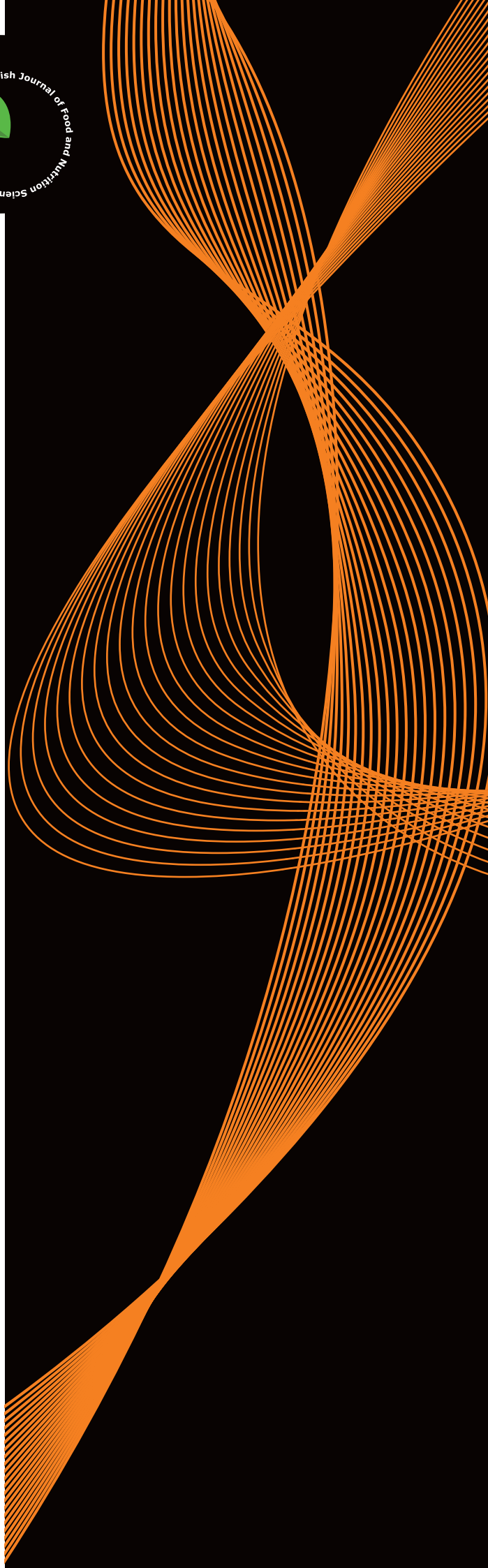
ISSN (1230-0322)

2 0 1 9, Vol. 69, No. 3

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 1 9, Vol. 69, No. 3

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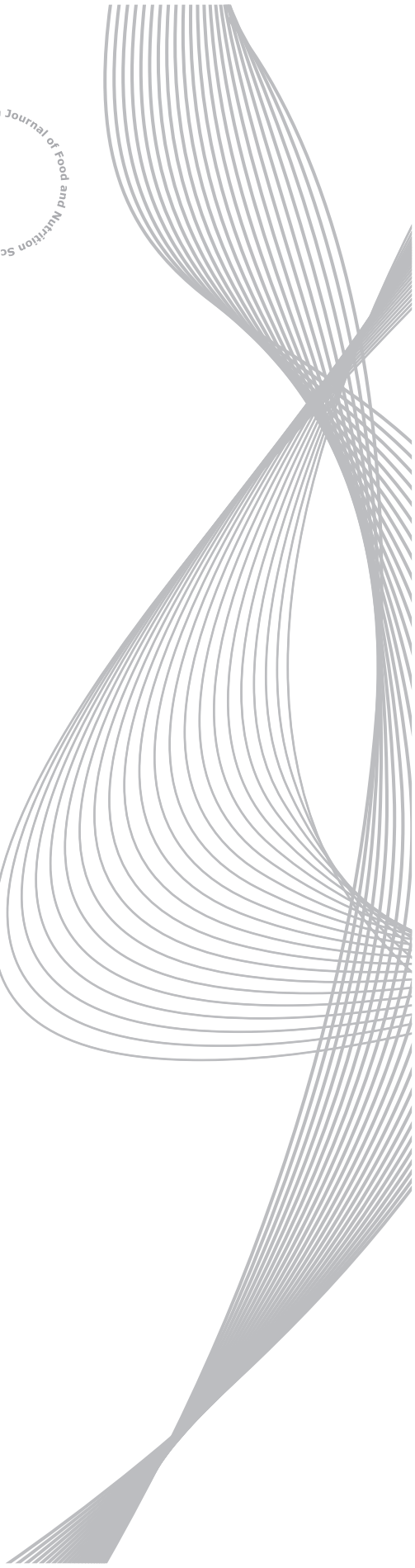

Published

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

Pol. J. Food Nutr. Sci. 2019, Vol. 69, No. 3

Advisory Board of PJFNS 2019–2022

Pol. J. Food Nutr. Sci. 2019, Vol. 69, No. 3

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
2019, VOL. 69, NO. 3

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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

2019 – 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. 15,5; Ark. druk. 15,5
Skład i druk: Mercurius, www.mercurius.com.pl

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 219–233
DOI: 10.31883/pjfns/110735
http://journal.pan.olsztyn.pl

Review article
Section: Nutrition Research

The Dose Makes the Poison: Sugar and Obesity in the United States – a Review

*Samir Faruque, Janice Tong, Vuk Lacmanovic, Christiana Agbonghae, Dulce M. Minaya, Krzysztof Czaja**

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Key words: sugar, obesity, sugar consumption, BMI, childhood obesity, adult obesity

Two-thirds of the US population is either overweight or obese. Obesity is one of the major drivers of preventable diseases and health care costs. In the US, current estimates for these costs range from $147 to $210 billion per year. Obesity is a multifactorial disease: genetics, lifestyle choices, metabolism, and diet. Low-fat diets have been suggested as the key to weight management. However, over the past 30 years, the calories from fat in people's diets have gone down, but obesity rates keep climbing. Evidence suggests that diets high in added sugar promote the development of obesity. However, the impact of sugar consumption on weight gain and body fat accumulation remains a controversial topic. Therefore, the aim of this review is to provide basic framework information about the prevalence of obesity and sugar consumption in the US over the last five decades. We also review the process by which sugar is converted to fat and stored in the human body. The relationship between sugar consumption and obesity was analyzed using United States Department of Agriculture (USDA) Sugar and Sweetener Outlook data, and obesity prevalence was analyzed using data from the Centers for Disease Control and Prevention (CDC). The analysis revealed a reduction in sugar consumption concurrent with a slowing down of the annual rate of increase of obesity. However, although the data show that the sugar consumption trend is going in the right direction (declining), the US population still consumes more than 300% of the recommended daily amount of added sugar.

OBESITY EPIDEMIC: THE ELEPHANT IN THE ROOM

What exactly defines obesity?

Obesity was first included in the international lists of diseases and causes of death in 1948 [WHO, 1948]. Currently, according to the Obesity Medicine Association obesity is defined as "a chronic, relapsing, multifactorial, neurobehavioral disease, wherein an increase in body fat promotes adipose tissue dysfunction and abnormal fat mass physical forces, resulting in adverse metabolic, biomechanical, and psychosocial health consequences." Therefore, better understanding of obesity as a disease would not only help people be more mindful and work towards a balanced daily diet on the personal level but would also improve the effort in curbing obesity on the national and global level.

Compared to the data from 1960 to 1962 from the National Health Examination Survey, there has been an overall increase in the prevalence of obesity in the United States. As of 2014, the age-adjusted prevalence of obesity in the United States was 35.0% for men and 40.4% for women among adults aged 20 to 74 [Flegal *et al*., 2016]. Similarly, the prevalence of obesity in children and adolescents has steadily risen in the past decades, surging from 5.2% (in 1971–1974) to 17.2% (in 2013–2014) [Fryar *et al*., 2014]. Consequently, obesity in children and adolescents was referred to as an "epidemic" by the American Academy of Pediatrics Committee on Nutrition [Caprio & Genel, 2005].

A first step in treating obesity is understanding what it is and how can it be measured. Throughout the years numerous methods have been developed and tested to accurately measure adiposity in order to diagnose obesity. Different from adults, practicality is a major deciding factor in choosing an appropriate measurement method for children because adhering to the testing protocols can be a challenge. Additionally, certain detailed body-composition measuring methods are based on adult models, and thus not suitable to be used on children [Alonso *et al*., 2018].

Currently, body mass index (BMI) is the most widely used method for diagnosing and staging obesity because it is more practical and easy to obtain compared to other methods. BMI estimates adiposity based on height and weight. The BMI data presented in the National Health Examination Survey are defined as weight in kilograms divided by height in meters squared. A high BMI is generally indicative of a high fat mass accumulation. Table 1 provides a breakdown of weight classification based on BMI [CDC, 2016]. Assessment of obesity in children and adolescents is commonly done by plotting BMI on a standard growth chart with defined BMI values and cut-off points relative to age and gender. Overweight is defined as BMI at or above the 85th percentile of the sex and age group, while obesity is defined as at or above the 95th percentile.

The accuracy of using BMI is often questioned because individuals of different heights or body build may have simi-

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TABLE 1. Weight classification based on BMI measurements (kg/m^2).

BMI	Weight classification
X < 18.5	Underweight
18.5 < x < 25	Normal
25 < x < 30	Overweight
30 < x < 35	Class 1 Obesity
35 < x < 40	Class 2 Obesity
X > 40	Class 3 Obesity

Data taken from the National Health and Nutrition Examination Surveys.

lar BMI values, yet different proportions of total body fat [Dietz & Bellizzi, 1999]. Because BMI is an indirect measure of the amount of tissue mass in an individual to then categorize that person, it is possible to misclassify individuals as BMI does not differentiate fat mass from lean mass [Romero-Corral, 2008]. Adult individuals who seem to have more body fat and decreased muscle mass may be classified as normal while lean individuals with high muscle mass, but low body fat percentage may be classified as obese [Rothman, 2008]. In addition, an assessment by the International Obesity Task Force (IOTF) showed that in order to circumvent such problem when using the BMI to classify children and adolescents, several factors must be considered. For the cut-off point used to define obesity to be valid, the growth chart must incorporate different ethnic, sex, and age groups to account for the variations in percent body fat with the same BMI. Prior undernutrition should also be taken into account [Dietz & Bellizzi, 1999]. Studies have shown that among short children, increased weight-for-height may be the result not of excessive body fat but rather lower body fat accompanied by greater lean tissue or lean tissue hydration [Trowbridge *et al*., 1987].

Besides BMI, other anthropometric measurements such as Waist circumference (WC), Waist to Hip ratio (WHR), Waist to Height ratio (WHtR), skinfold thickness, and bioelectrical impedance are indirect physiological measurements of central obesity. WC has been found to be correlated with the risk of acquiring chronic conditions such as type 2 diabetes. However, WC measurements are usually restricted to individuals labeled as normal or overweight so predicting disease risk using WC is generally not useful in patients with obesity [National Heart, 2013]. Men with a waist circumference above 102 cm and women with a WC above 88 cm are at a higher risk of developing an obesity-related disease. WC cut-off points usually apply to adults in all ethnic and racial groups. However, if the individual is very short or has a BMI above 30, WC cut-off points as a predictor for disease may not be as accurate. Although WC can be monitored with BMI, patients can also keep track of their WC independently as it can provide an estimate of changes in abdominal fat.

WHR and WHtR incorporate WC measurements and serve as additional measurements of body fat distribution. WHR is calculated by dividing WC by hip circumference. Similarly, WHtR is calculated by dividing WC by height. For these measurements, factors such as posture, stomach contents at the time of measurement, and tightness and type of tape may introduce measurement error [WHO, 2011]. Combining two or more of these methods could provide the most accurate classification [Liu *et al*., 2011]. Skinfold thickness is an anthropometric method that uses the total sum of skinfold to estimate body fat based on developed prediction models. This method uses a special caliper to measure subcutaneous fat at sites such as triceps, biceps, the subscapular region, abdomen, and thigh [Hu, 2008]. Equations used to estimate body fat from skinfold thickness are highly population specific and strongly dependent on age. The latter factor is because subcutaneous adipose tissue distribution changes during maturation. A notable limitation of this method is that skinfold thickness cannot be accurately measured in morbidly obese patients, both children and adults [Goran, 1998; Sarria *et al*., 1998]. Bioelectrical impedance analysis calculates fat mass by transforming total body water into fat-free mass. Compared to the hydration of fat-free mass in adults being relatively constant at approximately 73.2%, that of children varies. The variation complicates the conversion of total water into fat-free mass, imposing a limitation on the bioelectrical impedance analysis method [Talma *et al*., 2013].

Direct measure methods such as densitometry, Dual-energy X-ray absorptiometry (DXA), and X-ray computerized tomography are preferred for measuring visceral adipose tissue (VAT), yet are costly, and less practical for wide-scale use. Densitometry, also called underwater or hydrostatic weighing, uses measurement of total body density to estimate body composition. The method is based on the principle that fat is less dense than water and, consequently, an individual with more body fat will have lower body density. Densitometry weighs the subject in air as well as underwater. The recorded measurements are then used to estimate body volume, body density, and percent body fat using an established formula [Hu, 2008]. Although often referred to as the "gold standard" of measuring body composition, densitometry faces two limitations when applied to children. Besides the strict and time-consuming testing procedure that proved to be difficult for young subjects, densitometry also requires additional knowledge on densities of fat and fat-free masses in children, which are influenced by age, gender, and ethnicity [Lohman, 1986].

DXA is a commonly used standard to study VAT volume. DXA scans provide 2-dimensional images of different tissues and organs [Silver *et al*., 2010]. Photons are produced that travel through bone, fat, and non-bone lean tissue. Unique elements in each type of tissue, such as calcium and phosphorus in bone, allow for visualization and analysis of each tissue type. DXA scans typically last between 5–20 min and are advantageous because body fat distribution and regional bone mineral density can be determined simultaneously [Rothney *et al*., 2009]. Similar to densitometry, DXA has limitations. The maximum weight and width of the scanning area are approximately 136 kg (300 lb.) and 60 cm, respectively [Brownbill & Ilich, 2005]. To accommodate heavier subjects, iDXA was introduced with expanded capacity: 400 lb. and 66 cm. However, even with this improvement, morbidly obese individuals who are above the iDXA scanner weight limit are still not covered, making the total body composition of such subjects difficult to attain [Rothney *et al*., 2009]. In addition,

studies using animal carcasses have shown that confounding factors such as the animal size, the equipment, and the operation mode or calibration equation may affect the accuracy of DXA estimates [Brunton *et al*., 1993].

X-ray computerized tomography (CT) could be used as a diagnostic imaging technique for obesity but CT scans are hazardous to patients because of the ionizing radiation given off by the scans. For this reason, CT is typically limited to diagnosing patients with acute illnesses [Poonawalla *et al*., 2013]. T1-weighted magnetic resonance imaging (MRI) is a less harmful alternative to CT scans because it does not expose patients to any ionizing radiation. However, separating VAT from subcutaneous adipose tissue and non-adipose tissue by MRI is a costly and time-consuming process. This process is also highly likely to result in a systematic error. While small-scale research studies may use CT and MRI to measure obesity, anthropometric measures like BMI are still more widely used [Poonawalla *et al*., 2013].

In conclusion, obesity is a chronic disease manifested by an increase in body fat accumulation and resulting in adverse metabolic and psychosocial health consequences. Because recent studies show that body adiposity can be increased without dramatic increase in body weight by consumption of high-sugar diets [Do *et al.,* 2018; Sen *et al*., 2017], more specific methods determining body fat content described in this review should be used more frequently in diagnostics of obesity. This will allow us to identify metabolically obese normal weight people with normal BMI and metabolic syndrome [Ruderman *et al.,* 1998].

Today's overweight children. Tomorrow's obese adults?

Pediatric obesity is associated with increased prevalence of cardio-metabolic risk factors that predict early onset of serious diseases, such as hypertension and diabetes [Skinner *et al*., 2015]. Prevalence of the metabolic syndrome in obese adolescents may be as high as 30% [Daniels, 2009]. The number of additional cardiovascular events attributable to excess weight in adolescents is expected to be >100,000 by 2035, in the United States alone [Cote *et al*., 2013]. This prediction is plausible since approximately 75% of obese children have advanced vascular age similar to that of a 45-year old adult due to altered cardiac structure and function, which includes higher epicardial fat deposition, diastolic and systolic dysfunction at both rest and exercise, and greater atrial stiffness [Cote *et al*., 2013]. Consequently, the risk of having both fatal and nonfatal coronary heart disease event in adulthood is significantly elevated for children and adolescents with BMI higher than average, according to a cohort study on children aged seven to thirteen that evaluated the association between childhood BMI and adult coronary heart disease [Baker *et al*., 2007].

Growth and development during adolescence is associated with normal increase in insulin resistance [Daniels, 2009]. However, if the insulin resistance is further exacerbated by obesity, it can result in deleterious consequences, notably glucose intolerance followed by type 2 diabetes mellitus. The decreased insulin sensitivity and increased circulating insulin levels observed in many obese children often persist through the transition into young adulthood. Another metabolic complication observed in obese children and adolescents is dyslipidemia; more specifically, triglyceride levels are increased while high-density lipoprotein cholesterol level is decreased, often referred to as atherogenic dyslipidemia. The condition is known to accelerate atherosclerosis, marked by the buildup of plaque in the arteries that limits the flow of oxygen and further contributes to the coronary heart disease associated with pediatric obesity discussed earlier [Daniels, 2009]. Preventive and early intervention measures must be taken to prevent the comorbidity and mortality associated with obesity in children and adolescents. Besides having adverse effects on the well-being of the child, obesity may lead to other complications in the long run. Obese children and adolescents have a higher probability of being obese as adults and increased risk of obesity-related comorbidities in adulthood, *i.e.* cardiovascular disease, premature mortality [Reilly *et al.,* 2003]. Furthermore, individuals who were obese as a child showed elevated morbidity and mortality in adulthood despite losing the extra weight as adults [Deckelbaum & Williams, 2001].

Studies have shown that the energy imbalance causing obesity is in fact affected by other contributing or predisposing factors [Baranowski & Taveras, 2018]. Possible factors include obesogenic infectious agents, toxic chemicals, genetic influences, epigenetic influences, the gut microbiome, brown or beige fat, and prenatal and early childhood factors (such as the breast milk hormones and adipocytokines) [Fields *et al*., 2017]. These factors alone can influence energy balance or interact with one another to bring out a wide range of outcomes. To further complicate the equation, the contributing factors can also vary based on the stage of maturation. The interplay between obesity and underlying environmental factors is also observed, where different socioeconomic or cultural factors can make certain subpopulations more susceptible to obesity [Seidell & Halberstadt, 2016]. On a daily basis, family environment contributes to the increased prevalence of obesity through food supply/caloric intake and patterns of physical activity [Deckelbaum & Williams, 2001]. The existence and corresponding influence of contributing and predisposing factors were reflected in the result of obesity preventive trials. In an effort to curb the prevalence of adolescent obesity, numerous prevention trials (such as educational, behavioral, and health promotion interventions) have been designed and implemented to emphasize healthy diet, increase physical activity, decrease sedentary behaviors, and sleep. According to the Cochrane review of childhood obesity prevention research that evaluated 55 different studies, the majority of existing interventions had little to no effect on decreasing adiposity of the subject, mainly due to failure to consider and incorporate contributing or predisposition factors [Waters *et al*., 2011].

Prevalence of obesity

The National Health and Nutrition Examination Survey (NHANES) program of the National Center for Health Statistics used BMI as an indicator for the prevalence of obesity in the United States beginning in 1960. Although the cross-sectional health examination surveys began in 1960, it was not until 1999 that the NHANES continued without breaks

between each survey period [Flegal *et al*., 2016]. Information prior to that was collected by the National Health Examination II and III: 1963–1965 and 1966–1970, along with three less consistent NHANES I-III: 1971–1974, 1976–1980, and 1988–1994. The evolution of the national survey was mainly constituted by the shift in focus from growth and development to nutrition and health, the additional collection of environmental exposures and infectious diseases data, and the gradual inclusion of younger age groups as well as more ethnically diverse population groups.

The participants in the NHANES survey were selected using a complex, stratified, multistage probability cluster sampling design [Flegal *et al*., 2016]. Prior to the examination, a household interview was conducted in order to record the age of the participants. A mobile examination center with standardized techniques and equipment was used for all of the surveys. For every NHANES cycle, heights and weights of participants were measured by trained health technicians as part of the anthropometry component. Pregnant women and observations for participants missing valid height and weight measurements were excluded from the survey analysis [Flegal *et al*., 2016]. The surveys conducted by NHANES may have been subject to sampling error or non-sampling error. The surveys were also limited to detecting only small changes in the prevalence of obesity, especially among subgroups that were defined by sex, age, race, and ethnicity [Flegal *et al*., 2010].

To compare data across surveys, the data for subjects aged 20–74 were age-adjusted by direct method to the U.S. population estimates in 2000. The adult age groups were 20–39, 40–59, and 60–74 years old. For the data collected in the surveys, BMI was defined as indicated in Table 1. The National

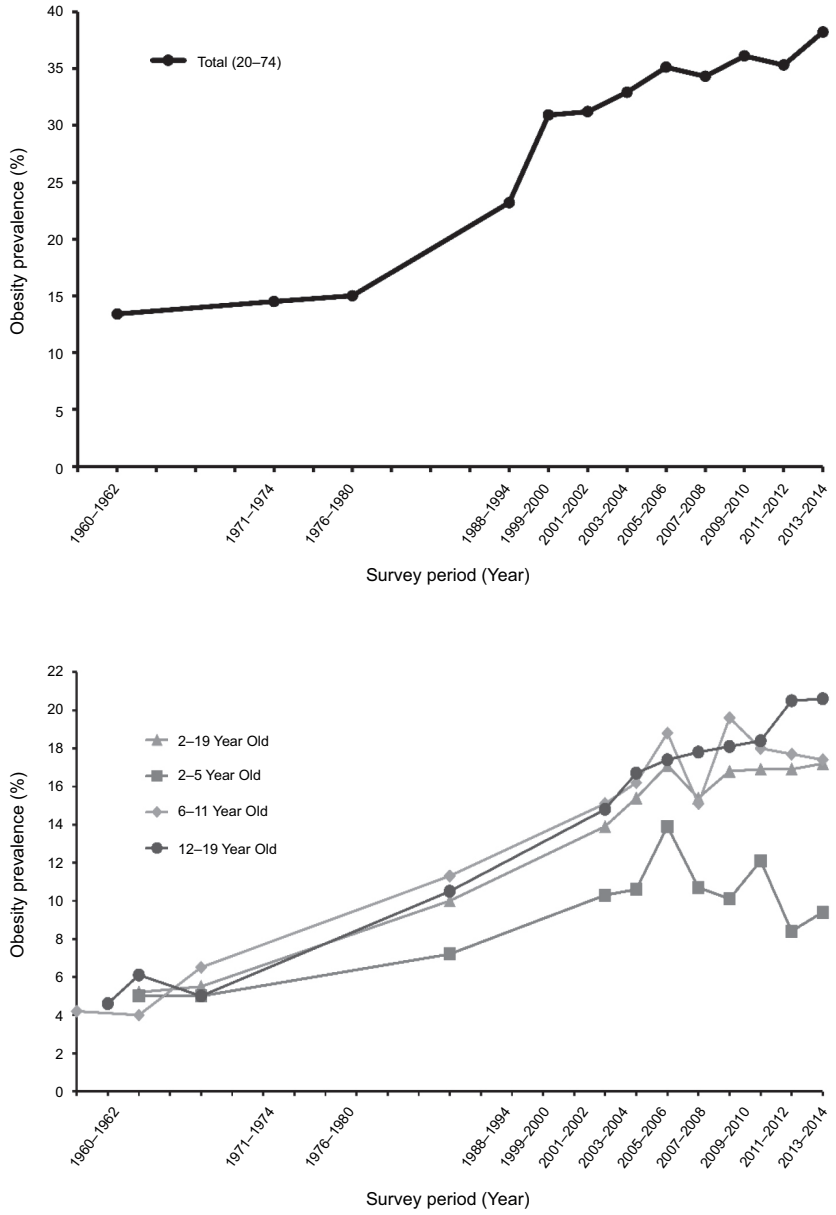

FIGURE 1. Prevalence of obesity in the United States. Top panel shows adult obesity level based on survey data collected 1960–2014. The bottom panel shows the prevalence of obesity in children and adolescents 1963–2014. Data from the National Health and Nutrition Examination Surveys.

Health Examination Survey (NHES) cycle I (1960 to 1962) included adults age 18–79. The National Health and Nutrition Examination Survey (NHANES) cycles I and II did not include adults over the age of 74 so trend estimates are made for adults between 20 and 74. Sample sizes for each of the surveys varied. NHES and NHANES also grouped data by race. Survey data were collected between 1960 and 2014 (Figure 1) [Flegal *et al*., 2010].

The US Centers for Disease Control and Prevention (CDC) BMI-for-age growth chart was used to determine obesity in participants aged 2–19. Similar to NHANES, the CDC growth chart has been revised to better reflect the populations [Kuczmarski, 2002]. The 1977 version of the growth chart used data from NHES II, NHES III, and NHANES I. The old chart was only applicable to prepubescent boys from 90 to 145 cm in height and girls from 90 to 137 cm. The revision of the growth chart was conducted in 2000, using all NHES and NHANES along with improved statistical smoothing procedures. The revision replaced the limited chart with the 2000 CDC BMI-for-age charts, applicable to all children ranging from two to twenty years. The pattern of the BMI curve shows a rapid increase from birth to the eighth month, then decrease until around six years of age, followed by a rebound, as seen in Figure 2. Adiposity rebound is the age where body masses begin to rise after reaching a minimum. Studies have shown that the younger the age at which the curve hits nadir, the greater the likelihood of higher level of adiposity during adolescence and early childhood [Kuczmarski, 2002]. Additionally, children at higher BMI percentiles tend to achieve earlier adiposity rebound.

Comparison of consecutive NHANES data have showed an increase in adult as well as childhood obesity in the United States. From 1960 to 1962, the percentage of overweight adults in the United States was 31.5% while the percentage of obese adults was 13.4% [Ogden & Carroll, 2010]. From 2013 to 2014, the percentage of overweight adults remained stable at 31.9% while the percentage of obese adults increased to 38.2% [Fryar *et al.,* 2016]. Between 1960 and 2014, the obesity level in the United States increased more than 100% (Figure 1, top panel). The increase in pediatric obesity is most evident in the 12–19 age subgroup (Figure 1, bottom panel). From 2001 to 2014, the prevalence of obesity in the 2–5 age group decreased by 1.2% while that in 6–11 year increased by 1.2%. The overall increased prevalence was mainly contributed by the 12–19 year old group, which experienced a dramatic 3.9% surge [Ogden *et al.,* 2016].

Although the percentage of both adult and childhood obesity has continued to rise during the past decades, the rate of increase has actually decreased. Four studies focusing on U.S. data showed stability or a leveling off in childhood obesity prevalence. The tendency towards stabilization of childhood obesity in the U.S. is analogous to that of Australia, Europe, and Russia [Rokholm *et al*., 2010]. As can be seen in Figure 2, the turning point toward stabilization in adult and childhood obesity both appeared to be predominantly in the early 2000s.

