their derivatives [Terpinc *et al.*, 2011; Rahman *et al*., 2018a].

The inhibition of the oxidation of β-carotene-linoleic acid emulsion by false flax extracts is reported in Figure 3. The antioxidant activity of the plant extracts at different growth stages decreases as follows: flowering > ripe seed-pod > early seed-pod > vegetative ≥ bud. The low antioxidant activity of the plant at bud stage extracts and the high antioxidant activity of the ripe seed-pod extract were not compatible with the previously discussed results from other antioxidant assays. This is probably the result of different activities of the antioxidant compounds present in the extracts under polar conditions (ABTS, FRAP and DPPH assays) and in the lipid emulsion system. Although the β-carotene bleaching assay of false flax seeds has already been performed previously [Matthäus, 2002; Terpinc *et al.*, 2012a, b; Rahman *et al.*, 2018b], it was difficult to compare our results with literature data due to differences in the emulsion oxidation conditions or the way the results were expressed.

Principal component analysis

The PCA was carried out separately for two data sets; the first one was for the values obtained on an extract basis (Figures 4A and B) and second one was for the values expressed on plant fresh matter (Figures 4C and D), both considering five growth stages. The variables were: antioxidant assays, four phenolic compounds, and TPC. In the case of the data set related to the extracts, the first two principal components (PC1 and PCA) accounted for 88.79% of the total variance. The growth stages differed from each other, as can be observed in plot B (Figure 4). Most of the variables (FRAP, compounds 1, 2, and 4) described the bud stage. The flowering stage was associated with TEAC, TPC,

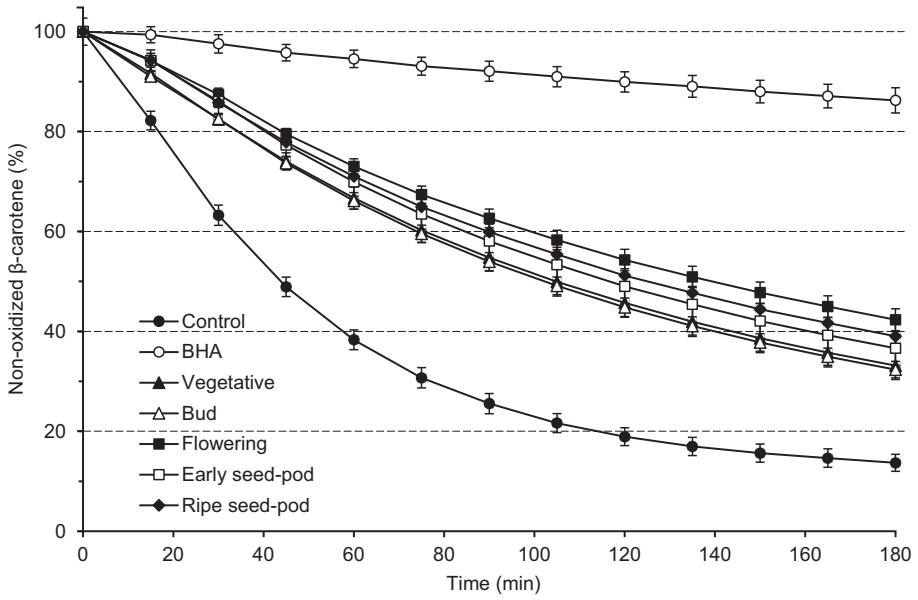


FIGURE 3. Inhibition of the oxidation of β-carotene-linoleic acid emulsion by false flax extracts at different growth stages; BHA, butylhydroxyanisole.