Of the environmental factors that contribute to obesity, sugar consumption, especially in the form of sugar-sweetened beverages, has gained the attention of epidemiologic studies

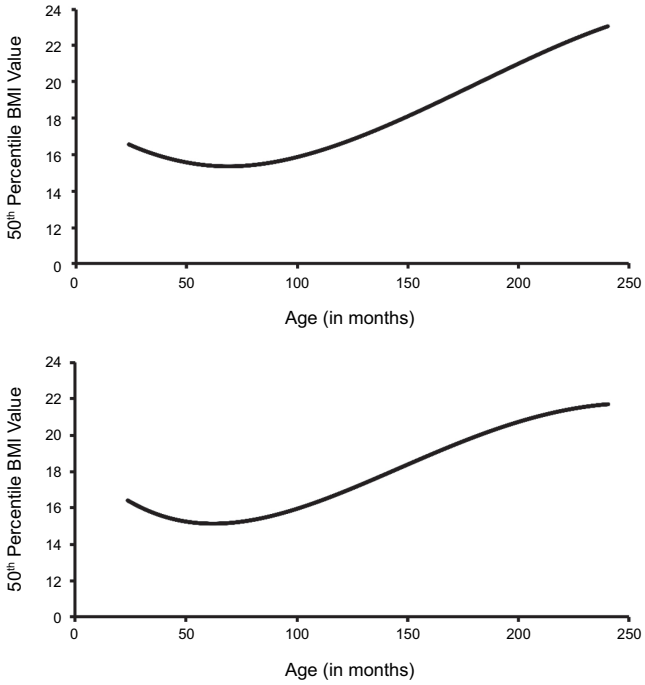

FIGURE 2. Average BMI-for-age charts for two to twenty-year-old (A) males and (B) females. Created from the CDC 2000 Growth Charts. The start of adiposity rebound is observed at 6 years of age in both male and female subjects.

in the past several decades. Soft drinks constitute the leading source of added sugar in everyday diet [Ludwig *et al*., 2001]. Findings from large cross-sectional studies along with cohort studies have shown a positive association between increased intake of sugar-sweetened beverages and weight gain as well as obesity in both children and adults [Malik *et al*., 2006]. **The US population consumes more than 300% of daily recommended amount of added sugar!**

Several theories attempt to explain the rising levels of obesity as described in the previous section. One posits that it is simply a balance of calorie intake and expenditure, with the former increasing to unprecedented levels and the latter reaching all-time lows with more sedentary lifestyles. Another concludes that increased amounts of fat consumption may induce obesity and its related comorbidities. These theories have validity, but their simplicity does not entertain the nuances or subtleties of the causes of obesity as they may disregard several factors and/or mechanisms that may be especially responsible for rising obesity rates. Altered patterns of sugar consumption may play a role as preceding and concurrent changes in sugar consumption rates in the United States since the 1970's occur simultaneously to changes in obesity rates.

In the United States, sugar consumption has been recorded and tracked by surveyed accounts conducted by the National Center of Health Statistics (NCHS) in the Center of Disease Control (CDC) and by deduction from imports and deliveries of sugar, which was done by the United States Department of Agriculture (USDA) [CDC, 2012; USDA, 2017]. The American Heart Association (AHA) recommends limiting daily sugar consumption to 25 g/day for an adult woman and 37.5 g/day for an adult man [Johnson *et al*., 2009]. These

recommendations were derived using the concept of discretionary calories. Discretionary calories was a concept developed to aid people in meeting their nutritional requirements while avoiding excess caloric intake. It is calculated by estimating a person's caloric need to meet nutrient requirements and subtracting this from their caloric need to maintain a stable body weight. The goal of the recommendation is to help people maintain a healthy body weight, decrease their risk of cardiovascular disease and other comorbidities, and meet their nutritional requirements [Johnson *et al*., 2009].

The NCHS uses the National Health and Nutrition Examination Survey (NHANES) to determine the sugar consumption per person. This survey assesses food and beverage consumption through an interview based on the self-reported recall of dietary information from a respondent from the last 24 hours [CDC, 2012]. These values are then converted into numerical values for caloric energy intake [Blair *et al*., 1992]. Although the NHANES has been used frequently to determine the *per capita* sugar consumption, it has methodological limitations. The majority of the caloric intake measured from this survey were found to be physiologically unlikely due to misreporting, suggesting that it may have a poor ability to estimate population trends in caloric intake, therefore making analyses using data from these surveys extremely limited in utility [Archer *et al*., 2013].

The Economic Research Service of the USDA has compiled information and deduced the annual *per capita* consumption of sweeteners, including refined cane and beet sugar, high fructose corn syrup (HFCS), and other sweeteners (molasses, honey, *etc*.) annually since 1970 [USDA, 2017]. The values for annual sugar consumption were found by estimating *per capita* sweetener deliveries for domestic food and beverage use at the market level and subtracting losses between the processing of the sugar to the ingestion by the consumer.

Data for the deliveries of the three categories of refined cane and beet sugar, high fructose corn syrup (HFCS), and other sweeteners (honey, molasses *etc*.) were gathered using different sources. Refined sugar data comes from the USDA's Farm Service Agency's Sweetener Market Data publication, where all US and Puerto Rican sugar beet processors, sugarcane millers, and cane sugar refiners report their information to the FSA in accordance to the Farm Security and Rural Investment Act. The ERS estimate HFCS deliveries with data from "various industry contacts and consulting firms" including the US Census Bureau. The "other" sweetener category includes delivery estimates from honey, molasses and syrups. Honey deliveries were estimated using production and stock data from the USDA's National Agricultural Statistics Service's (NASS) annual report on honey and the US Bureau's import and export of honey data, and the deliveries of other sugars are assumed to be about the same as maple syrup, which is also reported by NASS and US Bureau [Haley *et al*., 2005].

Sources of loss include loss from market to retail/institutional level, loss from retail/institutional to the consumer level, and loss at the consumer level, which includes uneaten foods and spoilage. ERS calculated the adjustment for loss

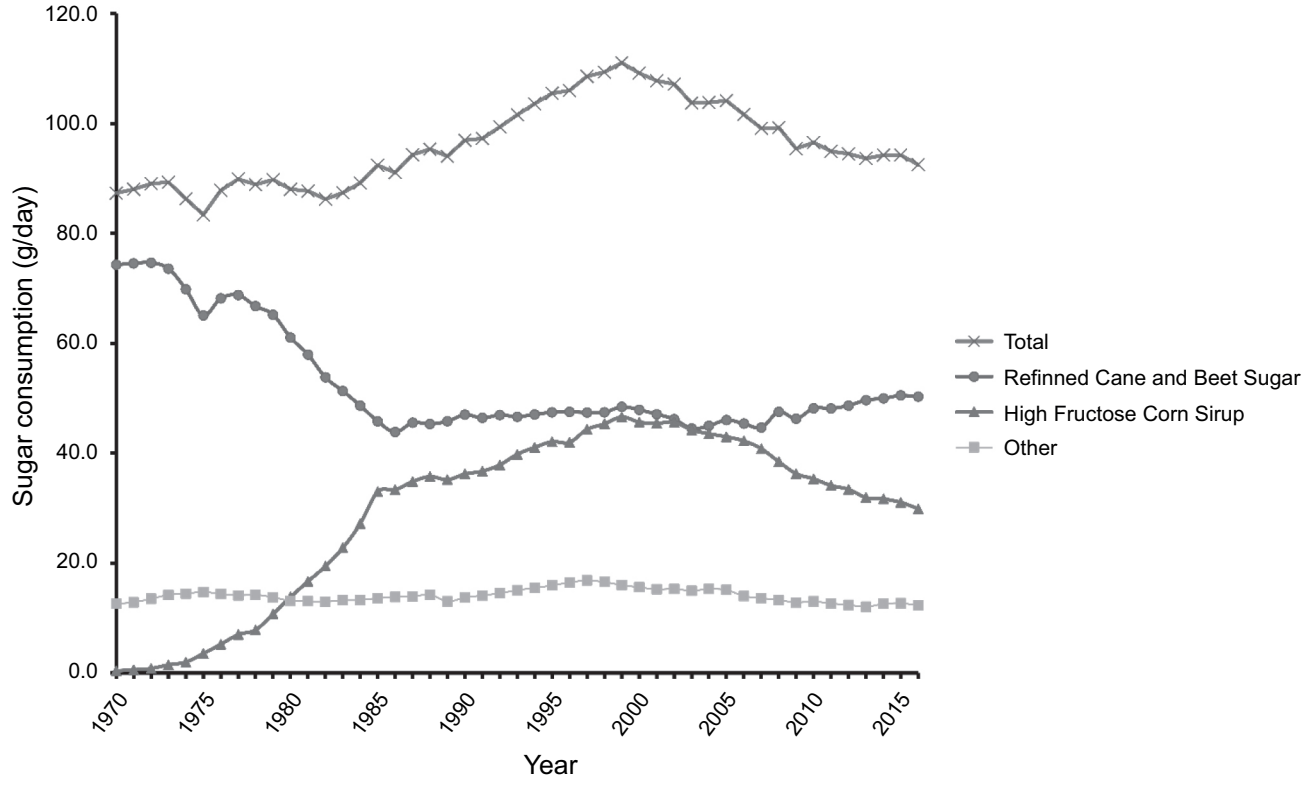

FIGURE 3. *Per capita* sugar consumption for total, refined cane and beet sugar, high fructose corn syrup, and other sugars (molasses, honey, *etc*.) in grams per day from 1970 to 2016. Total sugar consumption peaks in the year 1999 with 111.0 g/day and has declined slowly afterwards. Increase and subsequent decline of HFCS most likely plays major role in that trend. Data collected from the USDA Economic Research Service.

at these levels using analyzed survey data from the Food and Drug Administration [Kantor *et al.*, 1997].

By accounting for these sources of loss, the *per capita* consumption of sugar in grams per day was determined [USDA, 2017]. Since these estimations were made using food availability data and its loss at varied stages, they provide alternative data that may better represent *per capita* sugar consumption in the United States, analyzed below.

Total sugar consumption increased from 1970 to 1999, peaking in 1999 at 111.0 grams per day (Figure 3). Since 2000, total sugar consumption has had a steady state of decline to about 92.5 g/day in 2016. Refined sugar levels declined drastically from 59.8 g/day (1970) to 35.3 g/day (1986) and stayed steady, HFCS drastically increased from 1.0 g/day (1970) to about 37.5 g/day (1999) (Figure 3). Thus, the increase in total sugar consumption from 1970 to 1999 is likely due to the large influx of HFCS in the mid 1970's. After 2002, HFCS consumption dropped considerably to an estimated 24.0 g/day (2016), while consumption of refined sugar started to increase steadily and eventually leveled off in the last decade, with estimated levels at 40.5 g/day (2016). Other sources of sugar consumption have stayed steady since 1970 at around an estimated 14.1 g/day (Figure 3). Thus, the decline of total sugar consumption after 2002 can be attributed to the considerable decrease in the HFCS consumption [USDA, 2017].

The increase in total sugar consumption and, in particular, HFCS, roughly paralleled the increase in obesity in the United States, with some studies suggesting that it was causal [Bray *et al*., 2004; Havel, 2005]. With individual sugar consumption at levels over twice the recommended levels by the American Heart Association of 36 g/day for men and triple the recommended dose 20 g/day for women, the link between sugar consumption and obesity is plausible [Johnson *et al*., 2009]. However, research conducted in animals has shown that the relationship between sugar consumption and obesity is more nuanced, suggesting that a decrease in sugar consumption since the year 2000 has not led to a decrease in obesity [Schulze *et al*., 2004]. Although conflicting studies demonstrate some uncertainty on this topic, it is plausible that sugar consumption may be a strong factor that has driven changes in obesity rates in the United States. However, on average, the US population still consumes more than 300% of the daily recommended amount of added sugar.

HOW DOES TOO MUCH SUGAR MAKE YOU FAT?

Although increases in obesity have been attributed to a multitude of factors including a lack of physical activity and an increase in food portion sizes, dramatic changes in sugar consumption as reported and described in the previous section is a prime candidate that may independently contribute to the rise of obesity [Marteau *et al*., 2015; Morris *et al*., 2015].

Sugars are widely found in our food environment and are typically consumed as a naturally occurring component of many foods or as additive, *i.e.* sweeteners ae sometimes added to foods during processing or preparation for consumption. A healthy, well-balanced diet contains naturally occurring sugars since monosaccharides like fructose and disaccharides like sucrose and lactose are integral components of vegetables, fruits, milk and milk-derived products, and grains. Thus, understanding the process by which ingested sugars are broken down and then converted to fat and stored in the human body is of utmost importance to appreciate the harm that excess sugar consumption can cause. Sugar is initially consumed orally primarily as either glucose, fructose, lactose, sucrose, and as the polysaccharide starch. A small yet significant amount of starch is converted into maltose by amylase in the oral cavity [Stenesh & Stenesh, 1989]. These saccharides then move from the oral cavity to the stomach *via* the esophagus (Figure 4-A) [Marieb, 2001]. In the stomach, there is only partial digestion of sugars due to mechanical forces and low pH (Figure 4-A) [Miftahof, 2017]. The small intestine is where the majority of sugars are digested and absorbed. The important process of disaccharide hydrolysis occurs in the small intestine as well. Lactose is hydrolyzed by lactase whereas sucrose and maltose are hydrolyzed by sucrase and maltase respectively (Figure 4-A) [Levin, 1994]. The two primary monosaccharides: glucose and fructose, are then transported across the epithelium of the small intestine and into the circulatory system (Figure 4-A). Glucose crosses the apical membrane *via* a sodium-glucose symporter [Berne *et al*., 2010]. Before this movement of glucose and sodium into the epithelium, a Na^{2+}-K^{+} ATPase creates a favorable electrochemical gradient for Na^{2+} by pumping it out of the epithelium [Saha *et al*., 2015]. Fructose crosses the apical membrane *via* a GLUT5 transporter, however the exact process as to how fructose crosses the basolateral membrane has not been elucidated [Berne *et al*., 2010].

Following movement across the epithelium, glucose and fructose move through systemic circulation as they pass non-hepatic and hepatic tissue. The fructose capturing enzyme fructokinase is found exclusively in hepatic and kidney tissue [Watson, 2014]. Fructokinase also has a twenty-fold greater affinity for fructose than glucose [Raushel & Cleland, 1973]. Therefore, a majority of fructose is taken up by the liver and a majority of glucose bypasses hepatocytes and provides energy for non-hepatic tissue such as the brain and myocytes (muscle cells).

In the liver, both fructose and glucose are converted to an important intermediate; glyceraldehyde-3-phosphate (Figure 4-B). However, unlike glucose, fructose metabolism is not subjected to fructose kinase regulations. Therefore, energy status does not regulate fructose uptake by the liver and subsequent DNL, which means that a high level of consumed fructose enters the liver with little reaching the systematic circulation [Stanhope *et al*., 2013]. The increased rate of DNL induced by fructose generates fatty acids for hepatic triglyceride production and thus leads to postprandial hypertriglyceridemia. Fructose can yield greater amounts of fat when consumed in larger quantities than glucose [Berg *et al*., 2015]. Fructose consumption fosters an imbalance between hepatic lipid input and output, creating a net liver fat accumulation [Stanhope *et al*., 2013]. In addition, increased consumption of fructose leads to decreased secretion of insulin and leptin, hormones known to regulate energy homeostasis by decreasing food intake and increasing energy expenditure [Elliott *et al.,* 2002].

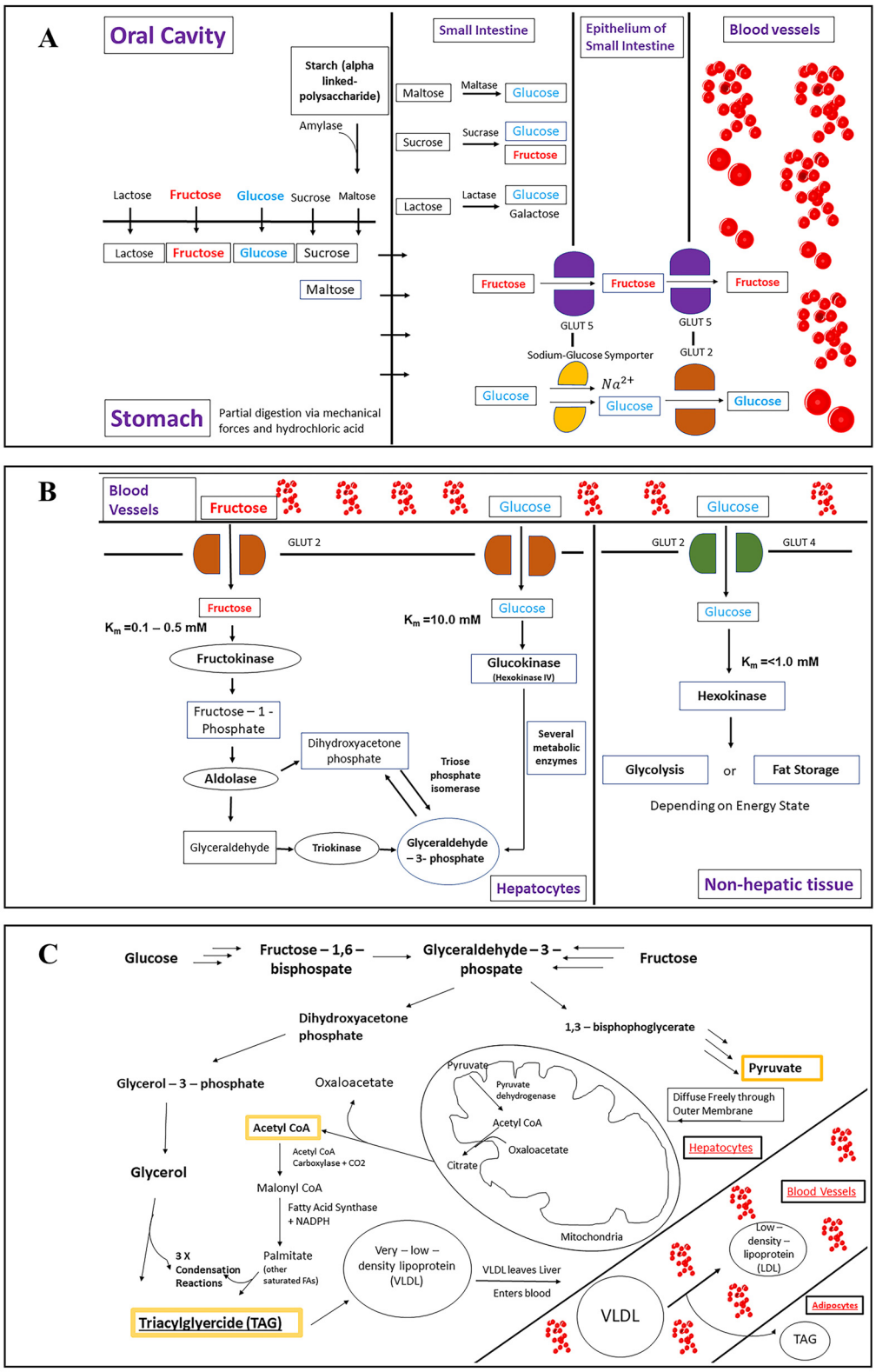

FIGURE 4. The sugars most commonly consumed are the monosaccharides glucose and fructose and the disaccharides sucrose, maltose, and lactose. Glycogen and starch are polysaccharides composed of the glucose disaccharide, maltose. The difference between glycogen and starch is that glycogen is more highly branched. A) Following ingestion of these sugars, they move down the esophagus into the stomach where there is minimal digestion. In the small intestine is where the majority of digestion is completed. Enzymes maltase, sucrose, and lactase all convert their respective disaccharides to their monosaccharide precursors. These sugars are then transported via channels to epithelial cells of the small intestine and ultimately to blood vessels. B) Fructose and glucose leave blood vessels and enter hepatic or non-hepatic tissue dependent on the affinities of those cells for the monosaccharides. Fructose almost exclusively moves into hepatic tissue due to fructokinase's high affinity (low Km) for the sugar. In hepatic tissue fructokinase is converted to glyceraldehyde and subsequently glyceraldehyde-3-phosphate both of which are involved in the process of glycolysis. C) The key steps in triglyceride synthesis shown in the pathway are the production of Acetyl CoA in the mitochondria which ultimately leads to the creation of fatty acids through a process of reduction, addition of the production of glycerol which serves as the backbone of triacylglycerides, and the production of TAGs via condensation reactions between glycerol and fatty acids.

In either pathway the steps following the production of glyceraldehyde-3-phosphate lead to the formation of endogenous triacylglycerides (TAGs). Acetyl CoA, which produces the fatty acid chains for TAGs, is synthesized from the regular pathway of glycolysis. However, unlike in energy deficient states, Acetyl CoA is not fed into the citric acid cycle but instead forms palmitate and other saturated fatty acids. Glycerol, which forms the backbone of TAGs is produced from a diverging pathway that is produced by conversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate by triose phosphate isomerase. This is followed by the conversion of dihydroxyacetone phosphate to glycerol–3–phosphate (Gro3P) by glycerol-3-phosphate dehydrogenase. Finally, glycerol is produced by the removal of a phosphate by glycerol-1-phosphatase (Figure 4-C) [Berg *et al*., 2015].

These endogenous TAGs are transported out of the liver by very low-density lipoproteins (VLDL) and travel through the blood stream binding to VLDL receptors on adipocyte surfaces. Once the VLDL particles are endocytosed, their cargo, which includes TAGs as well as cholesterol, are released *via* lysosomal degradation of VLDL membranes (Figure 4-C). Excess TAGs are then incorporated into existing lipid droplets found in adipocytes causing an increase in size of the droplet which triggers adipocytes to undergo hypertrophy [Berg *et al*., 2015; Stenesh & Stenesh, 1989].

Glucose and fructose have different regional adipose distribution: fructose promotes lipid deposition in visceral adipose tissue, while glucose favors subcutaneous adipose tissue deposition [Stanhope *et al.,* 2009]. Individuals who consumed high-fructose diets showed increased hepatic *de novo* lipogenesis (DNL), postprandial triglycerides, insulin resistance, and markers or altered lipid metabolism [Basciano, *et al.,* 2005].

REDUCTION IN SUGAR CONSUMPTION MAY SLOW DOWN THE ANNUAL INCREASE OF OBESITY

When comparing sugar consumption and obesity in the last half century, the data yields qualitative findings that show a trend indicative of sugar consumption's role in the obesity epidemic in the United States.

In order to analyze the relationship between sugar consumption and obesity, four variables need to be defined to describe the trends: obesity prevalence in percentage, sugar consumption *per capita* in grams per day, the annual change in obesity prevalence, and the annual change in sugar consumption *per capita*. The values of obesity prevalence and sugar consumption data are cited and described in the previous sections of this paper. The data was then used to estimate both the average obesity prevalence and average sugar consumption *per capita* for the following decades: 1970's, 1980's, 1990's, 2000's, and 2010's. The slope (rise over run) between these decades were then calculated, thus describing the respective annual rates of change (constant or average rate of change) per decade for those values. The slope is calculated by taking the differences in the average values between each of the decades and dividing them by the number of years elapsed, indicating how much obesity prevalence or sugar consumption either increased or decreased each year in their respective decades.

Figure 5 shows that sugar consumption *per capita* reached its peak in the 1990's and that obesity prevalence continues to grow, but at a slower rate since. In the 1970's and 1980's, sugar consumption increased drastically with an increasing pace reaching its peak in the 1990's; the annual rate of change for each decade were +0.26 g/day and +1.33 g/day, for 1970's and 1980's, respectively. In the 1990's and 2000's, sugar consumption fell, with the annual rate of change for those decades at – 0.08 g/day and - 0.91 g/day, respectively. Adult obesity prevalence also increased in the 1970's, 1980's, and 1990's, each decade with a higher annual rate of change of +0.05%, +0.82%, and +0.97%, respectively. In the 2000's, obesity increased by at a lower rate of only +0.365% each year, indicating a slowing in the annual rate of change in adult obesity. Childhood obesity followed a similar trend (Figure 5).

There are two main takeaways from the described data that suggest a strong link between sugar consumption and the obesity epidemic in the United States. First, sugar consumption's drastic rise from the 1970's to the 1990's is followed by the subsequent exponential growth in obesity prevalence from the late 1970's to the 2000's, and, even more indicative of this association, the drop in sugar consumption from the 1990's to 2010's preceding a slowing of the annual increase in obesity prevalence in the 2000's. Second, this general trend observed shows that over time, obesity prevalence may change as a response to changes in sugar consumption *per capita*, indicating a positive correlation between sugar consumption and obesity prevalence qualitatively observed (Figure 5).

SUGAR CONSUMPTION HIJACKS THE GUT-BRAIN COMMUNICATION SYSTEM: GUT RESIDENTS' LARGELY AT FAULT

Obesity has been recognized as a low-grade, chronic inflammatory disease [Ouchi *et al*., 2011]. Consumption of energy-dense diets strongly relates to obesity, induces detrimental changes in the gut microbiome, and triggers inflammation in the gut-brain neural communication [Sen *et al*., 2017; Vaughn *et al*., 2017]. Sensory information from the stomach to the brainstem is carried by gastric vagal afferents [Altschuler *et al*., 1989; Berthoud & Powley, 1992], and the significance of the integrity of this system for the control of ingestive behavior has been previously described [Norgren, 1983; Ritter, 2004; Schwartz, 2000]. Approximately 70–80% of vagal fibers are sensory fibers whose cell bodies are located bilaterally in the inferior ganglia of the vagus nerve (nodose ganglia; NG) [Czaja *et al*., 2006]. These afferents innervate the abdominal viscera [Berthoud & Powley, 1992; Prechtl & Powley, 1990] and make their first synapse in the *Nucleus Tractus Solitarius* (NTS) [Berthoud & Powley, 1992]. Visceral afferents in the NTS then project upstream to the parabrachial nucleus, the second central relay. Parabrachial nuclei neurons ultimately carry information to the hypothalamus, the amygdala, and the bed nucleus of the *stria terminalis* [Norgren, 1983]. In addition, NTS neurons project to the dorsal motor nucleus of the vagus (DMV) cells to provide preganglionic control of cholinergic excitatory as well as non-

adrenergic non-cholinergic inhibitory postganglionic neurons [Broussard & Altschuler, 2000]. The efferent innervation to the stomach originates from the DMV [Berthoud *et al*., 1991; Kirchgessner & Gershon, 1989; Moran *et al*., 1997] and the majority of DMV neurons project to the myenteric plexus, with the highest density of efferent fibers terminating in the stomach [Berthoud *et al*., 1991].

Appetite is an intrinsic driving force to search for, choose, and ingest food [De Graaf *et al*., 2004]. When we eat, a multi-tasking cascade of events is triggered to digest the consumed food and keep the organism informed of the nutrients and amounts being consumed. The gut microbiota plays a significant role in energy and nutrient harvesting as well as maintenance of a healthy mucosal barrier and stimulation of the host's immune system, and disturbances to the intestinal microbiome have been associated with inflammation and obesity [Gawey & Czaja, 2017; Cox *et al*., 2015; Sen *et al*., 2017; Tlaskalová-Hogenová *et al*., 2011; Vaughn *et al*., 2017]. In humans, the most abundant bacteria phyla are Bacteroidetes and Firmicutes [Johnson *et al*., 2017]. Bacteroidetes, also known as the good guys, are associated with production of healthy metabolites, like short-chain fatty acids. In contrast, Firmicutes are gram-negative bacteria typically labelled as the bad guys due to their role in glucose fermentation and its metabolites, mainly butyrate [Ismail *et al*., 2011]. In healthy individuals, Bacteroidetes are present in greater density than Firmicutes [Chakraborti, 2015].