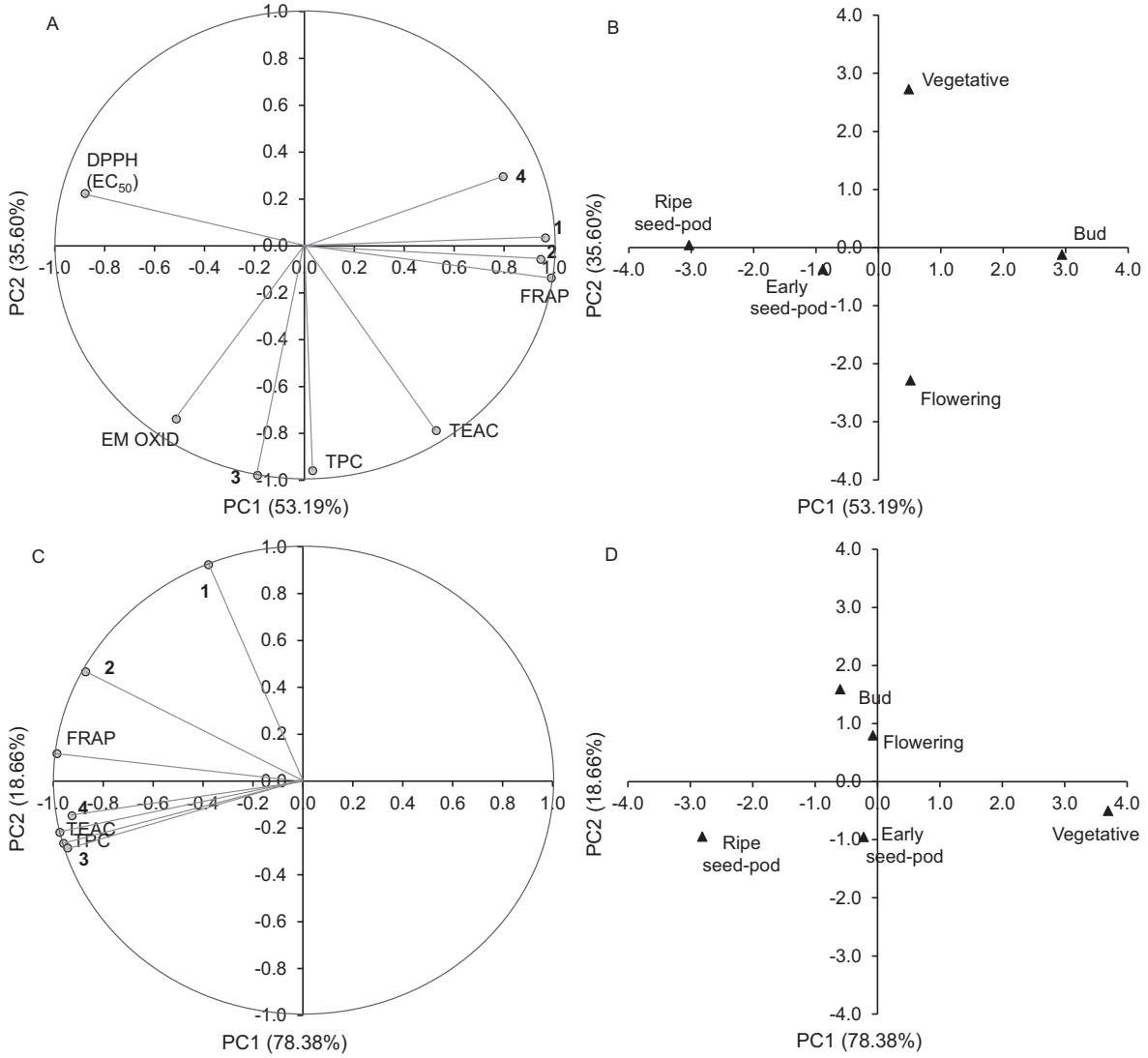


FIGURE 4. Principal component analysis (PCA) of the data set of the variables: total phenolic content (TPC), contents of the individual phenolic compounds 1–4, and results of the antioxidant assays (TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric-reducing antioxidant power; DPPH radical scavenging activity; EM OXID, β-carotene-linoleic acid emulsion oxidation) of false flax at different growth stages. A and B, distribution of the variables and growth stages, respectively, based on data expressed per extracts; C and D, distribution of the variables and growth stages, respectively, based on data expressed per plant FM.

and compound 3. As expected, the EC_{50} values of the DPPH assay were negatively correlated with FRAP and TEAC. They influenced the distribution of the ripe seed-pod stage, due to its low antiradical activity against DPPH$^{\bullet}$ with high EC_{50} value. The vegetative stage was not related to any variable.

The PCA of the second data set showed that the first two principal components were able to significantly explain up to 97.04% of the total variance. TEAC, FRAP, TPC and compounds 3 and 4 correlated with each other, as shown in plot C (Figure 4), and they also affected the ripe seed-pod stage. The bud and flowering stages were associated with compound 1. The vegetative stage was once again not associated with any variable.

CONCLUSIONS

Our research has shown that chlorogenic acid and quercetin glycosides are present in the aerial parts of *C. sativa* as the main phenolic compounds. Their contents changed in different ways during the growth cycle, although the chlorogenic acid content and the sum of the flavonoids reached the highest levels in the plant extracts at the bud stage. The high individual phenolic content in the plant extract at the bud and flowering stages determined their significant antioxidant activity under polar conditions (TEAC, FRAP and DPPH assay). As far as the antioxidant potential of fresh false flax matter is concerned, the best activity was observed for the plant at the ripe seed-pod stage. PCA indicated that quercetin 3-*O*-glucoside and quercetin glycoside were the main contributors to this potential. The extract and fresh matter of the youngest plant (vegetative stage) both showed a low antioxidant potential. The antioxidant activity of the extracts determined in the emulsion system was slightly different, the highest antioxidant activity was observed for the false flax extracts at the flowering and ripe seed-pod stages.

In short, the aerial part of *C. sativa,* which has a significant content of phenolic compounds, can be used as a source

of protective antioxidant activity ingredients. However, in order to obtain the best properties, the plant should be harvested at the bud, flowering or ripe seed-pod stage.