Diet is a major contributor to changes in the gut microbiome composition [Shoaie *et al*., 2015]. The underlying cause of obesity is, in its simplest explanation, an increase in caloric intake accompanied by a decrease in physical activity. However, it has been hypothesized that the gut microbiome of obese subjects harbors microbial communities that promote more efficient energy extraction from dietary sources. In recent years, there has been a surge in scientific evidence which shows that there is a correlation between changes in the gut microbiome and obesity. Ley *et al.* [2005] found that there is a difference in the ratio of Bacteroidetes to Firmicutes where the obese mice exhibit a decrease in Bacteroidetes with a concurrent increase in Firmicutes compared to their lean counterpart. This finding has been further shown in children [Bervoets *et al*., 2013] and adult human subjects [Turnbaugh *et al*., 2009].

Studies from our lab and others have shown that consumption of diets high in sugar, high in fat, or both triggers changes in the gut microbiome and favors the microbiota composition associated with an obese phenotype. Sen *et al.* [2017] showed that in rats, consumption of a high-fat, high sugar diet or a low-fat, high sugar diet led to microbiota dysbiosis characterized by a decrease in bacterial diversity and an increase in the ratio of Firmicutes to Bacteroidetes. This increase in abundance of the Firmicutes phyla was driven by an increase in members of the class Clostridia while consumption of a high sucrose diet led to a significant decrease in abundance of Bacilli. Noting this distinction is of particular importance because Lactobacillus, a member of the class Bacilli, is a known probiotic. However, the Ruminococcaceae and Lachnospiraceae families of the phyla Clostridia have been shown to exist in higher abundance in the gut microbiome of obese subjects [Cho *et al*., 2012; Gomez-Arango *et al*., 2016; Kim *et al*., 2012] and have been further associated with increased body fat accumulation [Duca *et al*., 2014]. In addition, it was further shown that consumption of a high sugar diet, independent of the fat content, significantly increases lipopolysaccharides levels – known endotoxins – and expression of the pro-inflammatory cytokines IL-6, IL-1β, and TNFα in the cecum [Sen *et al*., 2017]. In short, consumption of a diet high in sugar leads to gut microbiota dysbiosis, gut inflammation, and impaired gut function.

In addition to microbiota dysbiosis, consumption of high sugar diets leads to reorganization of the gut-brain vagal communication system. The responsiveness of vagal afferents to signals originating in the gut has been shown to be decreased in animals fed a high fat, high sugar diet [Covasa & Ritter, 1998; Little *et al*., 2007]. It is thus possible that the density and integrity of these afferents is negatively impacted by the hostile gut microbiome environment induced by these diets. Studies in our laboratory have shown that consumption of a high fat, high sucrose diet significantly decreased in the density of vagal afferents that synapse in the NTS [Sen *et al*., 2017; Vaughn *et al*., 2017]. It was further shown that this diet triggered activation of microglia cells, a marker of inflammation, in the NTS [Vaughn *et al*., 2017]. Thus, diet-driven changes in the gut microbiome disrupt gut-brain vagal communication, trigger brain inflammation, and increase accumulation of fat in the body.

PHYSICAL ACTIVITY: MORE IS BETTER, BUT NOT A SOLUTION

Besides energy consumption, energy expenditure constitutes the other important factor in the energy balance formula. Divided into occupational and leisure-time, physical activity is the only modifiable variable that affects energy expenditure, and thus body weight [Church *et al.*, 2011]. Physical activity is defined as any bodily movement produced by the skeletal muscles that elevates energy expenditure above basal levels [Katzmarzyk *et al,*, 2008]. Amongst its many health benefits, increasing physical activity resulting in increased energy expenditure has been shown to have a positive correlation with greater long-term weight loss and maintenance of a lower body weight [Jeffery *et al.*, 2003].

With the growing awareness of the importance of physical activity, efforts have been made to promote physical activity in the United States as well as globally in the past few decades. The second edition of the *Physical Activity Guidelines for Americans* recommends engaging in physical activity at least 3 h/d for children (3–5 *yo*), 1 h/d for children and adolescents (6–17 *yo*), and 2.5–5 h/w for adults. Following these recommendations, between 2008 and 2016, there was an increase in physical activity in adults, but no change in high school-age children. In 2008, ∼15% of women and 22% of men reported meeting the recommended guidelines for physical activity. This increased to ∼19% and 26% for women and men, respectively by 2016. In 2011, ∼12% of girls and 30% of boys met the requirements and there was no change by 2015 [Piercy *et al.,* 2018].

Occupation-related physical activity, in contrast, has decreased. Since 1960, occupation-related physical activity has been reduced in intensity due to the shift from good producing and agriculture to service, resulted in a drop of more than 100 calories in the estimated mean daily energy expenditure from work-related physical activity. Since work takes up a large portion of the day, this reduction in occupation-related energy expenditure contributed to the increase mean U.S. body weight over the last 5 decades [Church *et al.*, 2011]. The increased reliance on labor-saving technology, such as using motorized transport instead of walking or cycling, has also contributed to the reduction of physical activity in the general population [Fox & Hillsdon, 2007].

Nevertheless, increasing physical activity is not the sole answer to curbing the obesity epidemic given that increasing physical activity alone is not effective in treating obesity without a meal plan [Griera *et al.*, 2007].

CONCLUSION

The reviewed data show that since year 2000 the reduction in sugar consumption slowed down the annual increase of obesity in both the adults and children. While sugar is necessary for a healthy life and the consumption trend is going in the right direction, we still consume more than 300% of the daily recommended amount of added sugar. These

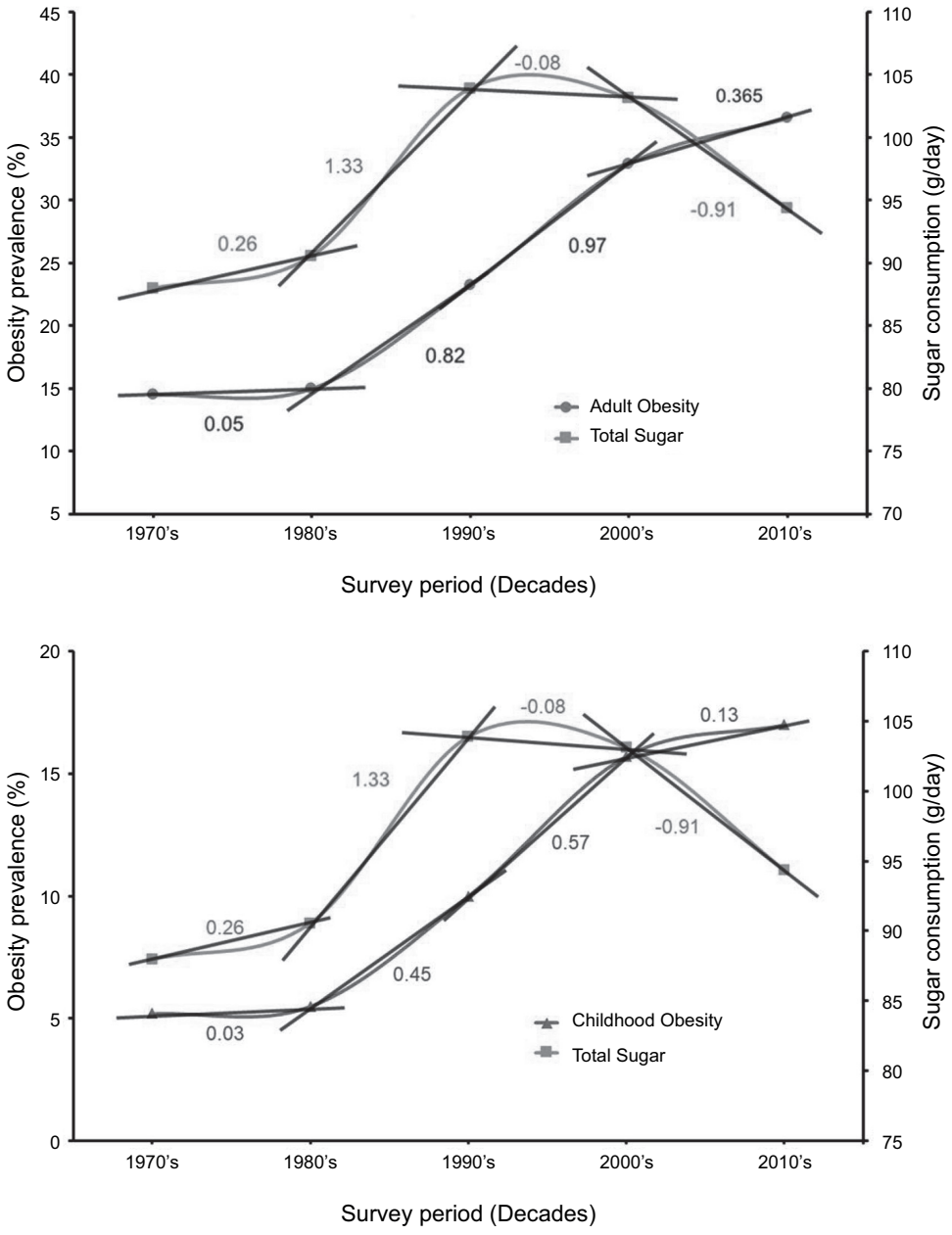

FIGURE 5. Total sugar consumption per decade in g/day compared to obesity prevalence per decade in percent for adults (top panel) and children (bottom panel). The rate of change per year between the decades for both sugar consumption and adult obesity were found by calculating the differences in the average values between each of the decades and dividing it by the number of years elapsed, as indicated by the slope lines and values between the decades.

trends may describe qualitatively the possible impacts of sugar consumption on rates of change in obesity prevalence, but this analysis is limited by a general lack of consistent data on obesity rates and the absence of statistical analyses and tests possible to better quantify and mathematically indicate the significance of these trends. Future studies can be done to better assess the limited data at hand in order to better determine and measure the described trends. It is important to not single out sugars as the enemy to be avoided at all costs. Sugars are a naturally-occurring, integral component of many unprocessed foods. Most importantly, the brain utilizes the monosaccharide glucose as its primary energy source [Mergenthaler, *et al.,* 2013]. In addition, systematic reviews and meta-analyses of cohort studies and randomized control trials have reported that the link between consumption of AHA-recommended amounts of dietary sugars and obesity does not exist [Khan & Sievenpiper, 2016; Rippe & Angelopoulos, 2016; Te Morenga *et al*., 2012; Khan & Sievenpiper, 2014]. A positive correlation between sugar consumption and obesity is only apparent when sugar is overconsumed, which is the topic of this review. It is worth recapitulating that obesity is a multifactorial disease. This review focuses on the association between sugar consumption trends and obesity prevalence. However, for a holistic understanding of the causes of obesity, we must also take into account such factors as physical activity, economic status, and consumption of other macronutrients, *i.e.* fat, and micronutrients, among others. A discussion of all the factors that play a role in the obesity epidemic would be beyond the scope of this review. Nonetheless, this investigation examines these trends using valid and measurable data that hints at the link between sugar consumption and obesity trends and may push further research that considers and further scrutinizes the role of excess sugar and its negative impacts on a population's overall health status.

ACKNOWLEDGEMENTS

Special thanks to Dr. Steven D. Holladay for proofreading and providing feedback for this paper.

FUNDING SOURCE

This work was supported by National Institutes of Health, grant no. 5R01DC013904–04.

CONFLICT OF INTERESTS

All authors declare no conflict of interests.

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Received: 13 March 2019. Revised: 10 June 2019. Accepted: 16 July 2019. Published on-line: 21 August 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 235–246
DOI: 10.31883/pjfns/109423
http://journal.pan.olsztyn.pl

Original research article
Section: Food Chemistry

Microwave-Assisted Extraction of Different Groups of Phenolic Compounds from Grape Skin Pomaces: Modeling and Optimization

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Key words: microwave-assisted extraction (MAE), grape skin pomace, phenolic compounds, antioxidant capacity, artificial neural network (ANN), response surface methodology (RSM)

A microwave-assisted extraction (MAE) technique was employed on grape skin pomaces to enable the extraction of different groups of phenolic compounds (total phenolics, tannins, flavonols, and hydroxycinnamic acids) and to obtain extracts with the highest antioxidant capacity (ORAC). The single-step extraction process was modeled and optimized by means of artificial neural network (ANN) and response surface methodology (RSM) coupled with full factorial design. Methanol concentration (20 to 100%, v/v), temperature (30 to 60°C) and duration (2 to 16 min) were MAE input parameters studied. Optimal parameters were further applied in multistep MAE cycles for the complete recovery of phenolic antioxidants. Results showed that methanol concentration was the most significant parameter influencing the extraction of all groups of phenolics and antioxidant capacity of extracts. Moreover, a significant effect of time and temperature was also noticed, except in the case of total hydroxycinnamic acids. The presented ANN model accurately predicted the effect of the three input parameters simultaneously on the output parameters (training R^2=0.9957; test R^2=0.9945; validation R^2=0.9965). Optimal parameters showed that higher methanol concentrations and lower temperatures (100%, v/v; at 40°C) were more convenient for the extraction of flavonols and hydroxycinnamic acids than for ORAC (78.1%, v/v; at 60°C) or total phenolics and tannins (62.7 and 65.3%, v/v; at 60°C). The number of MAE cycles was found to be a key factor for completing extraction of skin pomace phenolics and should always be considered prior to analytical determination.

LIST OF ABBREVIATIONS

MAE, Microwave assisted extraction; ANN, Artificial neural network; RSM, Response surface methodology; TP, Total phenolics; TT, Total tannins; THCA, Total hydroxycinnamic acids; TF, Total flavonols; ORAC, Oxygen radical absorbance capacity; GAE, Gallic acid equivalents; and TE, Trolox equivalents.

INTRODUCTION

Winemaking is one of the most important agricultural sectors worldwide, and according to the latest data collected by the OIV (International Organisation of Vine and Wine) 73.3 million tons of grapes (around 52% as wine grapes) in 2017, and about 279 million hectoliters of wine were produced in 2018 [OIV, 2018]. Grape pomace is the main solid organic waste from the wine industry, where large quantities are generated after fermentation and pressing, representing about 20% of the initial grape weight [Ky *et al.*, 2014; Laufenberg *et al.*, 2003]. Only 30 to 40% of phenolic compounds are extracted during vinification depending mainly on grape cultivar and applied technology of wine production [Deng *et al.*, 2011; Ky *et al.*, 2014; Tournour *et al.*, 2015; Valls *et al.*, 2017]. This means that grape pomace still exhibits high levels of bioactive compounds (60–70%) with strong antioxidant, antibacterial, and cytotoxic activities as well as favorable pharmacological properties [Bartolomé *et al.*, 2004; Peixoto *et al.*, 2018]. These compounds are known to contribute to human health and are particularly associated with reduced incidence of cardiovascular diseases as atherosclerosis and hypertension, neurodegeneration, and similar medical conditions [Auger *et al.*, 2004; De Sales *et al.*, 2018; Rodriguez-Rodriguez *et al.*, 2012]. Grape skins pomace proved to be a good source of anthocyanins, hydroxycinnamic acids, flavanols and flavonols, whereas flavanols are the most abundant seed polyphenols [Kammerer *et al.*, 2004; Ky *et al.*, 2014]. As a result of the increased concern over the sustainability of agricultural practices, efforts have been made to enable the use of grape pomaces bioactive extracts in various segments of food, pharmaceutical and cosmetic industry, resulting in applications such as natural antioxidant, source of natural pigments, additive in wine production, functional ingredient, *etc*. [Beres *et al.*, 2017; Ky & Teissedre, 2015]. Thus, it is necessary to have efficient extraction methods to achieve high recoveries of phenolic compounds that allow quality control of obtained extracts through their analysis and characterization.

Microwave-assisted extraction (MAE) has been investigated and proposed as better than conventional extraction

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in terms of extraction efficiency, time, and solvent consumption [Chan *et al.*, 2011]. Also, it has been introduced for a wide range of bioactive compounds from food by-products and natural sources, and likewise for the extraction of polyphenols from grape [Hong *et al.*, 2001; Karvela *et al.*, 2009; Krishnaswamy *et al.*, 2013; Liazid *et al.*, 2011] and grape pomace [Casazza *et al.*, 2010; Medouni-Adrar *et al.*, 2015; Pedroza *et al.*, 2015]. Nevertheless, in most of the aforementioned studies conducted on grape or pomace, target compounds were total phenolics, while the mode of operation was focused to the power level of microwave irradiation. This means that extraction was carried out at fixed power usually ranging from 300 to 550 W [Hong *et al.*, 2001; Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015] and in some cases even up to 900 W [Pedroza *et al.*, 2015] and at pre-determined extraction time ranging from 200 to 1003 s [Hong *et al.*, 2001; Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015; Pedroza *et al.*, 2015]. However, the mode of operation that focuses on the extraction temperature rather than microwave power (meaning that extraction temperature is set at desired point by regulating microwave power) is more suitable for the extraction of different groups of thermo-sensitive phenolics [Chan *et al.*, 2011], while temperatures lower than 60°C are recommended in order to avoid possible degradations [Liazid *et al.*, 2011; Pedroza *et al.*, 2015]. In addition, beside the microwave power and temperature, solvent nature and extraction time also showed to be important factors influencing the performance of MAE [Liazid *et al.*, 2011]. Ethanol is, by far, the most used solvent as a good microwave absorber [Chan *et al.*, 2011; Krishnaswamy *et al.*, 2013; Pedroza *et al.*, 2015], while on the other hand, there are only few studies of MAE of grape and pomace phenolics with methanol [Casazza *et al.*, 2010; Hong *et al.*, 2001]. Nevertheless, methanol compared to ethanol extracted higher concentrations of total phenolics, *o*-diphenols and flavonoids, in both grape skin and seed pomace [Casazza *et al.*, 2010], and showed to be a more selective solvent in conventional extraction of phenolic compounds [Pinelo *et al.*, 2005]. In addition, considering the time parameter, earlier studies showed that prolongation of extraction time beyond the optimal conditions was not useful to extract more phenolic compounds [Mané *et al.*, 2007; Medouni-Adrar *et al.*, 2015]. However, prolongation of the extraction time to ensure the completion of extraction and reduced risk of thermal degradation can be achieved through the repeating of the extraction step in multistep MAE [Chan *et al.*, 2011]. The effect of cycle number was only examined by Pedroza *et al*. [2015] revealing that two irradiation cycles (900 W, 1003 s, with temperature fluctuating up to 80°C) were necessary to achieve the equivalent yield of total phenolics with reference solid-liquid extraction; while to the best of our knowledge effects of sequential irradiation cycles with lower power and temperature on the matrix of grape skin pomace were not earlier studied. In addition, modeling and optimization of MAE extraction condition is usually performed through response surface methodology (RSM) [Krishnaswamy *et al.*, 2013; Medouni-Adrar *et al.*, 2015] and artificial neural network (ANN) methods [Ameer *et al.*, 2017]. RSM can demonstrate interaction effects of inherent MAE parameters on target responses, whereas ANN can reliably model the MAE process with better predictive and estimation capabilities.

The aim of the present study was to model and optimize single-step MAE (methanol concentration, temperature and time) of phenolic antioxidants (total phenolics, tannins, hydroxycinnamic acids, flavonols, and ORAC value) from grape skin pomaces by using ANN and RSM, and to further apply optimal parameters in multiple steps in order to study effects of sequential irradiation cycles and to develop a method for the complete recovery of phenolic antioxidants.

MATERIALS AND METHODS

Chemicals

Methanol, ethanol, and hydrochloric acid were purchased from Carlo Erba (Val del Reuil, France). Folin-Ciocalteu's phenol reagent was purchased from Reagecon (Shannon, Ireland), and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) from Acros (Gell, Belgium). Sodium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, caffeic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Grape skin pomace sample preparation

This study was conducted on grape skin pomaces from Cabernet Sauvignon, Merlot, and Teran cultivars (*Vitis vinifera* L.). Grape pomaces were provided from Agrolaguna winery (Poreč, Croatia), obtained as wine by-products after alcoholic fermentation and pressing; from grapes harvested in technological maturity in September 2014 and originating out of Istria vine-growing sub-region area (Croatia). Grape pomace samples were first frozen (-80°C) and then freeze-dried (vacuum 0.130 to 0.155 hPa, temperature -30 to 0°C for 24 h, isothermal desorption at 20°C for 12 h) using Christ Alpha 1–4 LSC Plus freeze-dryer (Osterode am Hatz, Germany). Freeze-dried skins were manually separated from seeds and pulp, and ground with an electric grinder. Grape skin powders particle size distributions measured by the laser particle size analyzer (Malvern, Mastersizer 2000, Germany) were: (i) Cabernet Sauvignon d(0.9)≤ 354.31 μm, d(0.5)≤ 123.03 μm, d(0.1)≤ 7.66 μm; (ii) Merlot d(0.9)≤ 376.54 μm, d(0.5)≤ 146.90 μm, d(0.1)≤ 8.37 μm; and (iii) Teran d(0.9)≤ 365.55 μm, d(0.5)≤ 130.70 μm, d(0.1)≤ 8.26 μm. Samples were stored at -20°C before subsequent analyses.

Microwave-Assisted Extraction (MAE)

Phenolic compounds from grape skin pomace were extracted using a professional single-mode microwave reactor (Milestone, Start S Microwave Labstation for Synthesis, Sorisole, Italy), with an adjustable microwave power output, operating at 2.45 GHz; equipped with an air and water reflux condenser and a magnetic stirrer. Parameters that were kept constant during extractions were: stirring (at 80%) and ventilation after extraction (1 min), as well as liquid to solid ratio (50:1), selected based on literature data [Hong *et al.*, 2001; Li *et al.*, 2011; Medouni-Adrar *et al.*, 2015] and preliminary experiment (data not shown). Operating extraction mode was focused to the extraction temperature that was set at the desired point, meaning that power was used to maintain the temperature in the reaction cell, rather than being

applied at continuous level, since this mode reduces the risk of thermal degradation, and is more suitable for the extraction of thermo-sensitive compounds [Chan *et al.*, 2011]. A portion of 0.5 g of freeze-dried grape skin pomace powder and 25 mL of the solvent were added to 50 mL round bottom flask with double neck and a cooling system. MAE was performed at first according to the experimental design shown in Table 1 in order to determine optimal variables (parameters) of single-step MAE. Secondly, the optimal parameters determined were applied in multiple steps (sequential repetitive extraction cycles) in order to define the necessary number of MAE cycles in the final multistep MAE method for the complete extraction of phenolic compounds.

Experimental design for modeling and optimization of single--step MAE

Full factorial design comprising 27 experiments was used to evaluate the effect of three independent variables and to obtain optimal conditions of a single cycle. Independent process variables were: solvent polarity (methanol concentration in methanol-water mixture, v/v), extraction time (min) and temperature (°C); named as X_1, X_2, and X_3, respectively (Table 1). Each experiment in experimental design was run in duplicate [54 (27 × 2) experiments in total]. Ranges of variables were: solvent polarity at 20%, 60% and 100% (v/v) methanol; time at 2, 9 and 16 min; and temperature at 30, 45 and 60°C, as listed in Table 1. The responses (output variables) determined were concentrations of extracted phenolics and antioxidant capacity of skin pomace extracts (Table 1). All experiments were conducted on grape skin pomace of Cabernet Sauvignon. After each MAE experiment (Table 1), the mixture was transferred to a centrifugation tube and centrifuged at 4000 rpm for 5 min on a Rotofix 32 instrument (Hettich Zentrifugen, Germany). Liquid (solvent) was evaporated at 30°C, the residue was dissolved in water and freeze-dried after which grape skin pomace extracts were obtained.

Application of sequential irradiation cycles (multistep MAE)

Optimal parameters of a single MAE cycle were performed in eight sequential consecutive cycles (eight-step MAE) in all three cultivars (Cabernet Sauvignon, Merlot, and Teran), following the earlier described protocol. In total, eight irradiation cycles were tested, since the significant increase in concentrations of phenolic compounds between 7th and 8th cycle was not established, while relative recovery for 8th cycle accounted for less than 1% (w/w, relative recovery was calculated relative to overall amount obtained after eight cycles). Average calculated power of each cycle was 47.3 W. After each MAE cycle, the mixture was transferred to a centrifugation tube and centrifuged as earlier mentioned. The solid part was separated and re-used for MAE with a fresh solvent in a recurring manner. Liquid (solvent) maintained after each cycle was separately evaporated at 30°C, and the residue was dissolved in water and freeze--dried. Concentration and relative recovery (%, w/w) of total phenolics were determined in extracts after each single cycle of multistep MAE and expressed cumulatively. The multistep MAE procedure was run in triplicate for each cultivar.

Spectrophotometric analyses

Spectrophotometric analyses were conducted with a double-beam UV-1600PC spectrophotometer (VWR International, China). Freeze-dried grape pomace skin extracts were solubilized in a wine model solution [Ćurko *et al.*, 2014; Ky & Teissedre, 2015] at concentrations of 3 g/L and 0.25 g/L for analyses of total phenolics (TP) and tannins (TT), respectively; as well as at 7 g/L for analyses of total flavonols (TF) and hydroxycinnamic acids (THCA). Total phenolics were determined with the Folin-Ciocalteu method [Singleton & Rossi, 1965] and results were expressed in mg of gallic acid equivalents (GAE) per g of dry weight (dw) grape skin pomace. Concentrations of total tannins were measured by acid hydrolysis and expressed in mg per g of dry weight (dw) grape skin pomace [Ribéreau-Gayon & Stonestreet, 1966]. Total hydroxycinnamic acids and flavonols concentrations were determined by measuring absorbance at 320 and 360 nm according to the method described by Mazza *et al.* [1999]. Results were expressed in mg of caffeic acid equivalents (CAE) per g of dw; and mg of quercetin equivalents (QE) per g of dw grape skin pomace, for the concentrations of THCA and TF, respectively. All spectrophotometric analyses were conducted in triplicate.

Oxygen Radical Absorbance Capacity (ORAC) Assay

The oxygen radical absorbance capacity (ORAC) was determined according to Ninfali *et al.* [2005], as briefly described by Mazor Jolić *et al.* [2011]. Results were calculated as ORAC values using the differences of areas under fluorescein decay curve between the blank and the sample. The results were expressed as μmol Trolox equivalent (TE) per g of dw grape skin pomace.

Data analysis

Statistical analysis of analytical data was carried out by the Analysis of Variance (ANOVA) using Statistica v.10.0 software (Statsoft Inc., Tulsa, OK, USA). Tukey's HSD Test was used as a comparison test when samples were significantly different after ANOVA ($p<0.05$). To test whether it is possible to predict phenolic and antioxidant characteristics of grape skin pomace based on three input variables (methanol concentration, temperature, and duration of process) artificial neural network modeling was applied. Multiple layer perceptron networks were developed in Statistica v.10.0 software (StatSoft Inc, Tulsa, OK, USA). Response surface methodology (RSM) was used to determine the optimal combination of process parameters which varied at three levels. Experimental data were analyzed using Design-Expert© software (Stat-Ease, Inc., MN, USA) and fitted to an empirical second-order polynomial regression model:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

where: y is the predicted response concentration of TP, TT, THCA, TF and ORAC; β_0, β_i, β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms respectively; and X_i and X_j are the actual levels of the independent variables. Based on the regression model, three-dimensional re-

sponse surface methodology (RSM) plots of optimal extraction conditions for TP, TT, THCA, TF and ORAC were designed.

RESULTS AND DISCUSSION

Effect of process variables on the extraction yields of phenolic compounds and antioxidant capacity of grape skin pomace extracts

Systematic study was carried out based on the experimental design presented in Table 1, in order to evaluate the effects of different MAE process variables, *i.e.* solvent (methanol) concentration, temperature and time, on the extraction yields of different groups of phenolics and antioxidant capacity of grape skin pomace extracts.

In addition, polynomial equations and statistical parameters describing the effect of operating process variables on the phenolic and antioxidant characteristics of grape skin pomace extracts are presented in Table 2.