ACKNOWLEDGEMENTS

The authors would like to express their thanks to the Italian National Research Council which, in the framework of a Short Term Mobility Program – 2019 (STM 2019), provided a visiting grant to Magdalena Karamać.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Abramovič, H., Butinar, B., Nikolič, V. (2007). Changes occurring in phenolic content, tocopherol composition and oxidative stability of *Camelina sativa* oil during storage. *Food Chemistry*, *104*(3), 903–909.
2. Amyot, L., McDowell, T., Martin, S.L., Renaud, J., Gruber, M.Y., Hannoufa, A. (2019). Assessment of antinutritional compounds and chemotaxonomic relationships between *Camelina sativa* and its wild relatives. *Journal of Agricultural and Food Chemistry*, *67*(3), 796–806.
3. Belayneh, H.D., Wehling, R.L., Reddy, A.K., Cahoon E.B., Ciftci, O.N. (2017). Ethanol-modified supercritical carbon dioxide extraction of the bioactive lipid components of *Camelina sativa* seed. *Journal of the American Oil Chemists' Society*, *94*(6), 855–865.
4. Benzie, I.F.F., Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, *239*(1), 70–76.
5. Berti, M., Gesch, R., Eynck, C., Anderson, J., Cermak, S. (2016). Camelina uses, genetics, genomics, production, and management. *Industrial Crops and Products*, *94*, 690–710.
6. Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a free-radical method to evaluate antioxidant activity. *LWT – Food Science and Technology*, *28*(1), 25–30.
7. Das, N., Berhow, M.A., Angelino, D., Jeffery, E.H. (2014). *Camelina sativa* defatted seed meal contains both alkyl sulfinyl glucosinolates and quercetin that synergize bioactivity. *Journal of Agricultural Food Chemistry*, *62*(33), 8385−8391.
8. Faure, J-D., Tepfer, M. (2016). Camelina, a Swiss knife for plant lipid biotechnology. *OCL – Oilseeds and Fats, Crops and Lipids*, *23*(5), D503.
9. Ferreres, F., Valentao, P., Llorach, R., Pinheiro, C., Cardoso, U., Pereira, J.A., Sousa, C., Seabra, R.M., Andrade, P.B. (2005). Phenolic compounds in external leaves of tronchuda cabbage (*Brassica oleracea* L. var. costata DC). *Journal of Agricultural Food Chemistry*, *53*(8), 2901–2907.
10. Ferreres, F., Sousa, C., Vrchovska, V., Valentao, P., Pereira, J.A., Seabra, R.M., Andrade, P.B. (2006). Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. *European Food Research Technology*, *222*, 88–98.
11. Gai, F., Peiretti, P.G., Karamać, M., Amarowicz, R. (2017). Changes in the total polyphenolic content and antioxidant capacities of perilla (*Perilla frutescens* L.) plant extracts during the growth cycle. *Journal of Food Quality*, 2017, art. no. 7214747.
12. Karamać, M., Orak, H.H., Amarowicz, R., Orak, A., Piekoszewski, W. (2018). Phenolic contents and antioxidant capacities of wild and cultivated white lupin (*Lupinus albus* L.) seeds. *Food Chemistry*, *258*, 1–7.
13. Karamać, M., Gai, F., Longato, E., Meineri, G., Janiak, M., Amarowicz, R., Peiretti, P.G. (2019). Antioxidant activity and phenolic composition of amaranth (*Amaranthus caudatus*) during plant growth. *Antioxidants*, *8*(6), 173.
14. Kirkhus, B., Lundon, A.R., Haugen, J.E., Vogt, G., Borge, G.I.A., Henriksen. B.I.F. (2013). Effects of environmental factors on edible oil quality of organically grown *Camelina sativa*. *Journal of Agricultural Food Chemistry*, *61*(13), 3179−3185.
15. Kosińska, A., Karamać, M., Penkacik, K., Urbalewicz, A., Amarowicz, R. (2011). Interactions between tannins and proteins isolated from broad bean seeds (*Vicia faba* Major) yield soluble and non-soluble complexes. *European Food Research and Technology*, *233*(2), 213–222.
16. Martinelli, T., Galasso, I. (2011). Phenological growth stages of *Camelina sativa* according to the extended BBCH scale. *Annals of Applied Biology*, *158*(1), 87–94.
17. Matthäus, B. (2002). Antioxidant activity of extracts obtained from residues of different oilseeds. *Journal of Agricultural and Food Chemistry*, *50*(12), 3444–3452.
18. Matthäus, B., Zubr, J. (2000). Variability of specific components in *Camelina sativa* oilseed cakes. *Industrial Crops and Products*, *12*(1), 9–18.
19. Miller, H.E. (1971). A simplified method for the evaluation of antioxidants. *Journal of the American Oil Chemists' Society*, *48*(2), 91.
20. Onyilagha, J., Bala, A., Hallett, R., Gruber, M., Soroka, J., Westcott, N. (2003). Leaf flavonoids of the cruciferous species, *Camelina sativa*, *Crambe* spp., *Thlaspi arvense* and several other genera of the family Brassicaceae. *Biochemical Systematics and Ecology*, *31*(11), 1309–1322.
21. Pavlović, J., Mitić, S., Mitić, M., Kocić, G., Pavlović, A., Tošić, S. (2019). Variation in the phenolic compounds profile and antioxidant activity in different parts of hawthorn (*Crataegus pentagyna* Willd.) during harvest periods. *Polish Journal of Food and Nutrition Sciences*, *69*(4), 367–378.
22. Peiretti, P.G., Meineri, G. (2007). Fatty acids, chemical composition and organic matter digestibility of seeds and vegetative parts of false flax (*Camelina sativa* L.) after different lengths of growth. *Animal Feed Science and Technology*, *133*(3–4), 341–350.
23. Peiretti, P.G., Karamać, M., Janiak, M., Longato, E., Meineri, G., Amarowicz, R., Gai, F. (2019). Phenolic composition and antioxidant activities of soybean (*Glycine max* [L.] Merr.) plant during growth cycle. *Agronomy*, *9*(3), 153.
24. Quezada, N., Cherian, G. (2012). Lipid characterization and antioxidant status of the seeds and meals of *Camelina sativa* and flax. *European Journal of Lipid Science and Technology*, *114*(8), 974–982.
25. Rahman, M.J., de Camargo, A.C., Shahidi, F. (2018a). Phenolic profiles and antioxidant activity of defatted camelina and sophia seeds. *Food Chemistry*, *240*, 917–925.
26. Rahman, M.J., Ambigaipalan, P., Shahidi F. (2018b). Biological activities of camelina and Sophia seeds phenolics: Inhibition of LDL oxidation, DNA damage, and pancreatic lipase and α-glucosidase activities. *Journal of Food Science*, *83*(1), 237–245.

27. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237.
28. Rice-Evans, C.A., Miller, N.J., Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*(7), 933–956.
29. Shahidi, F., Chandrasekara, A. (2010). Hydroxycinnamates and their *in vitro* and *in vivo* antioxidant activities. *Phytochemistry Reviews*, *9*, 147–170.
30. Salminen, H., Estévez, M., Kivikari, R., Heinonen, M. (2006). Inhibition of protein and lipid oxidation by rapeseed, camelina and soy meal in cooked pork meat patties. *European Food Research and Technology*, *223*, 461–468.
31. Salminen, H., Heinonen, M. (2008). Plant phenolics affect oxidation of tryptophan. *Journal of Agricultural and Food Chemistry*, *56*(16), 7472–7481.
32. Terpinc, P., Polak, T., Ulrih, N.P., Abramovič, H. (2011). Effect of heat treatment of camelina (*Camelina sativa*) seeds on the antioxidant potential of their extracts. *Journal of Agricultural and Food Chemistry*, *59*(16), 8639–8645.
33. Terpinc, P., Polak, T., Makuc, D., Ulrih, N.P., Abramovič, H. (2012a). The occurrence and characterisation of phenolic compounds in *Camelina sativa* seed, cake and oil. *Food Chemistry*, *131*(2), 580–589.
34. Terpinc, P., Čeh, B., Polak, T., Ulrih, N.P., Abramovič, H. (2012b). Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. *Industrial Crops and Products*, *39*, 210– 217.
35. Zubr, J., Matthäus, B. (2002). Effects of growth conditions on fatty acids and tocopherols in *Camelina sativa* oil. *Industrial Crops and Products*, *15*(2), 155–162.

Submitted: 5 February 2020. Revised: 24 March 2020. Accepted: 26 March 2020. Published on-line: 17 April 2020.

Pol. J. Food Nutr. Sci., 2020, Vol. 70, No. 2, pp. 199–201
http://journal.pan.olsztyn.pl

Instruction for Authors
Section: Editorial News

INSTRUCTIONS FOR AUTHORS

SUBMISSION. Original contributions relevant to food and nutrition sciences are accepted on the understanding that the material has not been, nor is being, considered for publication elsewhere. **All papers should be submitted and will be processed electronically via Editorial Manager system (available from PJFNS web site: http://journal.pan.olsztyn.pl). On submission, a corresponding author will be asked to provide: Cover letter; Files with Manuscripts, Tables, Figures/Photos; and Names of two potential reviewers (one from the author's homeland – but outside author's Institution, and the other from abroad).** All papers which have been qualified as relevant with the scope of our Journal are reviewed. All contributions, except the invited reviews are charged. Proofs will be sent to the corresponding author or to the first author and should be returned within one week since receipt. No new material may be inserted in the text at proof stage. It is the author's duty to proofread proofs for errors.