High values of R^2 presented in Table 2 indicated a very good correlation between experimental values and values of models that could explain more than 90% of the variation. Moreover, very low p-values indicated that each generated model was statistically significant, suggesting that MAE could be well described with presented models.

Analyses of experimental data revealed that all three process (input) variables, that is solvent, time and temperature, significantly influenced the MAE of TP and TT ($p<0.05$). In addition, analogous qualitative trends were detected for both output variables (TP, TT) indicating a similar behavior toward variation of solvent concentration and temperature. Experiments performed with 60% (v/v) methanol extracted significantly higher concentrations of TP and TT compared to the ones with the identical time and temperature using 20% (v/v) methanol. Interestingly, an increase in methanol concentration up to 100% (v/v) did not further increase the concentrations of target compounds in both cases. Contrary, concentrations of TP and TT extracted with 100% (v/v) methanol were lower than the ones obtained with 60% (v/v) methanol. A similar type of behavior was observed by Yilmaz & Toledo [2006] for the conventional extraction of grape seeds polyphenols, where the highest concentrations were obtained by 60% or 70% (v/v) methanol. Moreover, 60% (v/v) methanol is most commonly applied in two-step conventional extraction of grape and pomace tannins [Chira *et al.*, 2009; Ky *et al.*, 2014]. Results obtained may be attributed to the changes of solvent polarity, and consequently changes in solubility and diffusivity of TP and TT. In addition, significant differences between the 60% and 100% (v/v) methanol samples of identical extraction time were obtained at 45°C and 60°C, while the same trends among 60% and 100% (v/v) methanol samples of identical extraction time were not observed at the lower temperature (30°C) (Table 1). Furthermore, an increase in the applied temperature (30–45–60°C) resulted in a significant increase of both TP and TT concentrations among the experiments performed under identical conditions of solvent concentration and time. Exceptionally, in the case of 100% (v/v) methanol, an increase of the temperature caused only a slight increase of TP and TT concentrations. Namely, the increase in the temperature favored the extraction by enhancing both the solubility of solute and the diffusion coefficient [Pinelo *et al.*, 2005]; and as a consequence, the highest concentrations of TP and TT were determined at the highest temperature tested (60°C). However, temperature range was kept relatively low (maximum 60°C) and was not further increased in order to avoid possible degradation of phenolic compounds as well as denaturation of membranes [Liazid *et al.*, 2011; Pedroza *et al.*, 2015]. This showed to be particularly important for the extraction of flavonoids that were found to be more sensitive to the degradation caused by high temperature and long extraction time [Casazza *et al.*, 2010]. Furthermore, the time variable differently affected the extraction trends of TP and TT. Prolongation of extraction time from 2 to 9 min among the experiments performed under identical conditions positively affected the extraction of both TP and TT, particularly for the experiments conducted with 60% (v/v) methanol. However, further prolongation from 9 to 16 min only slightly increased concentration of TP, but at the same time negatively affected extraction of TT. Namely, concentrations of TT extracted after 16 min were lower than the ones obtained after 9 min under identical conditions, where a significant decrease was found for 60% (v/v) methanol at higher temperatures (45 and 60°C) as well as for 100% (v/v) methanol at 60°C. As earlier mentioned [Casazza *et al.*, 2010], results obtained confirmed the sensitivity of TT, indicating that temperatures higher than 60°C and extraction time longer than 9 min should be avoided in the case of single-step MAE of TT. This phenomenon could be explained by Fick's second law of diffusion, when the solvent oversaturates, and concentration gradient becomes null after a particular duration; while further augmentation of extraction time may favor degradation reactions and thus decrease in concentration of phenolic compounds [Medouni-Adrar *et al.*, 2015]. Hence, excessive extraction time was not useful to extract more phenolic compounds [Mané *et al.*, 2007; Pinelo *et al.*, 2005]. Finally, the highest values of TP were reached at the conditions of 60% (v/v) methanol and 60°C after 9 or 16 min of extraction, while the highest values of TT were extracted under identical conditions of methanol concentration and temperature but only after 9 min of MAE.

Likewise, methanol concentration also had a significant influence ($p<0.05$) on the extraction of THCA and TF, but trends established were quite different from those noticed for the TP. As it can be seen in Table 1, an increase in methanol concentration significantly promoted the extraction of THCA and TF, and the highest concentrations were thus extracted with 100% (v/v) methanol. In addition, temperature and time had no significant effect on the extraction of THCA, but did affect the extraction of TF. Concentrations of TF extracted at 45°C were higher than those extracted at 30°C or 60°C. Time variable influenced the extraction of TF in similar manner as earlier proposed for TT (Table 1), since the highest concentrations were obtained after 9 min of MAE, while prolongation up to 16 min led to a decrease in TF content. Decreased concentration of phenolic compounds induced by the prolongation of extraction time was previously reported in the literature [Liazid *et al.*, 2011]. This decrease could be ascribed to oxidative degradation of polyphenols, particularly the ones having a greater number of hydroxyl-type substituents in the B ring (like myricetin-3-*O*-

TABLE 1. Operating process variables of microwave-assisted extraction (MAE) experimental design and their effect on phenolic and antioxidant characteristics of grape skin pomace extracts.

Exp. no.	Operating input variables			Output variables				
	X_1	X_2	X_3	TP	TT	THCA	TF	ORAC
	Methanol conc. (%)	Time (min)	Temp (°C)	(mg GAE/g dw skin pomace)	(mg/g dw skin pomace)	(mg/g dw skin pomace)	(mg/g dw skin pomace)	(μmol TE/g dw skin pomace)
1	20	2	30	3.77 ± 0.12^{n}	1.00 ± 0.06^{m}	0.06 ± 0.04^{ef}	0.07 ± 0.01^{g}	70.18 ± 0.84^{n}
2	60	2	30	8.98 ± 0.28^{ij}	7.60 ± 0.20^{gh}	0.44 ± 0.13^{bcde}	0.47 ± 0.05^{f}	127.78 ± 2.77^{jk}
3	100	2	30	8.42 ± 0.17^{jk}	7.38 ± 0.23^{h}	3.22 ± 0.03^{a}	3.22 ± 0.25^{bc}	124.83 ± 3.63^{jk}
4	20	9	30	4.62 ± 0.29^{mn}	2.36 ± 0.09^{lm}	0.30 ± 0.05^{bcdef}	0.16 ± 0.03^{g}	80.71 ± 1.64^{mn}
5	60	9	30	10.68 ± 0.22^{fgh}	9.64 ± 0.21^{ef}	0.34 ± 0.09^{bcdef}	0.50 ± 0.06^{ef}	154.58 ± 7.64^{i}
6	100	9	30	10.08 ± 0.26^{fghi}	8.48 ± 0.24^{fgh}	3.04 ± 0.05^{a}	3.73 ± 0.06^{a}	149.76 ± 2.14^{i}
7	20	16	30	5.13 ± 0.12^{mn}	2.27 ± 0.19^{lm}	0.19 ± 0.11^{def}	0.13 ± 0.05^{g}	97.61 ± 0.47^{lm}
8	60	16	30	11.56 ± 0.44^{def}	9.14 ± 0.47^{efg}	0.33 ± 0.08^{bcdef}	0.47 ± 0.02^{f}	181.71 ± 5.20^{h}
9	100	16	30	10.66 ± 0.84^{fgh}	7.24 ± 0.74^{hi}	2.72 ± 0.11^{a}	3.67 ± 0.07^{a}	178.23 ± 4.06^{h}
10	20	2	45	5.86 ± 0.34^{lm}	2.95 ± 0.08^{kl}	0.02 ± 0.00^{f}	0.11 ± 0.01^{g}	102.44 ± 3.62^{l}
11	60	2	45	13.06 ± 0.44^{d}	12.47 ± 0.34^{d}	0.60 ± 0.05^{bc}	0.69 ± 0.04^{de}	198.85 ± 6.93^{gh}
12	100	2	45	9.79 ± 0.27^{ghij}	8.57 ± 0.35^{efgh}	2.86 ± 0.07^{a}	3.36 ± 0.10^{bc}	185.52 ± 7.59^{gh}
13	20	9	45	7.30 ± 0.48^{kl}	4.99 ± 0.16^{j}	0.11 ± 0.03^{ef}	0.24 ± 0.06^{g}	110.32 ± 1.20^{kl}
14	60	9	45	14.82 ± 0.54^{c}	15.15 ± 0.33^{c}	0.58 ± 0.07^{bcd}	0.75 ± 0.06^{d}	230.52 ± 3.63^{e}
15	100	9	45	10.99 ± 0.41^{efg}	9.15 ± 0.74^{efg}	3.07 ± 0.17^{a}	3.86 ± 0.07^{a}	206.53 ± 6.06^{fg}
16	20	16	45	8.48 ± 0.15^{ijk}	4.39 ± 0.20^{jk}	0.15 ± 0.03^{ef}	0.18 ± 0.04^{g}	115.69 ± 1.00^{kl}
17	60	16	45	16.20 ± 0.29^{c}	12.11 ± 0.23^{d}	0.40 ± 0.10^{bcdef}	0.58 ± 0.06^{def}	269.77 ± 4.43^{bc}
18	100	16	45	11.59 ± 0.45^{def}	8.45 ± 0.10^{fgh}	2.91 ± 0.18^{a}	3.74 ± 0.06^{a}	237.70 ± 1.58^{de}
19	20	2	60	9.30 ± 0.36^{hij}	5.77 ± 0.10^{ij}	0.17 ± 0.07^{ef}	0.16 ± 0.04^{g}	128.48 ± 2.83^{jk}
20	60	2	60	19.19 ± 0.14^{b}	15.93 ± 0.66^{c}	0.61 ± 0.09^{b}	0.61 ± 0.03^{def}	225.74 ± 5.38^{ef}
21	100	2	60	11.01 ± 0.49^{efg}	10.08 ± 0.57^{e}	2.73 ± 0.08^{a}	3.16 ± 0.04^{c}	199.37 ± 7.65^{gh}
22	20	9	60	10.78 ± 0.68^{efgh}	8.21 ± 0.64^{fgh}	0.25 ± 0.08^{bcdef}	0.19 ± 0.03^{g}	137.19 ± 2.33^{ij}
23	60	9	60	21.39 ± 0.29^{a}	21.66 ± 0.37^{a}	0.45 ± 0.05^{bcde}	0.65 ± 0.05^{def}	257.02 ± 5.57^{cd}
24	100	9	60	12.36 ± 0.26^{de}	13.02 ± 0.71^{d}	2.83 ± 0.26^{a}	3.39 ± 0.10^{b}	246.52 ± 9.28^{de}
25	20	16	60	11.54 ± 0.02^{def}	7.69 ± 0.04^{gh}	0.21 ± 0.09^{cdef}	0.17 ± 0.04^{g}	142.65 ± 5.72^{ij}
26	60	16	60	22.16 ± 0.68^{a}	19.61 ± 0.49^{b}	0.61 ± 0.08^{b}	0.59 ± 0.06^{def}	302.25 ± 9.36^{a}
27	100	16	60	12.99 ± 0.44^{d}	9.72 ± 0.14^{ef}	2.77 ± 0.01^{a}	3.28 ± 0.03^{bc}	280.87 ± 7.70^{b}

Data are expressed as average value over two replications ± standard deviation. ANOVA to compare data; different letters indicate significant difference between grape skin pomace extracts (Tukey's test, $p<0.05$). Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

-glucoside and procyanidins) that showed to be less stable to oxidation and more easily degradable [Crupi *et al.*, 2018].

Methanol concentration, time, and temperature significantly influenced the antioxidant activity (ORAC) of grape skin pomace extracts ($p<0.05$), while trends found were close to those earlier established for TP. The highest concentrations were extracted with 60% (v/v) methanol, while significantly lower ORAC values were generally found in experiments using 100% and particularly 20% (v/v) methanol (on average 2-fold lower) under identical conditions of temperature and time. Moreover, an increase in the temperature and time contributed to an increase in ORAC values of grape skin pomace extracts. Namely, the antioxidant activity of extracts significantly increased in the range of 30–45–60°C and 2–9–16 min for temperature and time, respectively. Results obtained are in accordance with findings from an earlier study of Pinelo *et al.* [2005] who reported a higher DPPH inhibition percentage for methanol extracts compared to water or ethanol ones. The same authors showed that temperature had

TABLE 2. Polynomial equations and statistical parameters describing the effect of operating process variables and on the phenolic and antioxidant characteristics of grape skin pomace extracts.

Output variables	2nd-order polynomial equation (quadratic model)	R^2	*p*-value
TP (mg GAE/g dw skin pomace)	$15.26 - 1.73X_1 + 1.16X_2 + 3.16X_3 - 0.02X_1X_2 - 0.91X_1X_3 + 0.08X_2X_3 - 6.19X_1^2 - 0.35X_2^2 + 0.47X_3^2$	0.9191	< 0.0001
TT (mg/g dw skin pomace)	$14.54 + 2.36X_1 + 0.49X_2 + 3.14X_3 - 0.44X_1X_2 - 0.53X_1X_3 + 0.21X_2X_3 - 6.94X_1^2 - 1.83X_2^2 + 0.57X_3^2$	0.9237	< 0.0001
THCA (mg/g dw skin pomace)	$0.55 + 1.35X_1 - 0.01X_2 + 0.02X_3 - 0.03X_1X_2 - 0.03X_1X_3 + 0.02X_2X_3 + 1.03X_1^2 - 0.07X_2^2 + 0.02X_3^2$	0.9911	< 0.0001
TF (mg/g dw skin pomace)	$0.76 + 1.67X_1 + 0.05X_2 - 0.01X_3 + 0.07X_1X_2 - 0.08X_1X_3 - 0.03X_2X_3 + 1.24X_1^2 - 0.13X_2^2 - 0.13X_3^2$	0.9954	< 0.0001
ORAC (μmol TE/g dw skin pomace)	$224.09 + 47.78X_1 + 24.63X_2 + 41.93X_3 + 11.02X_1X_2 + 9.51X_1X_3 + 3.12X_2X_3 - 61.21X_1^2 + 1.30X_2^2 - 12.73X_3^2$	0.9695	< 0.0001

Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

a critical role in the extraction efficiency, where the value of 50°C maximized the antiradical activity of phenolic extracts. Furthermore, solvent concentration, time, and microwave power, and interaction of power with time and solvent concentration, as well as interaction of time and solvent concentration showed to play a significant role in the antioxidant activity of grape seed extracts [Krishnaswamy *et al.*, 2013]. Our results demonstrated that the extract with the highest phenolics content, that was extracted under 60% (v/v) methanol and the highest temperature (60°C) and the longest process duration (16 min), exhibited the highest antioxidant activity.

Modeling single-step MAE by artificial neural network (ANN)

In order to test whether it is possible to predict the concentrations of total phenolics, tannins, flavonols, and hydroxycinnamic acids as well as the antioxidant capacity based on methanol concentration, temperature, and duration of the process, several AANs were developed. In all the cases, three variables were used as input data (methanol concentration, temperature, and duration of the process) and 5 variables were used as output data (TT, TP, THCA, TF and ORAC). The ANN training was performed with random separation of data into training, test, and validation sets as 60:20:20 ratio. Back error propagation algorithm available in Statistica v.10.0 was applied for the model training and model performance was evaluated based on R^2 and Root Mean Squared Error (RMSE) values for training, test, and validation [Benković *et al.*, 2015]. Examples of few developed ANNs are given in Table 3.

Almost all of the developed networks had a high linear correlation coefficient (R^2) for training, test, and validation. The five selected ones (Table 3) had the highest R^2 values for training, test, and validation with lowest RMSE values. It may be observed that there are basically two different ANNs regarding the number of neurons in the hidden layer (8 and 10) since all of them have 3 neurons in the input layer and 5 neurons in the output layer. Also the hidden activation and the output activation of the ANNs with same numbers of neurons in the hidden layer were different. When looking at the correlation coefficients for training, for all of the five networks,

TABLE 3. Characteristics of five selected artificial neural networks (ANNs) based on coefficients of determination and root mean square errors for the prediction of phenolic and antioxidant characteristics of grape skin pomace extracts obtained by microwave-assisted extraction (MAE).

Network number	1	2	3	4	5
Network name*	MLP 3–10–5**	MLP 3–10–5	MLP 3–8-5	MLP 3–10–5	MLP 3–8-5
Training performance	0.9957	0.9947	0.9951	0.9958	0.9919
Training error	0.0016	0.0020	0.0018	0.0015	0.0029
Test performance	0.9945	0.9918	0.9925	0.9939	0.9863
Test error	0.0020	0.0040	0.0022	0.0017	0.0040
Validation performance	0.9965	0.9954	0.9964	0.9965	0.9936
Validation error	0.0026	0.0034	0.0028	0.0031	0.0054
Training algorithm	BFGS103	BFGS66	BFGS100	BFGS128	BFGS69
Hidden activation	Logistic	Tanh	Tanh	Logistic	Logistic
Output activation	Exponential	Exponential	Exponential	Identity	Exponential

*In the network name, the first number describes the number of input variables, the second one the number of neurons in the hidden layer and the third one number of output variables. **The most suitable artificial neural network. Abbreviations: MLP, multi layered perceptron.

the highest value was observed for ANN 4 (R^2=0.9958) which also had the lowest training error (RMSE=0.0015). ANN 1 which had slightly lower training performance (R^2=0.9957) had the highest value for test performance (R^2=0.9945). The highest value for validation performance was observed for ANN 1 (R^2=0.9965) and ANN 4 (R^2=0.9965) which had the same values but the ANN 1 had lower validation error (RMSE=0.0026). Based on these results, ANN 1 was selected as the optimal one. The comparison between experimental and predicted values of TP, TT, THCA, TF, and ORAC for the most suitable ANN 1 (MLP 3–10–5) is presented in Figures 1a-d respectively, while correlation coefficients for prediction of TT, TP, THCA, TF, and ORAC are presented in Table 4.

From Figure 1 it is clearly visible that the ANN managed to achieve a high correlation between experimental data and ANN predictions for each parameter (TP, TT, THCA, TF,

TABLE 4. Correlation coefficients of the most suitable artificial neural network* for the prediction of phenolic and antioxidant characteristics of grape skin pomace extracts.

Output variables	Correlation coefficient (R^2)		
	Training	Testing	Validation
TP	0.9943	0.9893	0.9981
TT	0.9928	0.9900	0.9981
THCA	0.9973	0.9978	0.9916
TF	0.9991	0.9978	0.9988
ORAC	0.9952	0.9979	0.9962

*The most suitable artificial neural network: ANN 1 (MLP 3–10–5). Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity.

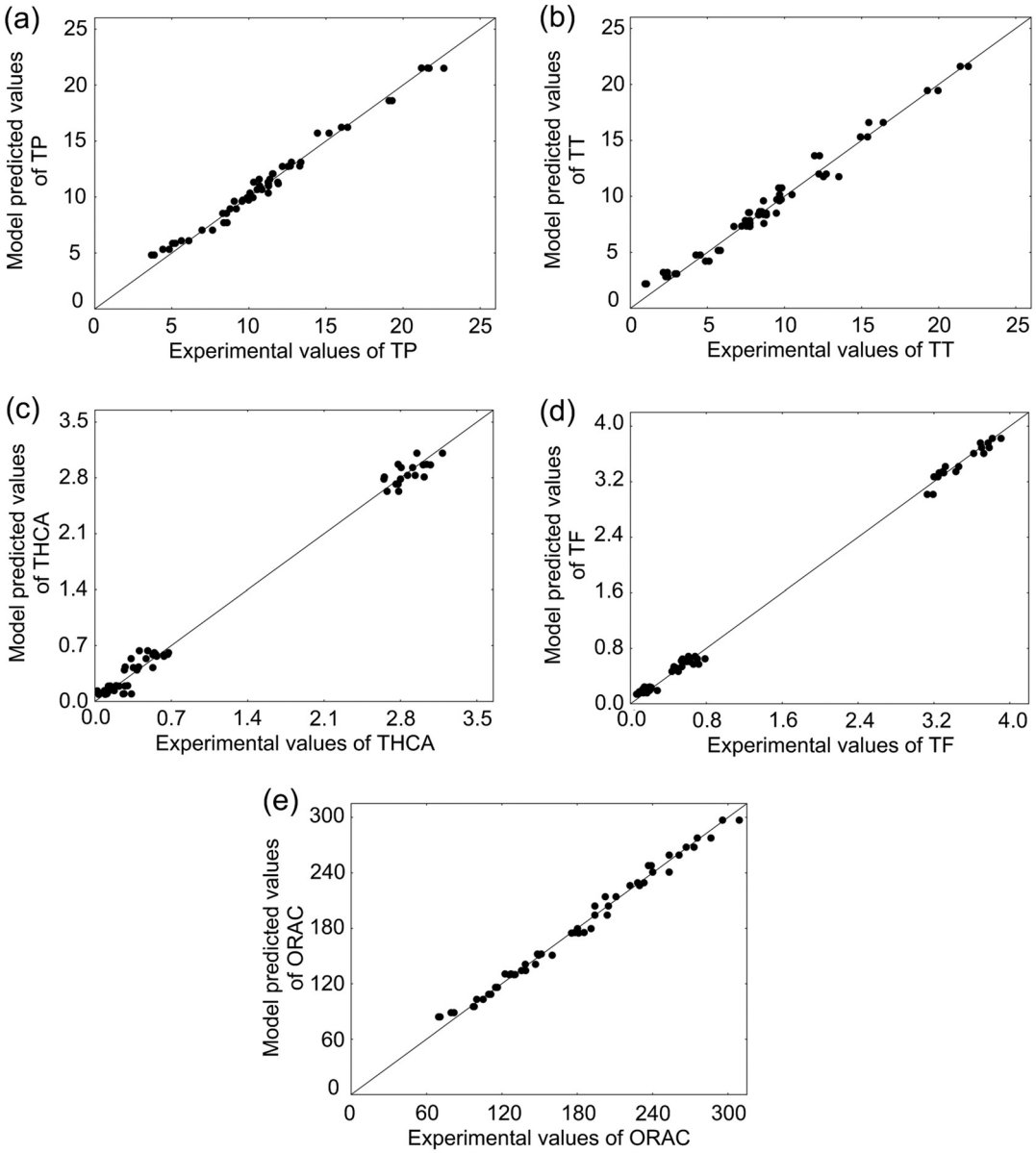

FIGURE 1. Comparison between experimental and predicted values of the most suitable ANN 1 (MLP 3–10–5) for: (a) total phenolics (TP), (b) total tannins (TT), (c) total hydroxycinnamic acids (THCA), (d) total flavonols (TF), and (e) oxygen radical absorbance capacity (ORAC).

and ORAC). From Table 4 it is visible that the best correlations between experimental data and the ANN predictions were obtained for TF with the R^2 values of 0.9991, 0.9978, and 0.9988 for training, test, and validation, respectively. The second highest value for validation was observed for TP (R^2=0.9981) and TT (R^2=0.9981) which had the same value, followed by ORAC (R^2=0.9962) and THCA (R^2=0.9916). Considering that those are very high values obtained for validations for all the tested parameters, these models could easily be used to monitor extraction processes since a good-fitting model or quantitative model would have R^2 values above 0.90 and quantitative models are compact representations where a single differential or difference equation may describe the performance of the system for a large set of input functions and initial states [Lunze, 1998]. This is not surprising since ANNs were proven to be one of the most useful tools in extraction processes for monitoring, predicting, and optimizing different compounds in microwave-assisted or ultrasound-assisted extractions like phenolic compounds from *Achillea berbresteinii* [Salarbashi *et al.*, 2014]; total polyphenolic compounds from chokeberries [Simić *et al.*, 2016]; as well as total extract, stevioside, and rebaudioside A from *Stevia rebaudiana* (Bertoni) leaves [Ameer *et al.*, 2017].

Optimization of single-step MAE by response surface methodology (RSM) and effects of sequential irradiation cycles

In order to provide overall optimal conditions of simultaneous and maximum extraction of phenolic antioxidants from grape skins, various output responses were first considered at the same time. However, desirability function (D), which is the most important and applied multicriteria methodology in optimization procedures [Bezerra *et al.*, 2008], of this joint model was importantly lower (D=0.7870) compared to D values obtained by each individual model. Also, earlier it has been shown that optimal conditions can vary significantly among the different phenolic groups [Karvela *et al.*, 2009]. Hence, the individual models developed (Table 2) were used to optimize the parameters of MAE process. Optimal conditions were selected using desirability for the maximum concentrations of phenolic compounds and antioxidant capacity of grape skin pomace extracts. Three-dimensional

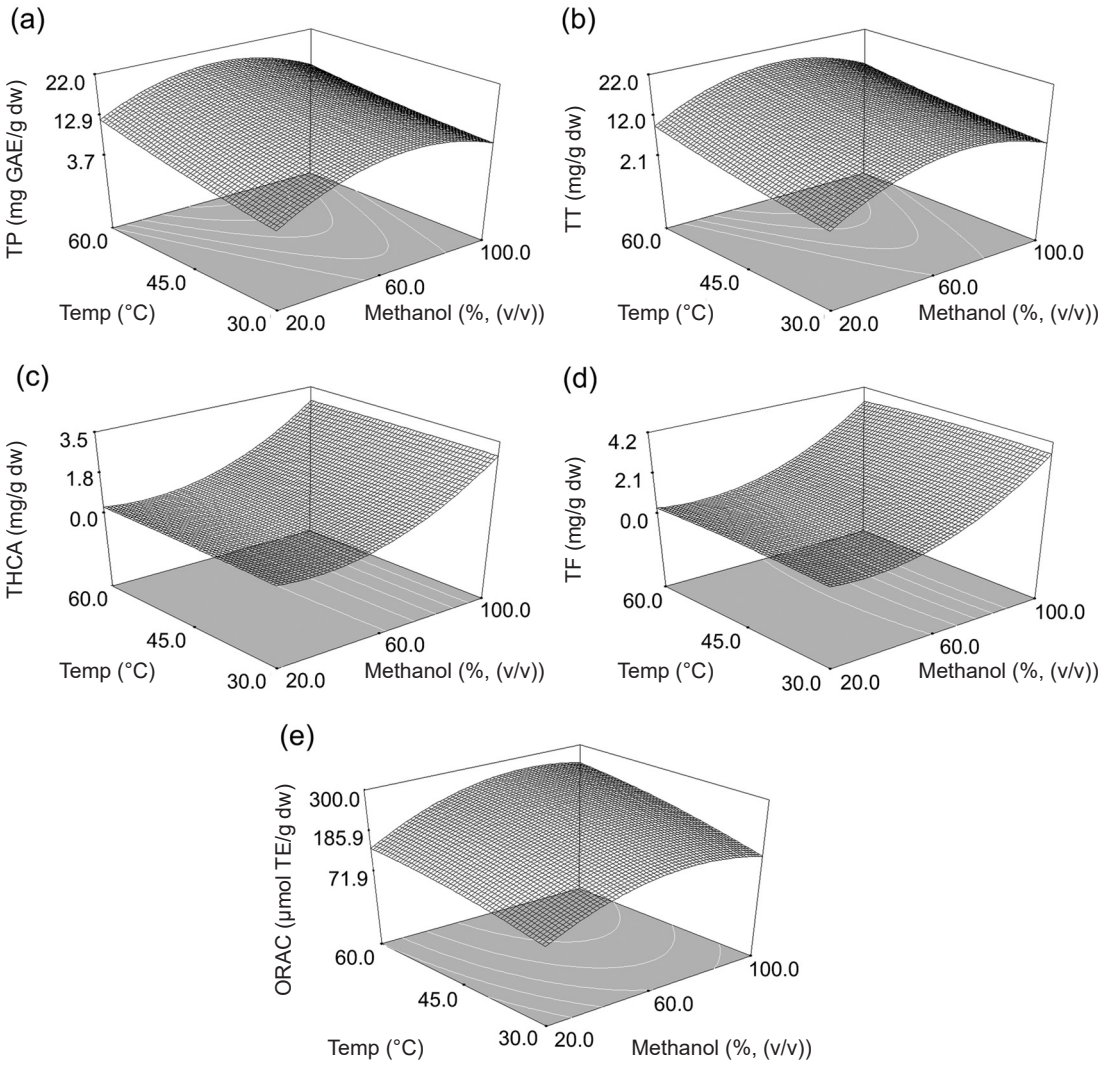

FIGURE 2. RSM plots of the models developed for single-step MAE of: (a) total phenolics (TP), (b) total tannins (TT), (c) total hydroxycinnamic acids (THCA), (d) total flavonols (TF), and (e) oxygen radical absorbance capacity (ORAC).

response surface plots were created for each variable individually and optimum conditions of single-step MAE for maximum response of TP, TT, THCA and TF, as well maximum antioxidant capacity of extracts (ORAC) are presented in Figures 2a-e. Given to the results that prolongation of single-step extraction time was not useful to extract more polyphenols as well as to optimize the overall time of multistep final method, all plots were generated by keeping the time variable to the fixed value (9 min) and plotting it against two other variables (methanol concentration and temperature). Optimal conditions for single-step MAE were 62.7% and 65.3% (v/v) methanol for TP and TT, respectively, at 60°C for 9 min with the predicted yields of 18.91 and 18.38 mg/g dw skin pomace for the first and the latter (Figures 2a and b). Optimal conditions for single-step MAE of THCA and TF were 100% (v/v) methanol, at 40°C for 9 min with the predicted yields of 2.94 and 3.68 mg/g dw skin pomace for THCA and TF, respectively (Figures 2c and d). Optimal conditions for the maximum antioxidant capacity of extracts (ORAC) by single-step MAE were 78.1% (v/v) methanol, at 60°C for 9 min with the predicted yields of 265.77 μmol TE/g dw skin pomace (Figure 2e). Validity of predicted optimal values for each output variable were experimentally confirmed. Experimental and predicted values of optimal conditions were given in Table 5. Experimental data were in accordance with the predicted ones, since predicted and experimental values were not significantly different within the 95% confidence interval.