Authors should very carefully consider the preparation of papers to ensure that they communicate efficiently, because it permits the reader to gain the greatest return for the time invested in reading. Thus, we are more likely to accept those that are carefully designed and conform the instruction. Otherwise, papers will be rejected and removed from the on-line submission system.

SCOPE. The Polish Journal of Food and Nutrition Sciences publishes original, basic and applied papers, and reviews on fundamental and applied food research, preferably these based on a research hypothesis, in the following Sections:

Food Technology:
– Innovative technology of food development including biotechnological and microbiological aspects
– Effects of processing on food composition and nutritional value

Food Chemistry:
– Bioactive constituents of foods
– Chemistry relating to major and minor components of food
– Analytical methods

Food Quality and Functionality:
– Sensory methodologies
– Functional properties of food
– Food physics
– Quality, storage and safety of food

Nutritional Research Section:
– Nutritional studies relating to major and minor components of food (excluding works related to questionnaire surveys)

"News" section:
– Announcements of congresses
– Miscellanea

OUT OF THE SCOPE OF THE JOURNAL ARE:
– Works which do not have a substantial impact on food and nutrition sciences
– Works which are of only local significance i.e. concern indigenous foods, without wider applicability or exceptional nutritional or health related properties
– Works which comprise merely data collections, based on the use of routine analytical or bacteriological methods (i.e. standard methods, determination of mineral content or proximate analysis)
– Works concerning biological activities of foods but do not provide the chemical characteristics of compounds responsible for these properties
– Nutritional questionnaire surveys
– Works related to the characteristics of foods purchased at local markets
– Works related to food law
– Works emphasizing effects of farming / agricultural conditions / weather conditions on the quality of food constituents
– Works which address plants for non-food uses (i.e. plants exhibiting therapeutic and/or medicinal effects)

TYPES OF CONTRIBUTIONS. *Reviews:* (at least: 30 pages and 70 references) these are critical and conclusive accounts on trends in food and nutrition sciences; *Original papers:* (maximally: 30 pages and 40 references) these are reports of substantial research; *Reports on post and forthcoming scientific events, and letters to the Editor* (all up to three pages) are also invited (free of charge).

REVIEW PROCESS. All scientific contributions will be peer-reviewed on the criteria of originality and quality. Submitted manuscripts will be pre-evaluated by Editor-in-Chief and Statistical Editor (except for review articles), and when meeting PJFNS' scope and formal requirements, they will be sent to a section Editor who upon positive pre-evaluation will assign at least two reviewers from Advisory Board Members, reviewers suggested by the author or other experts in the field. Based on the reviews achieved, Section Editor and Editor-in-Chief will make a decision on whether a manuscript will be accepted for publication, sent back to the corresponding author for revision, or rejected. Once a manuscript is sent back to the corresponding author for revision, all points of the reviews should be answered or rebuttal should be provided in the Explanation letter. The revised manuscripts will be checked by Section Editor and by the original reviewers (if necessary), and a final decision will be made on acceptance or rejection by both Section Editor and Editor-in-Chief.

Polish Journal of Food and Nutrition Sciences uses CrossCheck's iThenticate software to detect instances of similarity in submitted manuscripts. In publishing only original research, PJFNS is committed to deterring plagiarism, including self-plagiarism. Your manuscript may be screened for similarity to published materials.

COPYRIGHT LICENSE AGREEMENT referring to Authorship Responsibility and Acknowledgement, Conflict of Interest and Financial Disclosure, Copyright Transfer, are required for all authors, i.e. *Authorship Responsibility and Acknowledgement*: Everyone who has made substantial intellectual contributions to the study on which the article is based (for example, to the research question, design, analysis, interpretation, and written description) should be an author. It is dishonest to omit mention of someone who has participated in writing the manuscript ("ghost authorship") and unfair to omit investigator who have had important engagement with other aspects of the work. All contributors who do not meet the criteria for authorship should be listed in an Acknowledgments section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chairperson who provided only general support. Any financial and material support should also be acknowledged. *Conflict of Interest and Financial Disclosure*: Authors are responsible for disclosing financial support from the industry or other conflicts of interest that might bias the interpretation of results. *Copyright License Agreement*: Authors agree that papers accepted become the copyright of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences in Olsztyn, Poland, and may not be published elsewhere without the Editor's permission in writing.

A manuscript will not be published once the signed form has not been submitted to the Editor with the manuscript revised after positive reviews.

ETHICAL APPROVAL OF STUDIES AND INFORMED CONSENT. For all manuscripts reporting data from studies involving human participants or animals, formal approval by an appropriate institutional review board or ethics committee is required and should be described in the Methods section. For those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be followed. For investigations of humans, state in the Methods section the manner in which informed consent was obtained from the study participants (i.e., oral or written). Editors may request that authors provide documentation of the formal review and recommendation from the institutional review board or ethics committee responsible for oversight of the study.

UNAUTHORIZED USE AND COPYRIGHT AGREEMENT. Published manuscripts become the property of the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences (IAR&FR PAS) and may not be published elsewhere without written permission. Unauthorized use of the PJFNS name, logo, or any content for commercial purposes or to promote commercial goods and services (in any format, including print, video, audio, and digital) is not permitted by IAR&FR PAS.

MANUSCRIPTS. A manuscript in English must be single-sided, preferably in Times New Roman (12) with 1.5-point spacing, without numbers of lines. The Editor reserves the right to make literary corrections and to make suggestions to improve brevity. English is the official language. The English version of the paper will be checked by Language Editor. Unclear and unintelligible version will be sent to the author(s) for correction.

Every paper should be divided under the following headings in this order: a **Title** (possibly below 150 spaces), **Running title** (up to 50 spaces, submitted under the Title); the Names(s) of the author(s) in full. In paper with more than one author, the asterisk indicates the name of the author to whom correspondence and inquiries should be addressed, otherwise the first author is considered for the correspondence. Current full postal address of the indicated corresponding author or the first author must be given in a footnote on the title page; the Place(s) where the work was done including the institution name, city, country if not Poland. In papers originated from several institutions the names of the authors should be marked with respective superscripts; the **Key words** (up to 6 words or phrases) for the main topics of the paper; an **Abstract** (up to 250 words for regular papers and reviews and 100 words for Short Reports) summarizing briefly main results of the paper, no literature references; an **Introduction** giving essential background by saying why the research is important, how it relates to previous works and stating clearly the objectives at the end; **Materials and Methods** with sufficient experimental details permitting to repeat or extend the experiments. Literature references to the methods, sources of material, company names and location (city, country) for specific instruments must be given. Describe how the data were evaluated, including selection criteria used; **Results and Discussion** presented together (in one chapter). Results should be presented concisely and organized to supplement, but not repeat, data in tables and figures. Do not display the data in both tabular and graphic form. Use narrative form to present the data for which tables or figures are unnecessary. Discussion should cover the implications and consequences, not merely recapitulating the results, and it must be accomplished with concise **Conclusions**; **Acknowledgements** should be made to persons who do not fill the authorship criteria (see: Authorship forms); **Research funding** should include financial and material support; and **References** as shown below.

REFERENCES each must be listed alphabetically at the end of the paper (each should have an Arabic number in the list) in the form as follows: **Periodicals** – names and initials of all the authors, year of publication, title of the paper, journal title as in Chemical Abstracts, year of publication, volume, issue, inclusive page numbers; **Books** – names and initials of all the authors, names of editors, chapter title, year of publication, publishing company, place of publication, inclusive page numbers; **Patents** – the name of the application, the title, the country, patent number or application number, the year of publication.