Effects of sequential irradiation cycles were further studied on three different cultivars (Cabernet Sauvignon, Merlot, and Teran) by application of optimal conditions of a single MAE cycle in multiple steps (cycles) on the model of TP covering all phenolic compounds. Application of sequential irradiation cycles allowed the prolongation of the extraction time [Chan *et al.*, 2011] but without risk of degradation due to the joint effects of temperature and longer extraction time [Medouni-Adrar *et al.*, 2015; Pinelo *et al.*, 2005]. Also, it is important to note that this was manipulated by the addition of fresh solvent to the residue and repeating the extraction step. This procedure allowed us to avoid the solvent evaporation earlier reported [Pedroza *et al.*, 2015], and ensured the completion of extraction, so that the MAE method could be applied prior to analytical determination. Effects of eight sequential irradiation extraction cycles on the extraction of total phenolics (concentration and relative recovery (%, w/w) – calculated relative to the overall amount obtained after eight cycles) are presented in Table 6.

TABLE 5. Phenolic and antioxidant characteristics of grape skin pomace extracts obtained by optimized single-step microwave-assisted extraction conditions.

Output variables	Experimental concentrations	Predicted concentrations
TP (mg GAE/g dw skin pomace)	19.05±0.27	18.91
TT (mg/g dw skin pomace)	18.18±0.35	18.38
THCA (mg/g dw skin pomace)	3.02±0.09	2.94
TF (mg/g dw skin pomace)	3.70±0.05	3.68
ORAC (μmol TE/g dw skin pomace)	261.04±5.32	265.77

Abbreviations: TP, total phenolics; TT, total tannins; THCA, total hydroxycinnamic acids; TF, total flavonols; ORAC, oxygen radical absorbance capacity; GAE, gallic acid equivalents; TE, Trolox equivalents.

The major part of TP was extracted in the first extraction step (1st), and then less and less TP were extracted in each successive individual cycle. Concentrations in each cycle decreased in the order: Cabernet Sauvignon, Teran, Merlot. On the other hand, very similar values of relative recovery were found after the second cycle independently of cultivar, meaning that % (w/w) of extracted TP were quite similar for all three cultivars. For example, ~ 83% (w/w) of TP were cumulatively extracted after three cycles in all three cultivars, and around 90% and 94% (w/w) of TP, after four and five cycles, respec-

TABLE 6. Cumulative effect of sequential irradiation cycles on the extraction of total phenolics (concentration and relative recovery) from Cabernet Sauvignon, Merlot, and Teran grape skin pomaces.

Cycle number	Cabernet Sauvignon		Merlot		Teran	
	TP (mg GAE/g dw skin pomace)	TP (%)	TP (mg GAE/g dw skin pomace)	TP (%)	TP (mg GAE/g dw skin pomace)	TP (%)
1st	19.05^{m}	46.4^{j}	15.18^{o}	51.6^{h}	17.82^{n}	49.3^{i}
2nd	28.66^{hi}	69.7^{g}	21.17^{l}	71.9^{f}	25.96^{j}	71.8^{f}
3rd	34.05^{e}	82.9^{e}	24.30^{k}	82.5^{e}	30.22^{g}	83.6^{e}
4th	37.07^{c}	90.2^{d}	26.58^{j}	90.3^{d}	32.70^{f}	90.4^{d}
5th	38.66^{b}	94.1^{c}	27.82^{i}	94.5^{c}	34.14^{e}	94.4^{c}
6th	40.35^{a}	98.2^{ab}	29.03^{h}	98.6^{ab}	35.44^{d}	98.0^{ab}
7th	40.73^{a}	99.1^{ab}	29.21^{gh}	99.2^{ab}	35.89^{cd}	99.3^{ab}
8th	41.09^{a}	100.0^{a}	29.45^{gh}	100.0^{a}	36.16^{cd}	100.0^{a}

Data are expressed as average value of three replications ± standard deviation. ANOVA to compare data among three cultivars; different letters indicate statistical differences between extracts (Tukey's test, $p<0.05$). Abbreviations: TP, total phenolics; GAE, gallic acid equivalents.

tively (Table 6). In addition, results in Table 6 showed that concentrations cumulatively extracted by sequential irradiation cycles showed a significant increase with the prolongation of extraction cycle number up to six. Further extraction only slightly contributed to the concentrations of TP. Hence, there were no significant differences among the last three cycles. According to these results, the extraction process can finally be limited to six cycles for all three cultivars, that approximately extracted more than 98% (w/w) of total phenolics. Nevertheless, it is important to note that the number of cycles should not be considered as fixed. Namely, wide ranges in concentrations of different phenolics were detected in extensive studies of grape pomace over the years, comprising variations of cultivar and vintage as well as geographical origin, maturity, and winemaking technology [Deng *et al.*, 2011; Kammerer *et al.*, 2004; Ky *et al.*, 2014; Ky & Teissedre, 2015]. For instance, these differences can lead even up to ten times lower/higher concentrations of phenolic compounds and antioxidant activity of grape skin pomaces [Ky *et al.*, 2014; Ky & Teissedre, 2015]. Hence, our results demonstrated the importance of MAE with successive irradiation cycles, particularly for the conditions operating under lower power and temperature, where the exact number should always be examined in order to ensure the completion of the extraction process.

Comparison of our final MAE conditions to literature data for grape skin and pomace was difficult, due to great variation considering the operating systems and parameters studied (extraction solvent, temperature range, power, time, number of irradiation cycles, *etc*.), thus reflecting to the differences in selected or optimal conditions for extraction of TP [Chan *et al.*, 2011]. For instance, Pedroza *et al.* [2015] proposed extraction from Chardonnay grape skin pomace with 60% (v/v) aqueous ethanol solution, and liquid to solid ratio of 4 mL/g for 1033 s at 900 W, in two cycles; while successive irradiation under these conditions caused solvent evaporation and imbibition, and led to decreasing recovery. In our study, in order to avoid these negative effects of sequential irradiation cycles, multistep MAE was performed by the addition of fresh solvent in each repetitive cycle, while extraction was conducted with significantly lower irradiation power and longer time, as well as different solvent. Furthermore, optimal conditions for single-step MAE of TP from Ahmar Bou-Amar grape skin pomace obtained by optimization modeling were 51.45% (v/v) acetone, with solid to liquid ratio of 0.1 g/32.25 mL for 113.74 s and 384.44 W [Medouni-Adrar *et al.*, 2015]. Hong *et al.* [2001] also proposed single-step MAE of TP from grape skin with 90% (v/v) methanol, solid to liquid ratio of 1 g/15 mL for 200 s and 540 W. Overall extraction time (6×9 min) of our MAE methods was comparable to the study of Casazza *et al.* [2010] who also worked with a similar operating system, as well as lower power for longer time. Namely, Casazza *et al.* [2010] performed single-step MAE from Pinot noir grape skin pomace using 100% (v/v) methanol, with solid-liquid ratio of 0.2 g dw/mL for 60 min at 110°C and 60 W. Concentrations extracted with this single-step extraction at higher temperatures (110°C) in Pinot Noir skin pomace were slightly lower than those shown in Table 6, probably due to the differences in MAE parameters but also to grape cultivar, maturity, vintage, winemaking technology, *etc.* [Deng *et al.*, 2011; Kammerer *et al.*, 2004; Ky *et al.*, 2014; Valls *et al.*, 2017]. Concentrations of TP determined in grape skin pomaces of three cultivars decreased in the order: Cabernet Sauvignon, Teran, and Merlot, and were comparable with other studies regardless of the extraction method prior to the analysis. For instance, concentrations of TP found were in line with the values previously reported for Cabernet Sauvignon and Merlot or other red grape cultivars (11.8–54.8 mg GAE/g dw grape skin pomace) [Casazza *et al.*, 2010; Deng *et al.*, 2011; Ky *et al.*, 2014; Medouni-Adrar *et al.*, 2015; Yilmaz & Toledo, 2006]. Finally, results showed high efficiency of MAE method, which allowed completion of extraction in shorter time compared to time consumed during conventional solid-liquid extraction methods, that for processes with temperatures under 60°C can take from 6 to 24 h [Casazza *et al.*, 2010; Ky *et al.*, 2014].

CONCLUSIONS

The effects of methanol concentration (20, 60, and 100%, v/v), time (2, 9, and 16 min), and temperature (30, 45, and 60°C) on the extraction of phenolic antioxidants from grape skin pomace were studied using modeling and optimization by ANN and RSM. All input parameters significantly influenced the MAE of total phenolics, tannins, flavonols, and antioxidant capacity of extracts (ORAC), while extraction yields of hydroxycinnamic acids was markedly influenced only by methanol concentration. The ANN model was accurate to predict the extraction yields of phenolic antioxidants with high correlation coefficients for training (R^2=0.9957), test (R^2=0.9945), and validation (R^2=0.9965), thus confirming that ANN could be successfully used in MAE experiments for monitoring or prediction. The optimal parameters of a single-step MAE cycle for maximum yields of phenolic compounds and antioxidant capacity obtained by RSM were: (i) 62.7% and 65.3% (v/v) methanol for total phenolics and tannins, respectively, at 60°C for 9 min; (ii) 100% (v/v) methanol, at 40°C for 9 min for total flavonols and hydroxycinnamic acids; and (iii) 78.1% (v/v) methanol, at 60 °C for 9 min for ORAC. The number of extraction steps showed to be an important factor influencing extraction yields of phenolic compounds. Relative recovery of total phenolics (%, w/w) showed to be rather constant extraction parameter for all three cultivars, where six MAE cycles significantly contributed to the concentration of TP and extracted more than 98% (w/w) of total phenolics. Multistep MAE by optimal parameters proved to be a highly efficient method for the extraction of grape skin pomace phenolics prior to analytical determination.

RESEARCH FUNDING

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Submitted: 7 March 2019. Revised: 23 April 2019. Accepted: 16 May 2019. Published on-line: 25 June 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 247–254
DOI: 10.31883/pjfns/109666
http://journal.pan.olsztyn.pl

Original research article
Section: Food Quality and Functionality

Fat from Tenebrionidae Bugs – Sterols Content, Fatty Acid Profiles, and Cardiovascular Risk Indexes

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Key words: mealworm, superworm, cholesterol, stigmasterol, β-sitosterol, cholecalciferol

This work focused on analysing the content of selected sterols and profile of fatty acids of edible insect species – mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*), which are expected to increase their usability in human nutrition. Sterols content was determined using capillary gas chromatography method. Cholesterol content was determined at 1335 mg/kg in dry matter (DM) for the mealworm, which was less than for superworm (3224 mg/kg DM). Other sterols analysed were stigmasterol and β-sitosterol, which were once again higher in superworm (stigmasterol – 44 mg/kg DM and β-sitosterol – 414 mg/kg DM) than in mealworm (stigmasterol – 18 mg/kg DM and β-sitosterol – 171 mg/kg DM). From the nutritional point of view, the amount of cholecalciferol is also not negligible, which was 190 μg/kg DM in mealworm and 199 μg/kg DM in superworm. Atherogenic index (AI), thrombogenic index (TI), and cholesterol index (CSI) were calculated for both species and compared with the results of other authors. These indexes are often considered predictors of cardiovascular diseases. A potential benefit of both species could be the balanced proportion of sterols of animal and plant origin that could be nutritionally well-accessible and lower weight of dry matter necessary to consume to cover the daily dose of linoleic acid compared to dried beef.

INTRODUCTION

From the nutritional point of view, edible insects have become an increasingly discussed topic in both the professional and laic public, even in countries where their consumption is not common [Mlček *et al.,* 2014; Ramos-Elorduy *et al.,* 2011; van Huis, 2016]. In particular, it is possible to use edible insects as a valuable alternative source of proteins, especially in regions with a lack of conventional animal proteins [Mlček *et al.,* 2014; Ramos-Elorduy *et al*., 2011; van Broekhoven *et al.*, 2015]. Other benefits include higher feed conversions, low greenhouse gas emissions, better soil utilization, and the conversion of organic materials to valuable products. This strategy can lead to financial savings and environmental benefits [Cerritos, 2009, 2011; Fontaneto *et al*., 2011; Mariod *et al*., 2011; Premalatha *et al.,* 2011]. Insects have also a great potential as feed, for example, in aquaculture [van Huis, 2016]. This is due to the high content of proteins and sulphur amino acids that can be successfully used as feed for poultry [Józefiak *et al.,* 2016].

One of the most studied species of insects is the mealworm [Finke, 2002, 2004; Barroso *et al.*, 2014; Ravzanaadii *et al*., 2012; Sánches-Muros *et al*., 2016; Tzompa-Sosa *et al*., 2014; Zielińska *et al*., 2015]. It is a good source of protein and fat. The highest protein content (637.0–676.5 g/kg in dry matter (DM)) and lowest fat content (148.8–184.0 g/kg DM) was determined in adult specimen. However, larvae and pupae are more nutritionally beneficial because of their better digestibility and sensory properties. For larvae, the total protein content is usually reported in the range from 477.6 to 527.0 g/kg DM and total fat content from 189.0 to 382.9 g kg DM.

Sterols are key nutritional elements of insects. They are precursors of steroid hormones and development process regulators [Mondy *et al.,* 2006]. Phytosterols, typical plant sterols, were found in insect samples [Piironen, 2000], because insects cannot synthesize cholesterol *de novo* [Behmer & Nes, 2003] and have to use plant phytosterols (β-sitosterol, campesterol, stigmasterol) to synthesize cholesterol. Cholesterol is the most abundant sterol present in insects. Mealworm (*Tenebrio molitor*) sterols contain about 17% of 7-dehydrocholesterol and about 67% of cholesterol [Ikekawa *et al*., 2013]. Insects need cholesterol to synthesize vitamin D_3 and steroid hormones known as ecdysteroids. These hormones are indispensable for the individual developmental stages of the instar [Nation, 2001; Klowden, 2007].

Cholesterol is found in foods of animal origin, but it is also the only sterol the human body can synthetize by itself. Too high cholesterol intake causes increased levels of low-density

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lipoprotein (LDL) and very low-density lipoprotein (VLDL), which results in an increased risk of the development of the metabolic syndrome (obesity, diabetes mellitus, cardiovascular disease) [WHO, 2004]. The human body consumes about 2 g of cholesterol per day. The optimal intake is 0.15–0.3 g per day. This amount is sufficient because the rest is produced in the body through endosynthesis. However, common diet means an intake of 0.6–0.8 g of cholesterol. A suitable diet can reduce cholesterol by 10%, *e.g.* by consumption of fiber, antioxidants, and phytosterols [Pánek *et al*., 2002].

Vitamin D is synthesized in the human body from ergosterol and 7-dehydrocholesterol by exposure to sunlight. In the latitudes of the temperate zone, sunlight does not cover these needs and vitamin D endosynthesis is insufficient. For this reason, it is necessary to supplement it. Vitamin D, along with calcitonin and parathormon hormones, controls the calcium and phosphorus metabolism. Its deficiency leads to rachitis in children, while older people develop osteopenia and consequently osteoporosis [Pánek *et al.*, 2002]. For this reason, the recommended daily dose of vitamin D is 5 μg/day [Decree No. 225/2008 Coll, 2008].

Developmental stage of insect is one of the major factors influencing the amount of fat and the fatty acids [Nowak *et al.*, 2016; Finke, 2004; Adámková *et al.*, 2016]. Other factors include gender, diet and the environment [Chakravorty *et al*., 2011, 2014, 2016]. Fatty acid profile description can be simplified and expressed by various proportional numbers, including the cholesterol index (CSI), the atherogenic index (AI), and the thrombogenic index (TI). These indexes serve often in medicine as important predictors of cardiovascular risks [Dobiášová, 2006]. Regarding the *n*-3:*n*-6 ratio of fatty acids and the mentioned indexes, edible insect fat may have a protective effect on human health. One of the objectives of this study was to determine the ratio of *n*-3:*n*-6 fatty acids and CSI, AI, and TI in edible insect fat and to compare them with other commodities of animal origin.

The content of sterols in the mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) bred on farms in the Czech Republic, which are fed *ad libitum* with conventional feed, is not yet sufficiently known from available literature [Sabolová *et al*., 2016] and so far this issue has not been thoroughly explored. The amount of cholesterol and phytosterols can be affected by long-term nutritional stress (the insects do not have access to feed). Although insects bred in farms usually do not starve, access to feed may be restricted during longer transport or longer pre-treatment before killing.

This study was carried out to define initial information on the content of cholesterol, phytosterols, and fatty acids in *Tenebrioidae* larvae and their presumed impact on human health (AI, TI and CSI). Furthermore, the impact of the nutritional stress (inaccessibility of the feed) on the cholesterol content was evaluated, as the nutritional stress has a significant impact on the welfare of the breed.

MATERIAL AND METHODS

Material

Species used for analysis were mealworm larvae (*Tenebrio molitor*) and superworm larvae (*Zophobas morio*). Samples were purchased from breeder Radek Frýželka, Brno, Czech Republic. Insects were reared in optimum conditions for the development of individual species (mealworm – 25–28°C, 60–70% relative humidity (RH); superworm – 28–30°C, 60–70% RH) and fed with wheat bran and oat bran *ad libitum* till the beginning of the experiment.

At the beginning of the experiment, two groups of live larvae, weighing approximately 200 g, were taken from a breeding. The first group was left to starve for 12 h. According to the European Food Safety Authority (EFSA) recommendations [EFSA, 2015], the expiration time is 12–24 h. To shorten the processing, the lower limit was chosen. Subsequently, the insects were killed in boiling water (100°C) and dried at 105°C. Subsequently, the samples were homogenized and stored in a hermetically sealed aseptic box with a normal atmosphere at 4–7°C until analysis.

The second experimental group of larvae starved for 168 h (7 days) before killing, which led to nutritional stress. Furthermore, the larvae were killed and processed in the same way as the first experimental group of larvae.

Wheat bran with the following nutritional values (data per 100 g of product) were used as feed: energy 1210 kJ/292 kcal, fats 5.3 g, of which saturated fatty acids accounted for 0.88 g, carbohydrates 24.9 g, of which sugars were at 2.2 g, fiber 40.2 g and protein 16.2 g, and salt 0.1 g.

Oat bran with the following nutritional values (data per 100 g of product) were used as feed: energy 1518 kJ/ 361 kcal, fats 8.3 g, of which saturated fatty acids accounted for 1.4 g, carbohydrates 45 g, of which sugars were at 2.0 g, fiber 12 g and protein 21.0 g, and salt 0.01 g.

Determination of dry matter content of infrared scales

A homogenized sample of about 0.5 g was evenly distributed over the aluminium foil and subsequently analysed according to ISO 1442:1997 using Precisa HA 300 scales with infra radiator (Precisa Gravimetrics AG, Dietikon, Schweiz). In this method, the test sample is heated and the weight loss is measured. The basis of the method is the drying of the sample by infrared rays. Drying was carried out at 105°C until constant weight, where the difference of two consecutive weights was not less than 2 mg per min.

Crude protein content determination

The nitrogen and crude protein were analysed using the Kjeldahl's method [ISO 1871:2009]. The samples (1 g) and blank runs were mineralized at 420°C for 105 min. The distillation was performed on Kjeltec™ 2200 (FOSS, Denmark) for 4 min. The protein content was calculated using nitrogen-to-protein conversion factor of 6.25.

Fat content determination

The fat was extracted acc.to Soxhlet's method [Soxhlet, 1879] on the Gerhardt Soxtherm (C. Gerhardt GmbH & Co. KG, Germany). The sample (5 g) was placed in the extraction capsule and extracted (program selected: 70°C for 120 min) with 150 mL of petroleum ether (Ing. Petr Švec – PENTA s.r.o., Prague, Czech Republic). The extracted sample was then dried at 103°C and repeatedly weighed until a constant

weight (difference between two subsequent weighings of less than 10 mg) was achieved.

Sterols content determination

Sterols in fat of the samples were analysed and evaluated in an accredited laboratory Skúšobňa VETLAB, Ltd., Púchov, Slovakia. Determination of sterols content was done using capillary gas chromatography method according to ES no 213/2001 [Regulation (EC) No. 2013/2001, 2001].

Cholecalciferol (vitamin D_3) content determination

Cholecalciferol content determination was done according to EN 12821:2009 [EN 12821:2009, 2009]. Samples were extracted with hexane. Determination of vitamin D_3 content was performed using semi-preparative HPLC on normal phase followed by analytical reverse phase HPLC. Vitamin D was detected spectrophotometrically in the UV area. When determining vitamin D_3, vitamin D_2 was used as an inner standard.

Fatty acid profile determination

Weighted portions of fat (0.5 g) extracted from worms were esterified according to the ISO 12966-2: 2011 [ISO 12966–2:2011, 2011] using 0.25 mol/L of methanolic potassium hydroxide.

Fatty acid methyl esters in the samples were determined using gas chromatography with a flame ionization detector (GC/FID) on GC-2010 (Shimadzu, Kyoto, Japan), using a high polar chromatography column HP-88 (100 m × 0.25 mm × 0.2 μm) (Agilent Technologies, CO, Santa Clara, USA), which is intended for the identification of *cis/trans* fatty acid methyl esters. Chromatographic conditions were as follows: inlet volume – 1 μL; inlet temperature – 250°C; split ratio – 1:100; carrier gas – nitrogen; and temperature program – 80°C/5 min, 200°C/30 min, 250°C/15 min. Quantitative evaluation of the individual fatty acid contents in the samples was performed using the internal normalization method using FAME Mixture C4-C24 (Supelco Inc, Bellefonte, USA) containing 37 selected fatty acid methyl esters. The content of individual fatty acids was calculated as a percentage of the total methyl ester present.

Lipid nutritional quality indices evaluation

For each species, the atherogenicity index (AI) and thrombogenicity index (TI) were calculated according to the following formulas [Zhang *et al*., 2014; Kulma *et al*., 2016]:

$$AI = (C12:0 + 4 \times C14:0 + C16:0) / (\Sigma MUFA + \Sigma n\text{-}6 + \Sigma n\text{-}3), \quad (1)$$

$$TI = (C14:0 + C16:0 + C18:0) / (0.5 \times \Sigma MUFA + 0.5 \times \Sigma n\text{-}6 + 3 \times \Sigma n\text{-}3 + (n\text{-}3/n\text{-}6)), \quad (2)$$

where: MUFA stands for Mono Unsaturated Fatty Acids. These indexes were further calculated for both animal species from the fatty acid profiles from available literature.

The cholesterol-saturated acid index (CSI) was determined in larvae of mealworm fed *ad libitum* according to the following formula [Pánek, 2002]:

$$CSI = 1.01 \times \Sigma SFA\ (g/100\ g) + 50 \times \text{cholesterol}\ (g/100\ g), \quad (3)$$

where: SFA stands for Saturated Fatty Acids.

Statistical analysis

Each measurement was performed 4 times. All samples were from the same batch. The data was analysed using Excel 2013 (Microsoft Corporation, Redmond, USA) and STATISTICA Cz version 12 (StatSoft, Inc., Tulsa, USA). Results were expressed by average and standard deviation. Comparison of the results was performed using a Kruskal-Wallis test (α=0.05; α=0.01).

RESULTS AND DISCUSSION

To determine the basic characteristic and describe the material in mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) bred in the Czech Republic, the following basic components were evaluated: dry matter (DM), crude protein in DM, and fat in DM (Table 1). Further description of the material is given in Adámková [2017].

The cholesterol content in mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) fed *ad libitum* and these under nutritional stress (Table 2); and contents of stigmasterol, β-sitosterol, and cholecalciferol in the *ad libitum*-fed larvae (Table 3) were analysed.

The average cholesterol content in the *ad libitum*-fed mealworm larvae *(Tenebrio molitor)* was 1335 mg/kg DM. Superworm larvae (*Zophobas morio*) fed *ad libitum* contained higher amount of cholesterol (3224 mg/kg DM). Compared with the results of samples that have been subjected to nutritional stress, cholesterol levels were higher in the *ad libitum* fed larvae (Table 2). Statistically significant ($p<0.01$) difference was detected between the worms without nutritional

TABLE 1. Composition of material from mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) bred in the Czech Republic – dry matter (DM), crude protein in DM and fat in DM.

Components	*Zophobas morio*	*Tenebrio molitor*
DM (g/100 g)	47.9±0.6	32.6±0.7
Crude protein (g/100 g DM)	39.4±0.1	62.6±0.4
Fat (g/100 g DM)	39.1±0.4	16.7±0.1

DM – dry matter.

TABLE 2. Cholesterol content in dry matter of mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) fed *ad libitum* and under nutritional stress.

Nutrition of larvae	*Tenebrio molitor*	*Zophobas morio*
Ad libitum (mg/kg)	1 335±28^{Ab}	3 224±35^{Aa}
Nutritional stress (mg/kg)	1 124±24^{Bb}	2 985±31^{Ba}

^{A,B} – means with different letters in the same column are significantly different at $p<0.01$; ^{a,b} – means with different letters in the same row are significantly different at $p<0.01$

stress and these subjected to nutritional stress. The influence of insect species on sterol content was highly statistically significant. The level of significance was found to be $p<0.01$.