For papers published in language other than English, manuscript title should be provided in English, whereas a note on the original language and English abstract should be given in parentheses at the end.

The reference list should only include peer-reviewed full-text works that have been published or accepted for publication. Citations of MSc/PhD theses and works unavailable to international Editors, Reviewers, and Readers should be limited as much as possible.

References in the text must be cited by name and year in square parentheses (e.g.: one author – [Tokarz, 1994]; two authors – [Słonimski & Campbell, 1987]; more than two authors – [Amarowicz *et al*., 1994]). If more than one paper is published in the same year by the same author or group of authors use [Tokarz, 1994a, b]. Unpublished work must only be cited where necessary and only in the text by giving the person's name.

Examples:

Article in a journal:

Słonimski, B.A., Campbell, L.D., Batista, E., Howard B. (2008). Gas chromatographic determination of indole glucosinolates. *Journal of Science and Food Agriculture*, *40*(5), 131–143.

Book:

Weber, W., Ashton, L., Milton, C. (2012). *Antioxidants – Friends or Foes?* 2nd edition. PBD Publishing, Birmingham, UK. pp. 218–223.

Chapter in a book:

Uden, C., Gambino, A., Lamar, K. (2016). Gas chromatography. In M. Queresi, W. Bolton (Eds.), *CRC Handbook of Chromatography*, CRC Press Inc., Boca Raton, Florida, USA, pp. 44–46.

ABBREVIATIONS AND UNITS. Abbreviations should only be used when long or unwieldy names occur frequently, and never in the title; they should be given at the first mention of the name. Metric SI units should be used. The capital letter L should be used for liters. Avoid the use of percentages (% g/g,% w/w; Mol-%; vol-%), ppm, ppb. Instead, the expression such as g/kg, g/L, mg/kg, mg/mL should be used. A space must be left between a number and a symbol (e.g. 50 mL not 50mL). A small x must be used as multiplication sign between numeric values (e.g. 5×10^2 g/mL). Statistics and measurements should be given in figures, except when the number begins a sentence. Chemical formulae and solutions must specify the form used. Chemical abbreviations, unless they are internationally known, Greek symbols and unusual symbols for the first time should be defined by name. Common species names should be followed by the Latin at the first mention, with contracting it to a single letter or word for subsequent use.

FIGURES should be submitted in separate files. Each must have an Arabic number and a caption. Captions of all Figures should be provided on a separate page "Figure Captions". Figures should be comprehensible without reference to the text. Self-explanatory legend to all figures should be provided under the heading "Legends to figures"; all abbreviations appearing on figures should be explained in figure footnotes. Three-dimensional graphs should only be used to illustrate real 3-D relationships. Start the scale of axes and bars or columns at zero, do not interrupt them or omit missing data on them. Figures must be cited in Arabic numbers in the text.

TABLES should be submitted in separate files. They should be as few in number and as simple as possible (like figures, they are expensive and space consuming), and include only essential data with appropriate statistical values. Each must have an Arabic number and a caption. Captions of all Tables should be provided on a separate page "Table Captions". Tables should be self-explanatory; all abbreviations appearing in tables should be explained in table footnotes. Tables must be cited in Arabic numbers in the text.

PAGE CHARGES. A standard publication fee has been established at the rate of 450 EUR + tax (if applicable) irrespective of the number of pages and tables/figures. For Polish Authors an equivalent fee was set at 1950 PLN + VAT. Payment instructions will be sent to Authors via e-mail with acceptance letter.

Information on publishing and subscription is available from:

Ms. Joanna Molga
Editorial Office of Pol. J. Food Nutr. Sci.
Institute of Animal Reproduction and Food Research
Tuwima 10 Str., 10–748 Olsztyn 5, Poland
phone (48 89) 523–46–70, fax (48 89) 523–46–70;
e-mail: pjfns@pan.olsztyn.pl; http://journal.pan.olsztyn.pl

Nutrition