Cholesterol is supposed to be burnt up in nutritional stress to preserve life functions and eventually to build up the ecdysteroid needed for molting. Other available literature does not provide detailed information on cholesterol content depending on the nutritional stress and the stage of larval development in species we analysed. Sabolová *et al*. [2016] listed a number of selected sterols in farm insects from Sumatra Island and these kept under European conditions. In superworm from Sumatra, the authors demonstrated sterols content at 1784.1 mg/kg DM and in these from Czech Republic at 1594.9 mg/kg DM. These values are lower than in our work, but higher than those detected in the same species by Ramos-Bueno *et al*. [2016], *i.e.* 185 mg/kg DM. Different values can be caused by nutrition because cholesterol is not synthesized *de novo*, but the intake is dependent on the composition and dose of feed. Similar factors may influence the cholesterol levels in mealworm, in which Ramos-Bueno *et al*. [2016] determined cholesterol content at 726 mg/kg DM. In turn, Sabolová *et al*. [2016] reported 669.4 mg/kg DM for the same species bred in Sumatra. These values are again lower than in our work.

Ekpo *et al.* [2009] evaluated cholesterol content in fats of termites (*Macrotermes bellicosus*) and caterpillars (*Imbrasia Belina*). They found out that the average cholesterol content in their lipid fraction was up to 3.6%.

Cholesterol content in mealworm (*Tenebrio molitor*) detected in our work is comparable with some other commodities of animal origin, *e.g.* lobster (1460 mg/kg). Superworm *(Zophobas morio*) is comparable to carp (3540 mg/kg), *i.e.* an animal commodity considered a valuable source of nutrients and liver (up to 3450 mg/kg) [Velíšek, 2002; Venugopal & Gopakuma, 2017].

In comparison with other commodities, the species had higher cholesterol levels than most of the foods of animal origin. Cholesterol content in mealworm can be compared to beef tallow, mayonnaise, and Lobster (northern) and it is between the values for butter and whole eggs [Velíšek, 2002; Venugopal & Gopakuma, 2017]. The recommended dietary allowance for cholesterol consumption is 300 mg/day for adult man [EFSA, 2010]. To achieve this level, the amount of evaluated insects that would have to be consumed is 93 g for superworm and 224 g for mealworm. However, it is assumed that normally such a quantity is not consumed in the dried state, as it is not expected for a large quantity of eggs to be eaten each day. To fill the RDA (Recommended Dietary Allowances) when eating eggs, 75 g of the whole egg (about 1.5 eggs day) is needed.

On the other hand, the edible insects analysed contained phytosterols (stigmasterol and β-sitosterol) (Table 3), which serve as cholesterol antagonists, thus balancing the sterol levels. While comparing the species, a statistically significant ($p<0.01$) difference was detected for stigmasterol and β-sitosterol. Compared with the samples from Sumatra, which were analysed by Sabolová *et al*. [2016], the stigmasterol content was found to be up to 4 times higher. On the contrary, Sabolová *et al*. [2016] detected no stigmasterol in the edible insects from the Czech Republic. In the case of β-sitosterol in the mealworm, the content detected in this work was lower than that measured by Sabolová *et al*. [2016]. β-Sitosterol content of the superworm measured by Sabolová *et al*. [2016] was lower than the content detected in this work at both sites of origin. In general, nutritional values may depend on feed and living conditions [Ghosh *et al*., 2017]. *E.g.* Van Broekhoven *et al.* [2015] demonstrated a fat content in the range from 32.8 to 43.5%, depending on the mealworm feed. Oonincx [2015] reported the influence of feed on nutritional values (fat, protein, fatty acid profiles, and selected minerals) under the same breeding conditions of the same species. For this reason, the sterol content can be assumed to vary depending on nutrition and living conditions.

TABLE 3. Content of selected sterols and cholecalciferol in dry matter of mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) larvae fed *ad libitum*.

Compounds	*Tenebrio molitor*	*Zophobas morio*
Stigmasterol (mg/kg)	18 ± 6^{b}	44 ± 12^{a}
β-Sitosterol (mg/kg)	171 ± 20^{b}	414 ± 37^{a}
Vitamin D_3 (cholecalciferol) (μg/kg)	190 ± 20^{a}	199 ± 25^{a}

^{a,b} – means with different letters in the same row are significantly different at $p<0.01$.

Although common commodities of plant origin have a higher total phytosterol content, some commodities are comparable in their phytosterols content to edible insects (*e.g.* corn oil 590 mg/kg, palm oil 376-627 mg/kg) [Velíšek, 2002]. For this reason, edible insects may be found comparable with commodities of plant origin. The content of stigmasterol ranges from 0% to 20% of all sterols in vegetable oils, while that of β-sitosterol is up to 62% of all sterols in these commodities [Velíšek, 2002].

When comparing the amount of cholecalciferol, the values are comparable despite the difference in the total content of sterols – 190 μg/kg in mealworm and 199 μg/kg in superworm. Cholecalciferol content in the analysed samples is at least three to four times higher than that of other commodities of animal origin (except for fish), *e.g.* 3 μg/kg in meat, 10-20 μg/kg in butter, and 30-50 μg/kg in eggs [Velíšek, 2002]. A comparable content of cholecalciferol with the analysed samples is stated by Velíšek [2002] for sea fish (50–450 μg/kg). Edible insects can therefore be a good source of cholecalciferol and consequently meet the recommended daily dose for this compound. In practice, this means consuming approximately 25 g of dried mealworm or superworm to cover the required dose of cholecalciferol.

From the nutritional point of view, the profile of lipids and the content of individual fatty acids is important, as pointed out by the WHO [Zielinská et al., 2015]. Table 4 shows the fatty acid profile of the two insect species, where the superworm has a higher saturated fatty acid content of the total fatty acid content of more than 13% than mealworm. This material is therefore more advantageous in terms of technological processing. However, dried material from mealworm, which has higher MUFA and PUFA contents, is more suitable to prevent civilization diseases.

TABLE 4. Fatty acid composition in mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) bred in the Czech Republic (% of total fatty acids).

Fatty acid	*Zophobas morio*	*Tenebrio molitor*
C12:0	0.1±0.00	0.2±0.03
C14:0	1.3±0.04	3.5±0.03
C16:0	32.3±0.01	18.6±0.08
C17:0	0.9±0.04	0.3±0.02
C18:0	8.2±0.10	6.7±0.46
C20:0	0.3±0.02	0.3±0.08
SFA sum	43.0	29.7
C16:1 (*cis*-9)	0.6±0.05	1.4±0.07
C18:1 (*cis*-9)	32.4±0.01	36.9±1.53
MUFA sum	33.0	38.3
C18:2 (*cis*-9.12)	23.4±0.15	30.9±1.08
C18:3 (*cis*-9.12.15)	0.6±0.01	1.1±0.03
PUFA sum	24.0	32.0
n-3 sum	0.6	1.1
n-6 sum	23.4	30.9

SFA – saturated fatty acids, MUFA – monousaturated fatty acids, PUFA – polyunsaturated fatty acids

Differences in the fatty acid profile against other authors in the mealworm are, for example, in the content of oleic acid, which was determined in this work by almost 7% lower than in Ravzanaadii *et al*. [2012] and more than 13% than that found by Tzompa-Sosa *et al*. [2014]. These authors reported also a lower percentage of linoleic acid – up to 8% than in our study. In superworm, there are differences in the fatty acid profile, for example, in oleic acid content, where the difference is up to 7% and in linoleic acid content – up to 4% against the value reported by Finke [2002]. The sample was similar in values to that of Barroso *et al*. [2014]. Differences between the above nutritional values in this work and the other sources may be due to different breeding conditions.

The cholesterol-saturated acid index was 36.6 for mealworm and 59.5 for superworm. The CSI values for mealworm are comparable to the CSI value of goose and duck fat, and for the superworm these values correspond to the bovine tallow [Pánek *et al*., 2002]. Ramos-Bueno *et al.* [2016] analysed fatty acid profile and cholesterol content in seven insect species, and calculated CSI from these data, which reached 27.5 for mealworm and 46.3 for superworm. These values are lower than the values calculated in the samples analysed in our study. This may be due to different feeds administered.

The AI, TI, and *n*-3:*n*-6 ratio are determined in fats to evaluate their health effects. The atherogenic index was lower in mealworm than in superworm (Table 5). A similar trend was also found in the thrombogenic index, which is almost twice as low in mealworm.

The AI, TI and *n*-3:*n*-6 ratio were calculated from fatty acid profiles of other authors to enable comparison (Table 5),

TABLE 5. Atherogenicity index (AI), thrombogenicity index (TI) and *n*-3:*n*-6 ratio in mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*) bred in the Czech Republic with values calculated from the fatty acid profile measured by other authors (% of total fatty acids).

Stage	Origin	*n*-3:*n*-6	AI	TI	References
Zophobas morio					
Larvae	Czech Republic	0.027	0.7	1.4	This study
Larvae	Spain	0.045	0.7	1.4	Ramos-Bueno *et al.* [2016]
Larvae	USA	0.033	0.6	1.3	Finke [2002]
Larvae	Spain	0.062	0.6	1.2	Barroso *et al.* [2014]
Tenebrio molitor					
Larvae	Czech Republic	0.036	0.5	0.8	This study
Larvae	Spain	0.05	0.4	0.5	Ramos-Bueno *et al.* [2016]
Larvae	USA	0.04	0.4	0.6	Finke [2002]
Adult	USA	0.029	0.4	0.7	Finke [2002]
Larvae	Republic of Korea	0.045	0.4	0.5	Ravzanaadii *et al*. [2012]
Adult	Republic of Korea	0.023	0.4	0.7	Ravzanaadii *et al*. [2012]
Larvae	Spain	0.036	0.3	0.5	Sánches-Muros *et al*. [2016]
Larvae	Spain	0.036	0.3	0.5	Barroso *et al*. [2014]
Not specified	Netherlands	0.038	0.4	0.6	Tzompa-Sosa *et al*. [2014]
Larvae	Poland	0.054	0.4	0.6	Zielińska *et al*. [2015]

as the indices themselves have not yet been presented in the available literature. The AI and TI determined by other authors were lower than in our work. AI in our research was 0.7 for mealworm, while it was 0.6 acc. to both Finke [2002] and Barroso *et al.* [2014]. For superworm, the calculated AI ranged between 0.3 and 0.4 [Finke, 2002; Ravzanaadii *et al.,* 2012; Sánchez-Muros *et al.,* 2016; Tzompa-Sosa *et al*., 2014; Zielińska *et al.,* 2015]. Our results were slightly higher (0.5). The situation was similar for TI. It calculated values ranged from 1.2 to 1.3 for mealworm and from 0.5 to 0.7 for superworm [Finke, 2002; Barroso *et al.,* 2014; Ravzanaadii *et al.,* 2012; Sánchez-Muros *et al.,* 2016; Tzompa-Sosa *et al.,* 2014; Zielińska *et al.,* 2015]. In our work, TI was again slightly higher for both analysed species.

Edible insect fat can be compared to the fat of beef or pork or to vegetable margarine [Stajić *et al*., 2011]. The thrombogenic index for superworm (1.4) can be compared with these fats again, but the thrombogenic index of mealworm is similar to that of chicken meat fat. The calculated atherogenic index of the profiles reported by other authors can be compared with polyunsaturated acids of margarines, its value is similar to that of olive oil. Taking into account the risk of cardiovascular disease, the consumption of the mealworm is more favourable.

Another important factor describing the risk of the metabolic syndrome arising from fat consumption is the evaluation of the ratio of *n*-3 and *n*-6 fatty acids, which, according to WHO recommendations should be 1:2 to 1:6 [Mourek & Mourek, 2011; Jirák & Zeman, 2007]. The real *n*-3:*n*-6 ratio is normally 1:15 in the diet of the Western civilization, as reported by Simopoulos [2002]. The ratio determined for our samples (Table 5) as well as for the values calculated by other authors is higher than this. Although this ratio is not too favorable for the consumption of edible insect fats, the other parameters observed in this work balance this drawback.

The Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, *tran*s fatty acids, and cholesterol [EFSA, 2010] does not mention the recommendation for *n*-6:*n*-3 ratio. According to this material, linoleic acid intake should not fall below 4% and that of linolenic acid below 0.5% of total energy intake. An average man with light work has a total energy intake of 10,000 kJ and should receive 400 kJ of linoleic acid and 50 kJ of linolenic acid. Assuming an energy value of 37.6812 kJ/g, it is possible to calculate the amount which is necessary to be taken in the diet, *i.e*. 10.6 g of linoleic acid and 1.3 g linolenic acid. The amount of dry matter from mealworms required to cover the need for linoleic acid per day was calculated at 205 g and that need to cover for linolenic acid at 708 g. In the case of *Zophobas morio*, the respective amount of dry matter is 116 g for linoleic acid and 554 g for linolenic acid.

Detected contents of linoleic acid (5.16 g/100 g DM) and linolenic acid (0.18 g/100 g DM) for *Tenebrio molitor* were lower than these determined for *Zophobas morio* (linoleic acid content 9.15 g/100 g DM, linolenic acid content 0.23 g/100 g DM). In the case of dried meat, the content of linoleic acid is 1.02 g/100 g and that of linolenic acid is 0.39 g/100 g [Huis *et al*., 2013]. Therefore, to receive a daily dose of linoleic acid, up to 9 times less dry matter of superworm is needed in comparison to beef. On the other hand, for linoleic acid, less dry meat is needed than mealworm or superworm dry matter. Therefore, in order to minimise the weight of the dry matter as a nutritional dose (for example, in a person's special diet in places without access to a regular diet), a combination of both commodities can be recommended in the diet.

CONCLUSIONS

In this study, cholesterol, β-sitosterol, and stigmasterol were analysed in two edible insect species: mealworm (*Tenebrio molitor*) and superworm (*Zophobas morio*). When fed *ad libitum*, superworm had a higher content of sterols than mealworm. During the nutritional stress there was a statistically significant drop of the cholesterol content in both species. This suggests that cholesterol levels could be regulated in edible insects by proper nutrition. Although material of the animal origin is analysed, sterols of plant origin (β-sitosterol, stigmasterol) have been detected. For this reason, it is possible to assume that both species could serve as a source of cholesterol (zoosterol) and phytosterols simultaneously (two in one). Further analysis proved a significant content of cholecalciferol, which was the same for both species, although the fat content differed. Another benefit is the lower weight of dry matter necessary to consume to cover the daily dose of linoleic acid compared to dried beef. The linoleic acid content of mealworm (30.9 g/100 g) or superworm (23.4 g/100 g) is comparable with, for example, chicken lard, pork lard or goose lard.

Based on the legalization of edible insects as a novel food in Europe since 2018, it is not a problem to include this commodity in the diet both in the hidden form (dry matter for food fortification) and in the visible form as an experience food.

RESEARCH FUNDING

This research was supported by the internal grant of TBU in Zlín [No. IGA/FT/2019/004] and project BUT in Brno [No. FEKT S-17–3934].

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Submitted: Submitted: 19 December 2018. Revised: 27 February and 5 May 2019. Accepted: 27 May 2019. Published on-line: 3 July 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 255–265
DOI: 10.31883/pjfns/110134
http://journal.pan.olsztyn.pl

Original research article
Section: Food Quality and Functionality

Cytoprotective Effect of *Morchella esculenta* Protein Hydrolysate and Its Derivative Against H_2O_2-Induced Oxidative Stress

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Key words: *Morchella esculenta*, protein hydrolysate, Maillard reaction, oxidative stress, cytoprotective effect

Morchella protein hydrolysate (MPH) and its glycosylated derivative (G-MPH) may possess the potential as natural antioxidants. However, knowledge about the protective effects of MPH and G-MPH on cellular oxidative damage is limited. This study evaluated whether MPH and G-MPH protected Caco-2 cells from H_2O_2-induced oxidative injury and explored the potential mechanisms of protection. The results showed that, under H_2O_2 stress, both MPH and G-MPH significantly increased cell viability, suppressed intracellular ROS and MDA production, increased cellular antioxidant capacity, and activated Nrf2 signaling pathway. More importantly, MPH and G-MPH significantly inhibited the H_2O_2-induced apoptosis *via* restoring the loss of mitochondrial membrane potential and regulating the protein expressions of Bax, Bcl-2, and caspase-3. These data indicate that MPH and G-MPH can protect Caco-2 cells against oxidative injury by improving cellular antioxidant responses and inhibiting apoptosis. Therefore, MPH and G-MPH can have a broad application potential as promising ingredients of nutraceutical products or functional foods.

INTRODUCTION

It is well known that the production and elimination of reactive oxygen species (ROS) are in a delicate equilibrium in normal cell. Oxidative stress appears when ROS is generated beyond the scavenging capacity of the antioxidant defense system [Zhang *et al*., 2018d; Zhang *et al*., 2016]. Oxidative stress caused by excessive ROS production may disrupt the redox homeostasis, induce autophagy, trigger apoptosis, and cause irreversible tissue injury [Shen *et al*., 2017]. It has been confirmed that the damages caused by oxidative stress can lead to many chronic diseases such as cancer, diabetes, cardiovascular diseases, and neurodegenerative diseases [Wang *et al*., 2015; Zhang *et al*., 2016]. Therefore, how to protect the cell against oxidative stress-induced injury and enhance the cellular and tissue defenses against ROS require more attention.

Dietary intake of natural antioxidants has been demonstrated as a feasible way to eliminate the harmful effects of ROS and restore the body's antioxidant load [Seifried *et al*., 2007]. Hence, there is an increasing interest on natural antioxidants to prevent chronic diseases caused by oxidative stress. Food-derived protein hydrolysate, produced by the enzymatic hydrolysis of natural food proteins, is one of the numerous natural antioxidants. Due to a high antioxidant activity, satisfactory safety, and bioavailability, food-derived protein hydrolysates have received significant scientific attention in the food industry and healthcare field [Jin *et al*., 2013; Morifuji *et al*., 2010]. Protein hydrolysates from soybean [Zhang *et al*., 2018d], common carp [Zamora-Sillero *et al*., 2018], wheat germ [Zhou *et al*., 2016], and rice dreg [Zhang *et al*., 2016], exhibited potential *in vitro* antioxidant activity in chemical models or cell models. Additionally, our previous studies demonstrated that the protein hydrolysate (MPH) from *Morchella esculenta* (L.), an edible and medicinal fungus of high economic value, exhibited various antioxidant properties such as excellent reducing power, efficient free radical scavenging activity, and considerable H_2O_2 scavenging activity. Moreover, the glycosylated derivative of MPH (G-MPH) produced by conjugating with xylose *via* Maillard reaction exhibited higher antioxidant properties than MPH [Zhang *et al*., 2018b]. However, to the best of our knowledge, the cytoprotective effect of MPH and G-MPH against oxidative stress and its underlying mechanisms have never been reported.

It has been widely acknowledged that the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is a key mediator in oxidative stress [Li *et al*., 2017]. Under normal conditions, Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm to form a complex. To counteract oxidative stress, Nrf2 is released from Keap1 before being translocated into the nucleus, binds to the antioxidant-response elements (AREs), and promotes the expression of downstream genes including haeme oxygenase-1 (HO-1),

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NAD(P)H:quinone oxidoreductase 1 (NQO1), and some antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [Pyo *et al*., 2016; Xia *et al*., 2017]. These enzymes play a role in cell detoxification and maintenance of antioxidant capacity which in turn help regulate the redox balance [Xia *et al*., 2017]. It has been reported that Maillard reaction products of fish protein hydrolysate and ribose activate Nrf2 to protect HepG2 cells against oxidative stress [Yang *et al*., 2017]. Therefore, it is hypothesized that MPH and G-MPH may attenuate the H_2O_2-induced oxidative injury, potentially *via* the activation of Nrf2 signaling pathway.

Apoptosis (programmed cell death) plays an important role in regulating numerous physiological processes such as growth, development, and homeostasis maintenance [Mańdziuk *et al*., 2003]. Imbalance between cell proliferation and apoptosis can cause pathological phenomena such as cancer and Alzheimer's disease [Yoon *et al*., 2001]. Many reports have demonstrated that various natural antioxidants protect cells from oxidative damage by suppressing apoptosis [Chen *et al*., 2017; Zhou *et al*., 2016]. However, whether the underlying mechanisms by which MPH and G-MPH exert cytoprotective effects against oxidative damage is *via* apoptosis inhibition or not remains unclear.

The aim of this study was thus to evaluate the cytoprotective effects of MPH and G-MPH against oxidative stress using Caco-2 cells, which is an ideal tool to assess the cellular antioxidant response to food-derived antioxidants [Ruiz-Roca *et al*., 2011]. Moreover, we investigated whether MPH and G-MPH exert cytoprotective effects by activating Nrf2 signaling pathway and by inhibiting apoptosis in an attempt to explain its underlying molecular mechanisms.

MATERIALS AND METHODS

Reagents

The cell culture reagents were obtained from Gibco BRL Life Technologies (USA). Malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and catalase (CAT) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Kits for ROS assay, Annexin V-FITC detection, TUNEL assay, JC-1 assay, total protein extraction and quantification, and enhanced chemoluminescence (ECL) detection were purchased from Keygen Biotech Co., Ltd. (Nanjing, China). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents, unless otherwise stated, were purchased from Sigma-Aldrich.

Preparation of MPH and G-MPH

The strain of *M. esculenta* (ACCC 50537) used in this study was purchased from the Agricultural Culture Collection of China. Morchella protein was produced by alkaline extraction followed by acidic precipitation from Morchella mycelium obtained by liquid fermentation according to our previously described method [Zhang *et al*., 2018c]. Briefly, the Morchella mycelia were homogenized and the proteins were extracted with NaOH solution (pH 12.0) by incubating in a water bath at 45°C for 1 h. The solution was centrifuged at 4,000×*g* for 20 min, the supernatant was adjusted to pH 4.1 with 2 mol/L HCl, and further centrifuged at 4,000×*g* for 20 min. After removing the supernatant, the precipitate was lyophilized to obtain the Morchella protein.

MPH and G-MPH were prepared based on our previous report [Zhang *et al*., 2018b]. Briefly, the Morchella protein was hydrolyzed using papain at an enzyme/protein ratio of 2% in a shaking water bath at 45°C for 3 h, at pH 6.0. Subsequently, the enzyme was inactivated in a boiling water bath for 10 min, followed by centrifugation at 4,000×*g* for 15 min. The supernatant was lyophilized to obtain MPH. G-MPH was produced by the Maillard reaction. MPH was mixed with xylose at a mass ratio of 1:3.7 in distilled water, adjusted to pH 11.8, and incubated in a boiling water bath for 60 min. The resulting solution was dialyzed (molecular weight cut-off, 200 Da) against distilled water for 48 h to remove unreacted xylose and the retentate was freeze-dried to obtain G-MPH. The MPH and G-MPH have been preliminarily characterized by infrared spectroscopy, fluorescence spectroscopy, and scanning electron microscopy in our previous study [Zhang *et al*., 2018a].

Cell culture and treatment

Caco-2 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Caco-2 cells were cultured in DMEM medium containing 10% FBS and 1% streptomycin/penicillin at 37°C in a humidified 5% CO_2 incubator. To explore the protective effects of MPH and G-MPH against H_2O_2-induced oxidative stress, cells were grouped as follows: control group (Caco-2 cells without any treatment), model group (Caco-2 cells treated with 300 μM H_2O_2 for 6 h), MPH group (Caco-2 cells pretreated with 250 μg/mL MPH for 1 h followed by incubation with 300 μM H_2O_2 for 6 h), G-MPH group (Caco-2 cells pretreated with 250 μg/mL G-MPH for 1 h followed by incubation with 300 μM H_2O_2 for 6 h), and Vc group (Caco-2 cells pretreated with 250 μg/mL Vc for 1 h followed by incubation with 300 μM H_2O_2 for 6 h).

Cell viability assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, Caco-2 cells (1×10^5/mL) were seeded in 96-well plates (100 μL culture media/well), the cultures were maintained at 37°C for 24 h in a humidified 5% CO_2 incubator, and treated with specified concentrations of ligands for designated time. After the treatment, 20 μL of MTT (5 mg/mL) solution was added to each well and incubated for 3 h at 37°C. Thereafter, the supernatant was discarded from the wells, washed with phosphate-buffered saline (PBS; 1×, pH 7.4), and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were placed on a shaker for 10 min and the absorbance was measured at 490 nm with an ELx800 microplate reader (Bio-Tek, USA).

Measurement of intracellular ROS

Intracellular ROS levels were detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA), a cell permeable non-fluorescent probe. After experimental treatment, the cells were

washed once with PBS, harvested, incubated with 10 μM DCFH-DA (diluted with serum-free culture media) at 37°C for 20 min, and washed three times with a serum-free cell culture solution. The fluorescence was measured by flow cytometry at λex=488 nm and λem=530 nm.

Determination of MDA and GSH contents, T-AOC, and SOD and CAT activities

After the experimental treatment, cells were washed once with PBS, harvested, lysed in 1000 μL of cold PBS by sonication, and centrifuged at 13,000×*g* for 10 min at 4°C. The resulting supernatant was used for subsequent measurements. Total protein was quantified by a BCA protein assay kit. MDA and GSH contents, T-AOC, and SOD and CAT activities were detected using the commercial kits according to manufacturer's instructions. Each experiment was repeated 3 times. MDA and GSH are expressed as μmol/g protein and T-AOC, SOD, and CAT as U/mg protein.

Immunofluorescence staining

After the experimental treatment, the culture media were carefully discarded; the cells were washed three times with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were then washed with PBS (3 washes of 10 min each) and endogenous peroxidases were blocked with 3% H_2O_2-methanol solution for 10 min. Cells were then washed with PBS (3 washes of 10 min each), blocked with 10% goat serum for 20 min, followed by incubating with primary anti-Nrf2 antibody (1:100) at 37°C for 2 h. Cells were then washed with PBS (3 washes of 10 min each) and incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:200 dilution) at 37°C for 1 h. After washing 3 times with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 5 min in the dark. Stained cells were washed with PBS (3 washes of 10 min each) and blotted to remove any residual liquid. Cells were mounted onto glass slides with antifade mounting medium and were analyzed using an inverted fluorescence microscope (Olympus IX51, Japan).

TUNEL assay

Apoptotic cells were detected using TUNEL assay kit following the manufacturer's protocol. Briefly, after the experimental treatment, the cells were fixed in 4% paraformaldehyde for 30 min and washed three times with PBS. The cells were permeabilized with 1% Triton X-100 for 15 min at room temperature and rinsed three times with PBS. The cells were then incubated with 100 μL of TUNEL reaction mixture at 37°C for 60 min, rinsed three times with PBS, and stained with 100 μL of DAPI solution at 37°C in the dark for 5 min. The apoptotic cells were observed under an inverted fluorescence microscope (Olympus IX51, Japan).

Annexin V-FITC/PI double staining assay

Apoptotic cells were quantified using the Annexin V-FITC/PI apoptosis detection kit. After the experimental treatment, cells were harvested by trypsinization and washed twice with PBS. Then, the cells were collected by centrifugation at 1000×*g* for 5 min and approximately 5×10^5 cells were resuspended in 500 μL of binding buffer. They were then incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) at room temperature for 15 min in the dark prior to flow cytometry (Becton Dickinson FACSCalibur).

Measurement of mitochondrial transmembrane potential (MMP)

JC-1 assay kit was used to detect MMP according to manufacturer's instructions. Briefly, Caco-2 cells from different treatments were rinsed with 1× incubation buffer and incubated with 0.5 mL of JC-1 working solution at 37°C for 20 min in the dark. After rinsing twice with 1× incubation buffer, the cells were resuspended in 0.5 mL of 1× incubation buffer and the cell fluorescence was detected with a flow cytometer (Becton Dickinson FACSCalibur) (λex=488 nm, λem=530 nm). Data analysis was performed using CellQuest software (Becton-Dickinson).

Western blot analysis

Total proteins from Caco-2 cells were extracted with a total protein extraction kit and quantified using the BCA protein assay kit. After separation by SDS-PAGE (12%), proteins were transferred onto nitrocellulose (NC) membrane at 300 mA for 90 min, saturated with a blocking solution containing 5% non-fat milk for 1.5 h, and incubated with appropriate primary antibodies overnight at 4°C. The membranes were washed three times (10 min each) with TBST (Tris-buffered saline and Tween) and incubated with secondary antibody for 1.5 h at room temperature. The protein bands were visualized on a gel imaging system (Syngene G: BOXChemiXR5, Cambridge, UK) using an ECL kit. The relative protein expression levels were quantitated by the Gel-Pro32 software (MediaCybernetics Inc., USA) and normalized with GAPDH.

Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from the treated Caco-2 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA concentration was measured and quality was detected by measuring the absorbance at 260 and 280 nm using a UV-vis spectrophotometer (Shimadzu UV-2450). cDNA was synthesized in a 20-μL reaction volume from 2 μg of total RNA using the Reverse Transcription kit (K1622; Thermo Fisher Scientific). The qPCR reactions were performed with SYBR® Premix Ex TaqTM II (TaKaRa, Dalian, China) on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primer sequences were as follows: Nrf2, F: 5'-CAACTACTCCCAGGTTGCCC-3', R: 5'-AGTGACTGAAACGTAGCCGAA-3'; NQO1, F: 5'-GCTGCCATGTATGACAAAGGAC-3', R: 5'-CATGTCCCCGTGGATCCCTT-3'; HO-1, F: 5'-TCTTGGCTGGCTTCCTTACC-3', R: 5'-GGATGTGCTTTTCGTTGGGG-3'; GAPDH, F:5'-TGTTGCCATCAATGACCCCTT-3',andR:5'-CTCCACGACGTACTCAGCG-3'. The gene expression data were normalized to the housekeeping gene (GAPDH) and expressed as $2^{-\Delta\Delta CT}$ values.

Statistical analysis

Data are represented as mean ± SD of triplicate experiments. Data were analyzed by one-way ANOVA using

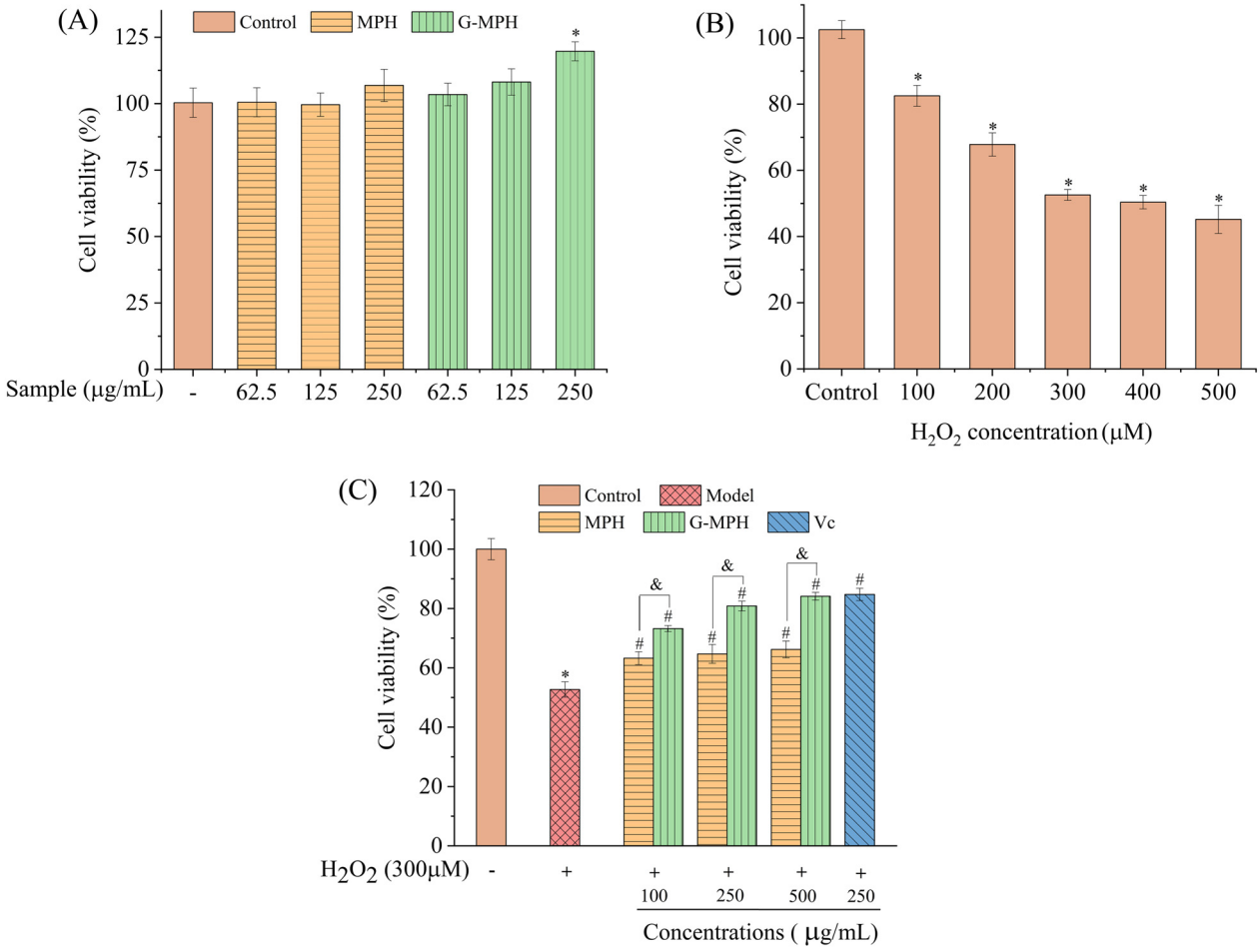

FIGURE 1. Protective effects of MPH and G-MPH against H_2O_2-induced cytotoxicity in Caco-2 cells. (A) Cells treated with various concentrations of MPH or G-MPH for 7 h. (B) Cells treated with different concentrations of H_2O_2 for 6 h. (C) Cells incubated with various concentrations of MPH or G-MPH for 1 h before exposure to 300 μM H_2O_2 for 6 h. Vc at a concentration of 250 μg/mL was used as a positive control. Cell viability was quantified by MTT assay. The data are presented as means ± SD, n = 3. $^{*}p<0.05$ *versus* control, $^{\#}p<0.05$ *versus* model, $^{\&}p<0.05$ *versus* MPH. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

SPSS 19.0. The differences between groups were performed by Duncan's multiple range test and considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

MPH and G-MPH protected Caco-2 cells from H_2O_2--induced injury

To examine the cytotoxic potential of MPH, G-MPH, and H_2O_2, cell viability of Caco-2 cells under different treatments was measured by MTT assay. MPH and G-MPH had no negative effects at the tested concentrations (62.5–250 μg/mL) (Figure 1A). However, H_2O_2 (100–500 μM) significantly ($p<0.05$) decreased cell viability after incubating for 6 h in a concentration-dependent manner (Figure 1B). At a concentration of 300 μM, H_2O_2 moderately decreased cell viability (52.58%). Therefore, the cells were treated with 300 μM H_2O_2 for 6 h in the following experiments.

Next, we evaluated the protective effects of MPH and G-MPH against H_2O_2-induced oxidative injury. As shown in Figure 1C, exposure of Caco-2 cells to 300 μM H_2O_2 for 6 h decreased cell viability by 47.31% compared to the control group, whereas pretreatment with MPH and G-MPH both significantly ameliorated the decrease in cell viability ($p<0.05$); G-MPH showed significantly more protective effect on cell proliferation than MPH ($p<0.05$). The protective effect of 500 μg/mL G-MPH was almost the same as that of 250 μg/mL Vc. This result implies that MPH and G-MPH can attenuate H_2O_2-induced cytotoxicity and exhibit significant protective effects against H_2O_2-induced oxidative injury. Furthermore, both MPH and G-MPH at 250 and 500 μg/mL concentrations caused similar protective effects. Therefore, 250 μg/mL was selected as the optimal concentration (MPH and G-MPH) in H_2O_2-treated Caco-2 cells for subsequent experiments. Similar study was conducted by Zha *et al.* [2015] who reported that neither the shrimp by-product protein hydrolysate nor its glycosylated derivative had any cytotoxic effect at 1000 μg/mL concentration, but protected human HepG2 cells against AAPH-induced oxidative injury.

MPH and G-MPH inhibited H_2O_2-induced ROS generation and lipid peroxidation in Caco-2 cells

Growing evidences support that oxidative damage plays a crucial role in the pathophysiology of various diseases [Fer-

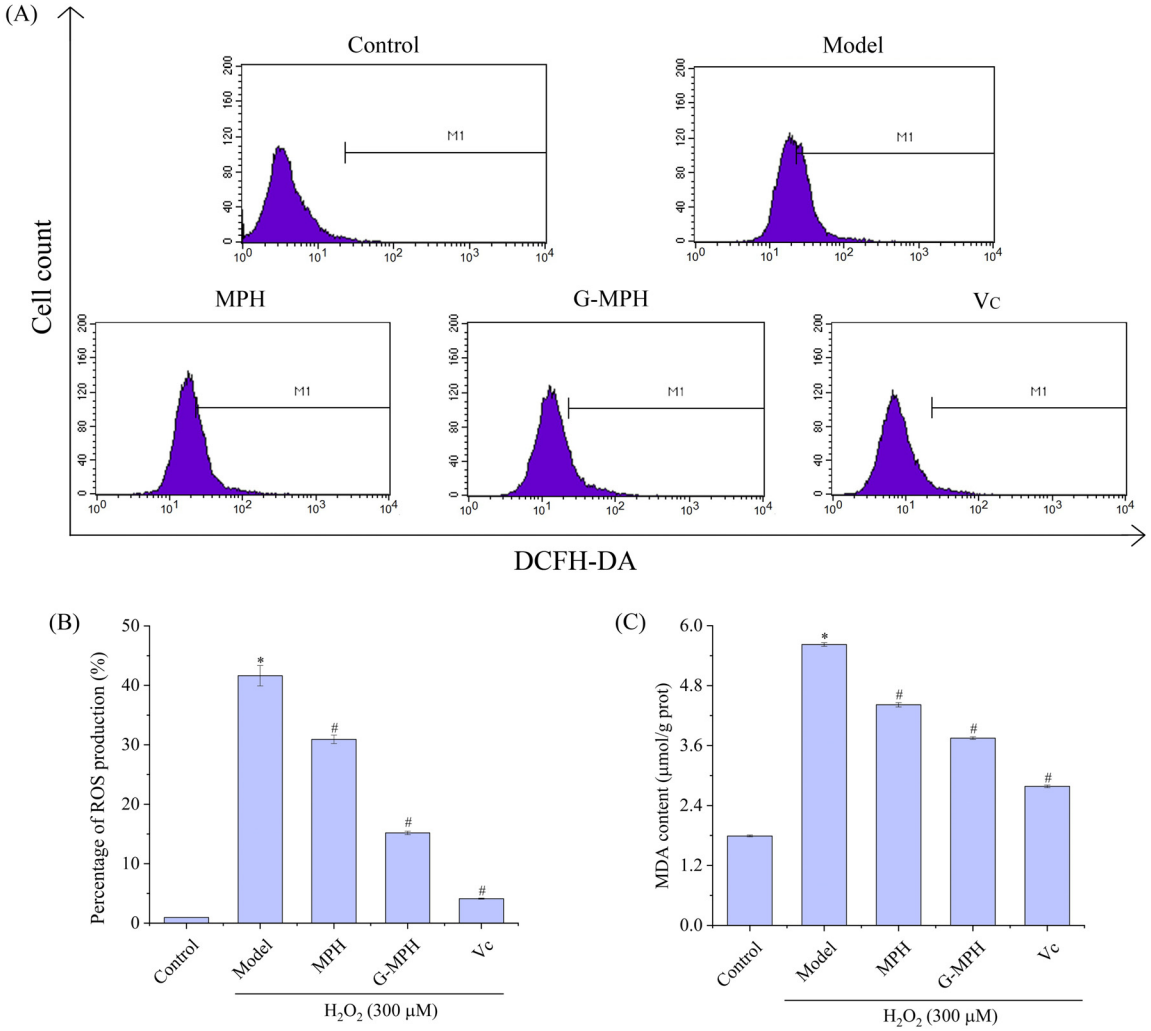

FIGURE 2. Effects of MPH and G-MPH on ROS generation and lipid peroxidation by H_2O_2 in Caco-2 cells. (A) and (B) ROS identified using DCFH-DA staining combined with flow cytometry and the corresponding data quantified. (C) Lipid peroxidation assessed by MDA assay. Data are means ± SD from 3 independent experiments. $^*p<0.05$ *versus* control, $^\#p<0.05$ *versus* model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

nandez-Checa *et al.*, 2010]. When the body is under oxidative stress, there is a shift in the redox balance between oxidants and antioxidants, resulting in an increase in the amount of ROS in the cells [Mariani *et al*., 2005]. Excessive ROS can cause oxidation of proteins and lipids in cells, destroying the integrity of nuclear DNA and mitochondria, and ultimately leading to cell death [Yamaguchi *et al*., 2015]. The levels of ROS in Caco-2 cells were detected by DCFH-DA probe combined with flow cytometry. As shown in Figure 2A to B, H_2O_2 treatment notably increased production of ROS in Caco-2 cells compared to control. However, pretreatment with MPH, G-MPH, or Vc significantly reduced H_2O_2-induced ROS generation and the ROS levels in Caco-2 cells reduced to 30.92±0.71%, 15.18±0.29%, and 4.12±0.09%, respectively, which were significantly lower than that in the model group ($p<0.05$).

Lipid peroxidation is one of the major events in free radical-induced cell oxidative damage. MDA, a major by-product of membrane lipid peroxidation, is considered as a biomarker of cell membrane injury. MDA can further amplify the effect of ROS causing a cascade of chain reactions, destroying biological macromolecules such as nucleic acids and proteins, and causing various diseases in the body [Je & Lee, 2015; Lee *et al*., 2004]. The effects of MPH and G-MPH on intracellular MDA levels are presented in Figure 2C. Along with ROS generation, MDA level in model group was also significantly more than that in the control group ($p<0.05$). Pretreatment with MPH, G-MPH, or Vc significantly attenuated H_2O_2-induced MDA production ($p<0.05$). Collectively, these results reveal that MPH and G-MPH may prevent the formation of ROS, inhibit lipid peroxidation, and accordingly protect the cells from H_2O_2-induced oxidative damage. These findings are similar to the earlier study which reported the antioxidative activities of rice dreg protein hydrolysate [Zhang *et al*., 2016]. The protective effect may be due to the wide range of antioxidant activities MPH and G-MPH possess and the roles they play as H_2O_2 and free radical scavengers, which have been confirmed in our previous studies [Zhang *et al*., 2018b].

MPH and G-MPH enhanced antioxidant defense capacity of H_2O_2-treated Caco-2 cells

There are both enzyme and non-enzyme antioxidant defense systems in the body. Excess ROS in the body is con-

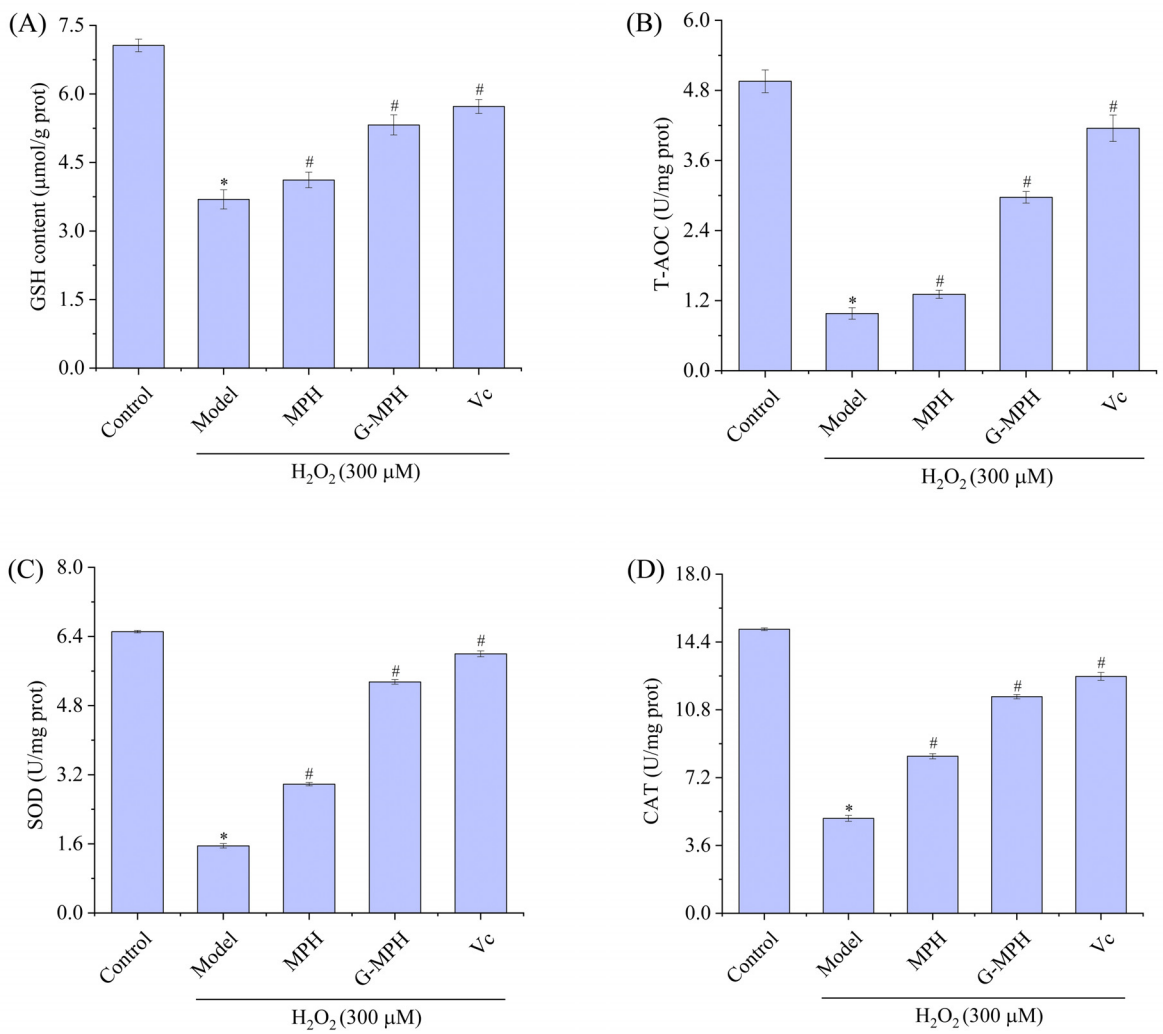

FIGURE 3. Effects of MPH and G-MPH on (A) glutathione (GSH) level, (B) total antioxidant capacity (T-AOC), (C) superoxide dismutase (SOD) activity, and (D) catalase (CAT) activity in H_2O_2-induced Caco-2 cells. All tested components were determined using the corresponding commercial assay kits. Data are means ± SD from 3 independent experiments. *$p<0.05$ *versus* control, #$p<0.05$ *versus* model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

sumed by this defense system, which in turn protect cells from damage and maintain a stable state [Xu *et al*., 2016]. GSH, T-AOC, and antioxidant enzymes such as SOD and CAT play an extremely important role in the antioxidant defense system of cells. Therefore, these components were evaluated to investigate whether the protective effects of MPH and G-MPH were associated with an improvement in antioxidant defense capacity of Caco-2 cells. After exposure to 300 μM H_2O_2 for 6 h, GSH level, T-AOC, and SOD and CAT activities were markedly decreased by 47.72%, 80.23%, 76.12%, and 66.57%, respectively, as compared to the control group ($p<0.05$) (Figure 3). As expected, compared with the model group, pretreatment of the cells with MPH and G-MPH significantly attenuated the decrease in the levels/activities of these components in H_2O_2-treated cells. Particularly, pretreatment of the cells with G-MPH reversed the H_2O_2--induced decrease in GSH levels by 1.44 folds (Figure 3A), T-AOC by 3.03 folds (Figure 3B), SOD activity by 3.44 folds (Figure 3C), and CAT activity by 2.28 folds (Figure 3D). However, the effects were not comparable to Vc. These data collectively indicate that MPH and G-MPH can exert their protective effects by improving the antioxidant defense capacity of Caco-2 cells. Results of the current study were similar to those of Shi *et al*. [2014], who found that eggshell membrane peptides protect Caco-2 cells from H_2O_2-induced oxidative damage by improving antioxidant enzyme activity and glutathione synthesis.

Protective effects of MPH and G-MPH against oxidative injury involving activation of Nrf2 signaling pathway

The Nrf2-antioxidant response element signaling pathway is a main endogenous antioxidant stress pathway that plays a key role in enhancing the antioxidant defense system of cells [Nguyen *et al*., 2009]. Nrf2, the main component of the signaling pathway, is normally located in the cytoplasm and combines with its cytosolic inhibitor, Keap-1. Various stimuli including oxidants, electrophiles, certain disease processes, and exogenous small (natural) molecules can activate Nrf2 through its disassociation from Keap-1. Activated Nrf2 translocates into the nucleus where it regulates the gene expression of phase II detoxifying and antioxidant enzymes to protect the organism from oxidative stress

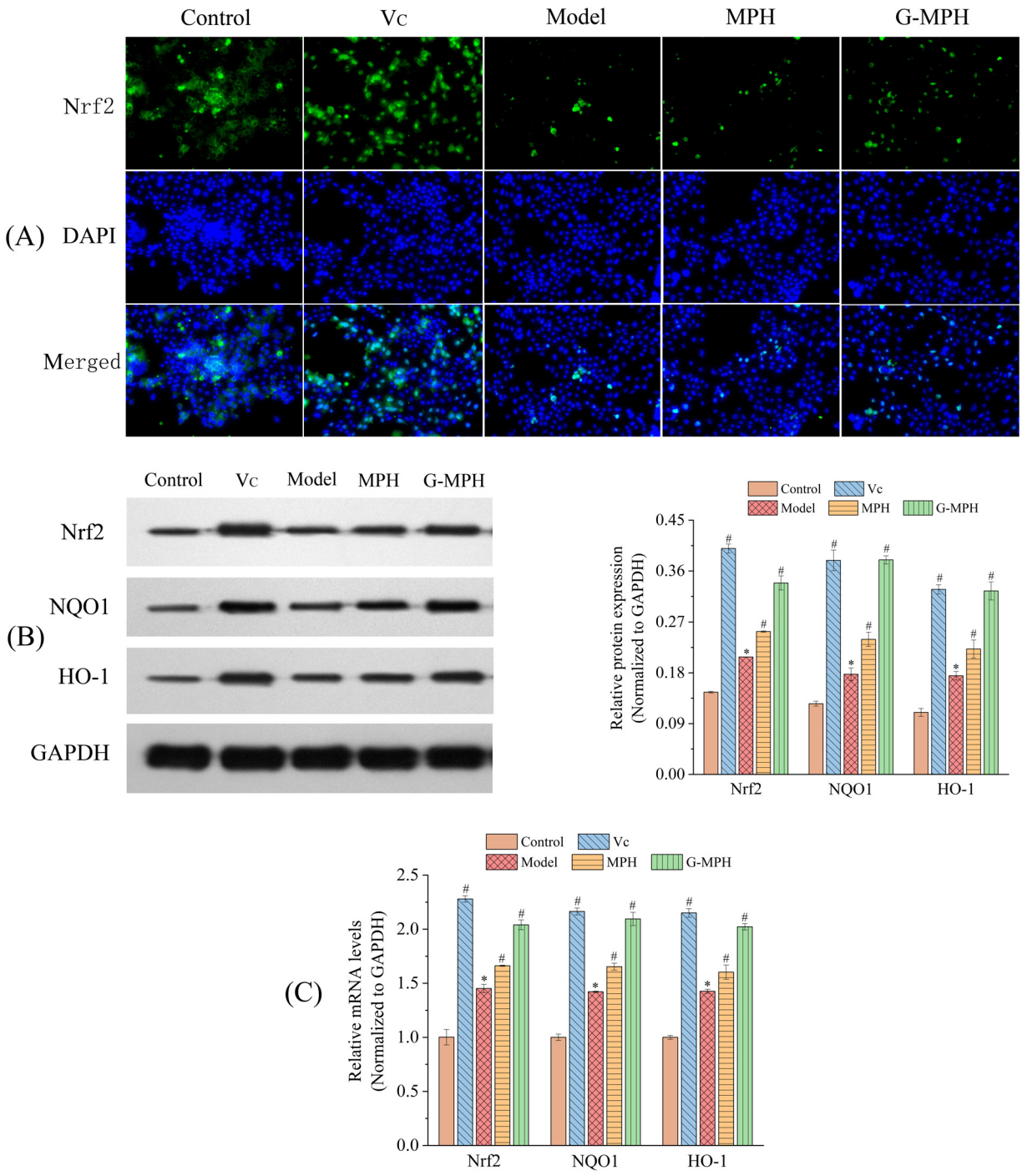

FIGURE 4. MPH and G-MPH activated Nrf2 signaling pathway in H_2O_2-treated Caco-2 cells. (A) Nrf2 nuclear translocation presented by immunofluorescence staining. Nrf2 was visualized using anti-Nrf2 antibody staining (shown in green) and nucleus was visualized using DAPI staining (shown in blue). Magnification: ×200. (B) Protein levels of Nrf2, NQO1, and HO-1 analyzed by Western blot. All data were normalized to the loading control GAPDH. (C) mRNA levels of Nrf2, NQO1, and HO-1 quantified by qPCR. The results were normalized with GAPDH. Data are means ± SD from 3 independent experiments. $^{*}p<0.05$ *versus* control, $^{\#}p<0.05$ *versus* model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

and related injuries [Chang *et al*., 2018; Zhang *et al*., 2018a]. Activation of Nrf2 is considered as a therapeutic target for neurodegenerative and cardiovascular diseases [Cuadrado *et al*., 2009; Li *et al*., 2009].

To further elucidate the mechanisms underlying the cytoprotective effects of MPH and G-MPH, the impact on Nrf2 signaling pathway was investigated. The Nrf2 nuclear translocation detected by immunofluorescence (Figure 4A) showed that Nrf2 was mainly present in the cytoplasm of normal cells. H_2O_2-induced oxidative stress promoted partial translocation of Nrf2 into the nucleus, while pretreatment with MPH, G-MPH, or Vc significantly promoted the nuclear translocation of Nrf2. This result was coincident with those reported by Yang *et al*. [2017], who demonstrated that glycosylated fish protein hydrolysates induced nuclear translocation of Nrf2 to activate the Nrf2 signaling pathway.

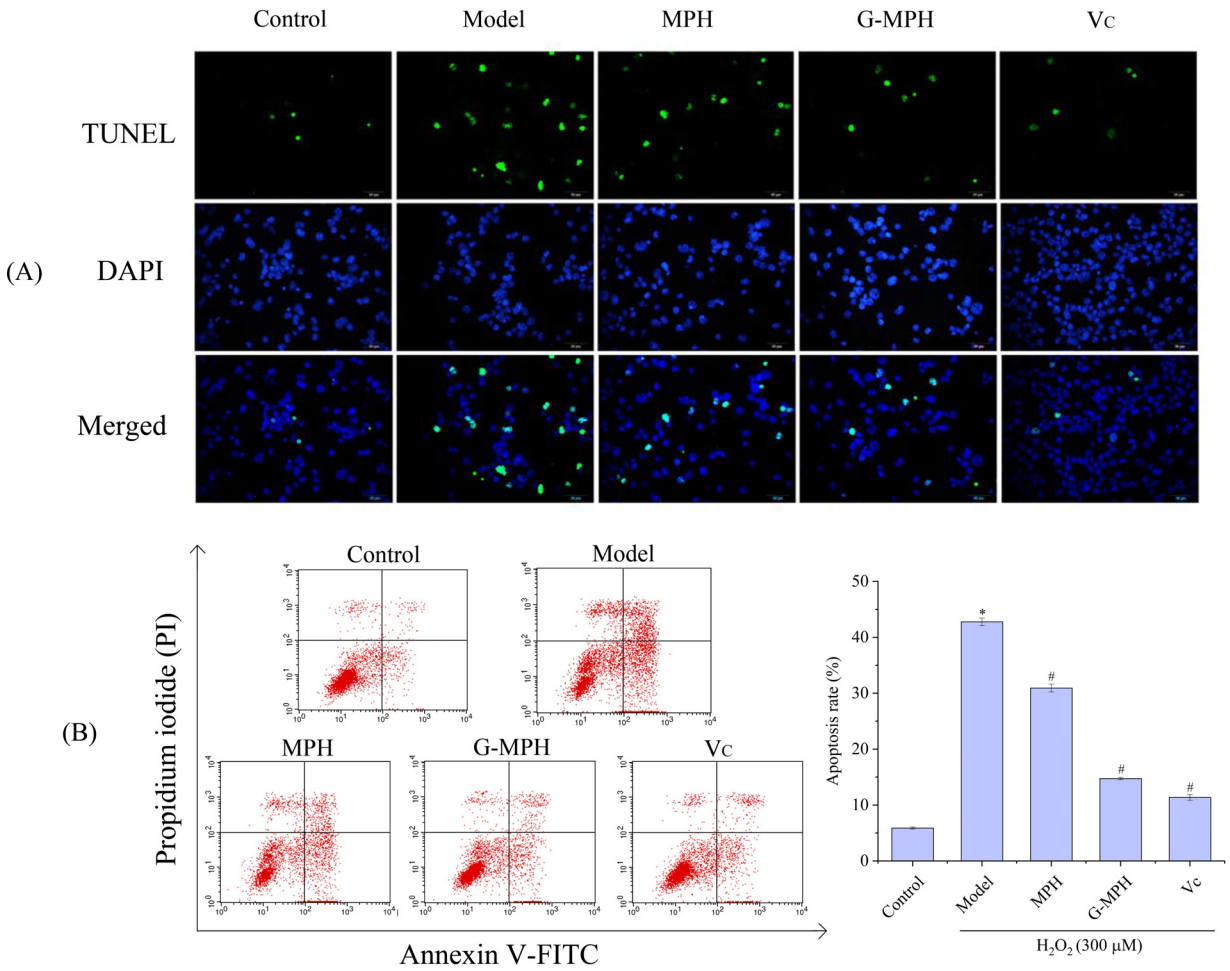

FIGURE 5. Effects of MPH and G-MPH on H_2O_2-induced apoptosis in Caco-2cells. (A) Cell apoptosis characterized by TUNEL assay (×200). TUNEL (green) represents apoptotic cells, DAPI (blue) represents living cells. (B) Cell apoptosis detected by flow cytometry following Annexin V-FITC/PI test. The upper and lower right quadrants of the dot plot indicate early apoptotic and late apoptotic cells, respectively. Apoptosis rate is the sum of early and late apoptosis values. The values are presented as means ± SD, n = 3. $^{*}p<0.05$ *versus* control, $^{\#}p<0.05$ *versus* model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

The effects of MPH and G-MPH on protein and mRNA expression of several components related to Nrf2 signaling pathway including Nrf2, NQO1, and HO-1 were evaluated by Western blotting and qPCR, respectively. As shown in Figure 4B to C, the expressions of Nrf2, NQO1, and HO-1 at both protein and mRNA levels were significantly higher in the model group than in the control group after H_2O_2-induced oxidative damage ($p<0.05$). This indicates that H_2O_2 activates Nrf2 antioxidant pathway while inducing oxidative stress. Compared with the model group, pretreatment with MPH, G-MPH, or Vc further increased the protein and mRNA expression levels of Nrf2, NQO1, and HO-1 in the H_2O_2-treated Caco-2 cells. These data suggest that MPH and G-MPH may activate the Nrf2 signaling pathway which might serve as a crucial mechanism for MAP and G-MAP to exert their protective effects on cell oxidative stress. Similarly, Pyo *et al*. [2016] reported that glycosylated whey protein concentrate activated the Nrf2-dependent pathway and induced expressions of antioxidant enzymes and phase II enzymes that had cytoprotective effects against oxidative injury in HepG2 cells.

MPH and G-MPH ameliorated H_2O_2-induced apoptosis *via* restoring MMP and regulating apoptosis-related protein expression in Caco-2 cells

Apoptosis is a mechanism which enables eukaryotic organisms to eliminate unwanted or defective cells through an orderly process of cell decomposition that is essential for the development of normal tissues [Li *et al*., 2016]. To maintain tissue homeostasis, a proper regulation of cell proliferation and apoptosis is critical [Pan *et al*. 2018]. To determine whether the protective effects of MPH and G-MPH against H_2O_2-induced injury were by counteracting apoptosis, the TUNEL staining and Annexin V/PI assay were performed to detect the apoptotic cells. TUNEL staining revealed that the number of TUNEL positive cells in the model group was notably increased in comparison with the control group. However, pretreatment with MPH, G-MPH, or Vc markedly decreased the number of TUNEL positive cells when compared with the model group (Figure 5A). At the same time, Annexin V/PI assay showed that the apoptosis rate was higher in the model group than that in the control group (42.78±0.68% *versus* 5.88±0.19%). Moreover, when compared with model group, pretreatment with MPH, G-MPH,

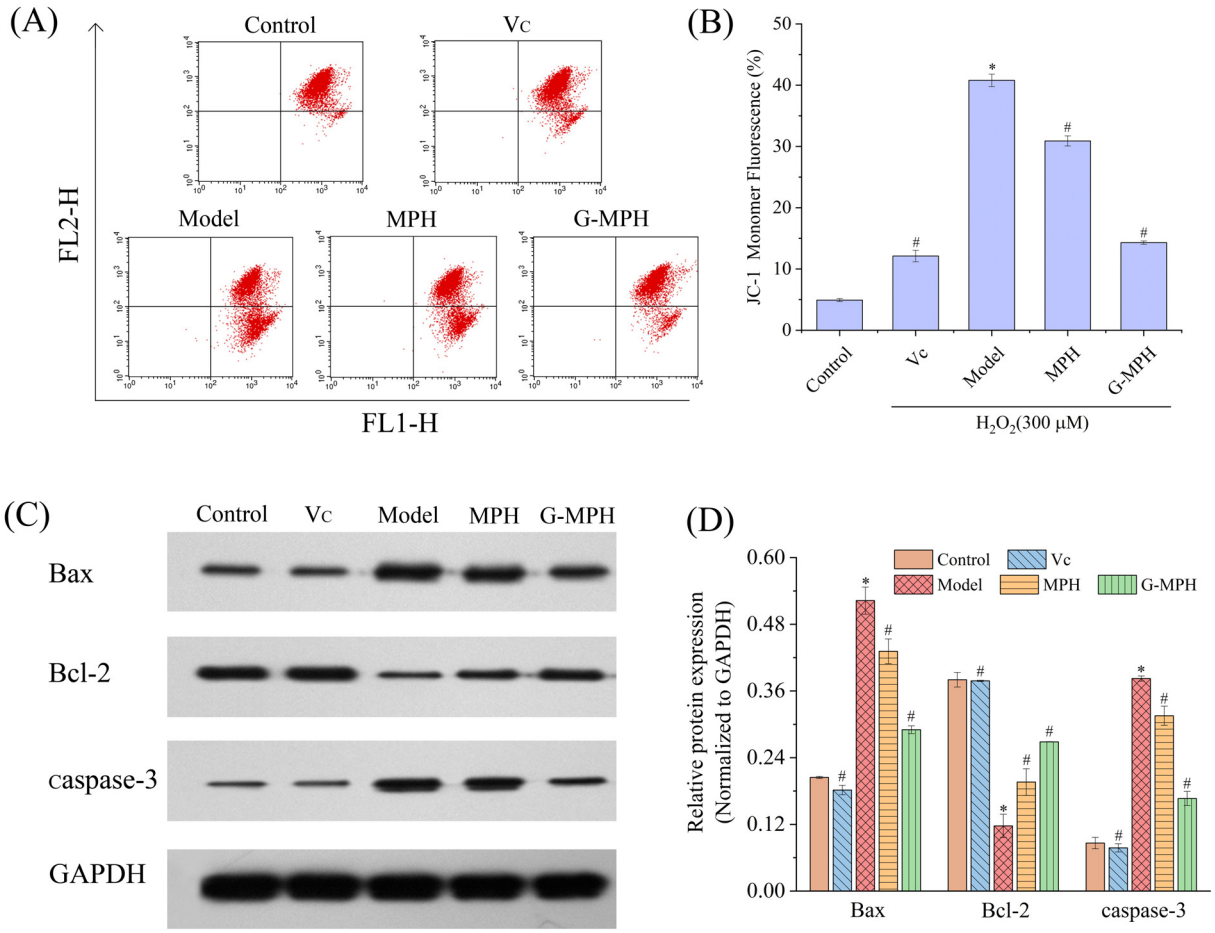

FIGURE 6. Effects of MPH and G-MPH on mitochondrial membrane potential (MMP) and expression levels of apoptosis related proteins. (A) The loss of MMP detected using flow cytometry after JC-1 staining. (B) The percentage of positive cells quantified. The increased JC-1 monomer ratio represents the loss of MMP. (C) Protein expressions of Bax, Bcl-2, and caspase-3 in Caco-2 cells analyzed by Western blot. (D) Quantification of Bcl-2, Bax, and caspase-3 protein levels. The results were normalized with GAPDH. Data are means ± SD from 3 independent experiments. $^{*}p<0.05$ *versus* control, $^{\#}p<0.05$ *versus* model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.

or Vc significantly decreased the apoptosis rate to 30.92±0.71, 14.74±0.21, and 11.37±0.51%, respectively (Figure 5B). Apparently, these results suggest that MPH and G-MPH can exert potential protective effects against H_2O_2 injury through the inhibition of apoptosis.

Mitochondria play a crucial role in the process of cell apoptosis. The loss of MMP is regarded as an important signal for damage to mitochondrial structure. Therefore, we assessed whether MPH and G-MPH exerted anti-apoptotic effects by restoring MMP in Caco-2 cells using JC-1 assay. As shown in Figure 6A to B, a marked increase in the number of cells with low MMP was observed in the model group compared with the control group; however, the number of cells with low MMP was significantly decreased on pretreatment with MPH, G-MPH, or Vc (30.90±0.63% for MPH, 14.30±0.28% for G-MPH, and 12.11±0.95% for Vc) compared with the model group ($p<0.05$). This indicated that MPH and G-MPH can restore MMP loss triggered by H_2O_2, stabilize mitochondrial function, and accordingly attenuate H_2O_2-induced apoptosis.

Apoptosis is regulated by the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins [Hu *et al*., 2015]. Increased Bax/Bcl-2 ratio (*i.e*., increased expression of Bax and reduced expression of Bcl-2) is considered as a reliable indicator of apoptosis [Xue *et al*., 2014]. In addition, Caspase-3 is the primary executioner of apoptosis. Activation of caspase-3 triggers DNA fragmentation and chromatin condensation leading to an irreversible cascade of events progressing towards cell death [Liu *et al*., 2007; Neuzil *et al*., 2004]. To further understand the mechanisms *via* which MPH and G-MPH regulate cell apoptosis, the expression levels of apoptosis-related proteins including Bax, Bcl-2, and caspase-3 were investigated using Western blot analysis. Figure 6C shows the expression levels of proteins Bax, Bcl-2, and caspase-3 in each group presented by Western blots using GAPDH as an internal control. The corresponding quantitative results showed that the levels of Bax and caspase-3 were evidently increased, whereas the expression of Bcl-2 was significantly reduced in the model group after H_2O_2 treatment when compared with the control group ($p<0.05$). However, pretreatment with MPH, G-MPH, or Vc remarkably inhibited Bax and caspase-3 expression and elevated Bcl-2 expression in comparison with the model group ($p<0.05$) (Figure 6D). Thus, it suggests that the anti-apoptotic effects of MPH and G-MPH are associated with

the regulation of Bax, Bcl-2, and caspase-3 protein expressions. As well, Jiao *et al.* [2018] confirmed that the cocaine- and amphetamine-regulated transcript (CART) peptide can decrease the expression of Bax and caspase-3 and increase the expression of Bcl-2 to inhibit neuronal apoptosis while attenuating oxidative injury in rat hippocampal neurons.

CONCLUSIONS

In the present study, the cytoprotective effects of MPH and G-MPH against oxidative stress and their underlying mechanisms were assessed in Caco-2 cells. MPH and G-MPH attenuated H_2O_2-induced cytotoxicity in Caco-2 cells and exhibited protective effects by inhibiting the productions of ROS and MDA, enhancing antioxidant defense capacity, and activating Nrf2 signaling pathway under H_2O_2-induced oxidative stress. Moreover, MPH and G-MPH prevented H_2O_2-induced apoptosis by restoring the loss of MMP and regulating the expression of apoptosis-related proteins. Collectively, MPH and G-MPH can protect Caco-2 cells against H_2O_2-induced oxidative injury *via* elevating antioxidant response and inhibiting apoptosis. Therefore, the current research suggests that MPH and G-MPH can be excellent nutraceutical/functional food components or potential therapeutic agents to prevent or treat oxidative stress-induced diseases.

RESEARCH FUNDING

This work was supported by the grant from the Doctorate Fellowship Foundation of Nanjing Forestry University (2014), the Natural Science Foundation of Anhui Provincial Department of Education (Project No. KJ2017A515), the Postgraduate Research & Practice Innovation Program of Jiangsu Province (Project No. KYLX15_0916), the Natural Science Foundation of Jiangsu province (Project No. BK20150883), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Submitted: 17 April 2019. Revised: 12 June 2019. Accepted: 19 June 2019. Published on-line: 5 July 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 267–278
DOI: 10.31883/pjfns/109899
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Composition and Significance of Bacterial Microbiota and Volatile Organic Compounds of Swiss-Dutch-Type Cheese as Determined by PCR-DGGE and HS-GC

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Key words: Swiss-Dutch-type cheese, microbiota, VOCs, PCR-DGGE, HS-GC, seasonal variation

This study aimed to determine seasonal differences in the composition of bacterial microbiota and volatile organic compounds (VOCs) in Swiss-Dutch-type cheese (manufactured between 2012 and 2014). Bacterial diversity and VOCs (acetaldehyde; ketones: acetone, diacetyl, acetoin; alcohols: methanol, ethanol; esters: ethyl acetate, ethyl propionate, ethyl butyrate; fatty acids: acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid) were determined by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE), and headspace gas chromatography (HS-GC), respectively. Season influenced the composition of both bacterial microbiota and VOCs in cheese. Counts of starter bacteria (*Lactococcus*, *Leuconostoc* and *Propionibacterium* – 6.51–7.14, 3.6–3.96 and 2.88–4.72 log CFU/g, respectively) were higher in the first year of the study, likewise these of the non-starter *Lactobacillus* (4.12–5.69 log CFU/g). The total VOC content was substantially lower in the summer-autumn 2012 (0.73228–3.34111 mg/g) than in the other seasons (63.28810–131.27690 mg/g). Differences in bacterial microbiota and the VOC profiles were observed between cheeses manufactured in winter-spring and summer-autumn seasons. Winter- and spring-manufactured cheeses were also characterized by a lower number of bacterial species (average 8.7–10.5 species/sample) than the cheeses produced in the summer and in the autumn (average 10–13 species/sample). The results of the study indicate that the cheese-making process has to be continuously monitored to minimize differences across manufacturing seasons.

INTRODUCTION

Ripened cheeses are manufactured with the use of microorganisms, which results in the transformation of raw material (milk) into the final product (cheese). The processes that take place during cheese making and ripening are affected mainly by the composition and activity of microorganisms. Cheeses, including those that are manufactured industrially from pasteurized milk with starter cultures, are characterized by high microbial biodiversity because starter cultures (selected species of *Lactococcus*, *Lactobacillus,* and *Propionibacterium*) are always accompanied by other bacteria, including nonstarter lactic acid bacteria (NSLAB) such as *Lactobacillus plantarum* and *L. brevis*, fecal bacteria (coliforms, enterococci), spore-forming bacteria (*Bacillus*, *Clostridium*), and others [Johnson, 2017; Ogier *et al.*, 2004; Ricciardi *et al.*, 2015; Rehfeld *et al.*, 2017; Santiago-Lopez *et al.*, 2018]. All these microorganisms synthesize a wide range of metabolites, including volatile compounds (aldehydes, ketones, esters, fatty acids) that are responsible for the flavor, aroma and consistency of cheese, and contribute to the development of desirable final product attributes [Felicio *et al.*, 2016; Franciosi *et al.*, 2009].

However, cheese production is a highly complex process; therefore, interdisciplinary methods are required to analyze the relationships between microorganisms, their metabolic activity, and changes in the physicochemical properties of cheese. Molecular biology methods and instrumental analytical techniques can be used to study these processes in greater detail. For instance, polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE) is one of such methods which supports the evaluation of various microbial genera and species, their semi-quantitative composition, changes over time or the presence of viable but nonculturable (VBNC) cells and cells that are suppressed by starter bacteria [Ercolini *et al.*, 2001; Joux & Lebaron, 2000]. In turn, the metabolic activity of cheese microbiota can be determined by analyzing the content of metabolites, such as volatile organic compounds (VOCs), by using chromatographic techniques, *e.g.*, headspace gas chromatography (HS-GC) [Ayad *et al.*, 1999].

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Therefore, the aim of this study was to assess the diversity of bacterial microbiota by PCR-DGGE and to identify and quantify VOCs by HS-GC in Swiss-Dutch-type cheeses manufactured in different seasons. Seasonal differences in VOC profiles and the potential links between the identified microorganisms and the VOC profiles of the examined cheeses were determined as well.

MATERIALS AND METHODS

Cheese manufacture and sampling

Swiss-Dutch-type cheeses were manufactured and obtained from a dairy plant in the Region of Warmia and Mazury in the north-eastern Poland. The cheeses were produced from 10,000 L (each) of premium cow milk which was pasteurized (72.5°C for 15 s) and standardized (to 3.0% fat), inoculated with calcium chloride, a coloring agent, rennet (Chymax, Ch. Hansen, Cząstków Mazowiecki, Poland), and a deep-frozen cheese starter (CSK food enrichment, Toruń, Poland). The applied starter was composed of *Lactococcus lactis* spp. *lactis*, *Leuconostoc mesenteroides* spp. *cremoris* (SLAB), and *Propionibacterium freudenreichii* spp. *shermanii* (PAB). The inoculum levels were 0.7% (by volume) for SLAB and 0.007% (by volume) for PAB. Every stage of the production process was consistent with industrial standards and followed the Swiss-Dutch-type cheese technology. After brining, 5 kg cheese blocks were wrapped in FCC type (Fesco Pack, Malbork, Poland) heat shrink, oxygen barrier bags and stored (ripened) under controlled conditions at 12°C for 10 days and then at 21°C for 42 days at 85% relative humidity. Cheese samples were collected over a period of two years between June 2012 and July 2014, and two cheese pieces of 0.5 kg each were sampled at one "time-point" (month). Three samples of each "time-point" cheese were taken in accordance with ISO 707:2008 [IDF 50:2008]. The samples for testing were packed in sterile bags. Cheeses manufactured between July and September were regarded as summer samples, cheeses produced in October-December – as autumn samples, cheeses produced in January-March – as winter samples, and cheeses produced in April-June – as spring samples.

Chemical composition of cheeses

Cheeses were subjected to chemical composition analyses to determine their sodium chloride, moisture, and fat contents. Salt content was determined according to ISO 5943:2006 [IDF 88:2006], moisture content was determined by oven drying at 102°C [AOAC 2005, 926.08], and fat content was determined according to ISO 3433:2008 [IDF 222:2008].

Determination of the counts of selected bacterial groups by the culture-dependent method

The counts of bacteria of the *Lactobacillus, Lactococcus, Leuconostoc, Propionibacterium, Enterococcus, Staphylococcus, Clostridium,* and *Bacillus* genera and of coliforms were determined by the standard plate count method on the appropriate culture media which are presented in Table 1. All media, except Reinforced Clostridial Medium (RCM), were supplied by Merck (Warsaw, Poland). RCM was supplied by Oxoid (Poznań, Poland).

TABLE 1. Culture media and incubation conditions applied in the study.

Microorganism	Medium	Incubation conditions
Lactococcus	M17 agar according to Terzaghi & Sandine [1975]	30°C, 48 h
Leuconostoc	Sucrose agar: (sucrose – 50 g/L, yeast extract – 10 g/L, agar – 15 g/L; pH 7.2–7.4)	30°C, 72 h
Propionibacterium	Sodium lactate (SLA) agar [Drinan & Cogan, 1992]	30°C, 72–96 h, anaerobic
Lactobacillus	Rogosa agar	30°C, 48 h, anaerobic
Coliforms	Violet Red Bile Lactose (VRBL) agar	37°C, 24–48 h
Enterococcus	Stanetz-Bartley agar	37°C, 48 h
Staphylococcus	Rabbit plasma fibrinogen (RPF) agar	37°C, 48 h
Clostridium	Reinforced Clostridial Agar (RCM agar)	37°C, 48 h, anaerobic
Bacillus	Nutrient agar	30°C, 48 h

Anaerobic conditions were obtained with the use of Anaerocult C bags (Merck, Warszawa, Poland).

DNA isolation and polymerase chain reaction (PCR)

Bacterial DNA was isolated directly from cheese samples with the Genomic Mini AX FOOD Kit (A@A Biotechnology, Gdańsk, Poland) in accordance with the manufacturer's instructions. The isolated DNA was stored at a temperature of −80°C until further analysis. Amplification was carried out in the MJ Mini Gradient Thermal Cycler (Bio-Rad, Warszawa, Poland). The applied primers were U968-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGC

GAAGAACCTTAC-3') and L1401-r (5'-CGGTGTGTACAAGACCC-3') [Randazzo *et al.*, 2010] which amplify the V6-V8 region of the 16S rRNA coding gene. The Master mix (25 μL) consisted of 1×reaction PCR buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 50 μmol/L deoxyribonucleotides (dNTPs), 5 pmol/L of each primer), 1.25 U *Taq* polymerase (all reagents were supplied by Thermo Fisher Scientific, Warsaw, Poland) and 10−40 ng of the DNA template. The PCR profile was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of: denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 68°C for 40 s. Final extension was carried out at 68°C for 7 min [Randazzo *et al.*, 2010]. The presence of PCR products was analyzed by electrophoresis on 1% agarose gel in a 0.5×Tris-borate-EDTA buffer in the MultiSub Choice system (Clever Scientific Ltd., Rugby, UK).

Denaturing gradient gel electrophoresis (DGGE)

PCR products (~450 bp) were analyzed by denaturing gradient gel electrophoresis (DGGE) with urea and formamide (Sigma, Poznań, Poland) as denaturing agents. Electrophoresis was carried out in 8% polyacrylamide gel (acrylamide:bis-acrylamide, 37.5:1) (Sigma, Poznań, Poland) where the denaturing gradient was increased from 35% to 57.5%. Electrophoresis was conducted in a 0.5×Tris-ace-

tate-EDTA buffer (Sigma, Poznań, Poland) at 60°C and 85 V for 16 h [Randazzo *et al.*, 2010] in the DCode Universal Mutation System (Bio-Rad, Warszawa, Poland). Gels were stained in SybrGreen I (1:10,000) (Sigma, Poznań, Poland) solution for 15 min and documented in G-Box (Syngen, Wrocław, Poland).

Biological diversity of cheese microbiota

DGGE band patterns of the analyzed cheeses were compared with the previously developed markers [Nalepa & Markiewicz, 2017] composed of 24 reference strains: *Propionibacterium freudenreichii* ssp. *shermanii* DSM 4902, *P. thoenii* DSM 20276, *Lactococcus lactis* ssp. *lactis* DSM 4366, *Leuconostoc mesenteroides* DSM 20346, *Lactobacillus acidophilus* DSM 9126, *L. plantarum* ATCC 8014, *L. brevis* DSM 1267, *L. casei* ATCC 334, *L. delbrueckii* DSM 20080, *L. fermentum* DSM 200052, *L. helveticus* DSM 20075, *Escherichia coli* ATCC 8739, *Enterobacter aerogenes* ATCC 13048, *E. cloacae* ATCC 13047, *Citrobacter freundii* ATCC 8090, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6051, *Clostridium butyricum* ATCC 10702, *C. tyrobutyricum* ATCC 2637, *C. perfringens* ATCC 13124, *Listeria monocytogenes* ATCC BAA-751, *Streptococcus thermophilus* ATCC 19258, *Staphylococcus xylosus* ATCC 29971, and *S. aureus* ATCC 43300. Bacterial species were identified and given a score of 0 to 4 points based on band brightness. Electrophorograms were subjected to 1D analysis in the Doc-It LS Image Analysis Software (UVP Ltd., Cambridge, UK). The results were used to calculate the Shannon-Wiener diversity index with the use of the following formula:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$ [Sienkiewicz, 2010],

where p_i is the proportion of individuals found in species *i*.

Analysis of volatile organic compounds (VOCs) by headspace gas chromatography

Selected volatile compounds, including aldehydes (acetaldehyde), ketones (acetone, diacetyl, acetoin), alcohols (methanol, ethanol), esters (ethyl acetate, ethyl propionate, ethyl butyrate), and fatty acids C_2-C_7 (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid), were identified by headspace gas chromatography (HS-GC) in the Headspace Turbomatrix 40 autosampler (Perkin Elmer, Waltham, USA) and the Clarus 500 gas chromatography system (Perkin Elmer, Waltham, USA) with a flame ionization detector (FID). The content of metabolites was expressed in mg/g. The chromatograph was calibrated for quantitative identification of selected metabolites based on external standards. Calibration curves were generated for every compound within the relevant concentration range. Equilibrium between the sample and the headspace was achieved by heating 3 g of cheese to 70°C for 40 min in tightly closed 22 mL vials. The sample was pressurized for 1 min and injected into the column (split ratio of 2:1) within 0.08 min. Needle and transfer line temperature was 100°C and 120°C, respectively. Volatile compounds were separated in an HP-INNOWAX (Agilent Technologies, Palo Alto, USA) column (60 m × 1.00 μm×0.537 mm) with the following temperature gradient: 40°C (5 min) →ΔT 10°C/min →220°C (5 min.). Injector and FID temperature was 230°C. The carrier gases were: helium (5 mL/min), synthetic air (400 mL/min), and hydrogen (40 mL/min). Column pressure was 130 kPa.

Statistical analysis

The results of chemical composition and bacterial counts were verified for normal distribution and homogeneity of variance. The significance of differences between means was analyzed by Duncan's test ($p \leq 0.05$). The interactions between factors were determined by ANOVA for the completely randomized design. The results of PCR-DGGE profile analyses were expressed as arithmetic means for each month (24 timepoints). To determine whether the presence of the identified VOCs in Swiss-Dutch-type cheeses was correlated with season, principal component analysis (PCA) was performed with VOCs as quantitative variables and seasons as qualitative variables. Before, VOCs were subjected to hierarchical cluster analysis (HCA) [Granato *et al.*, 2018] for assessment of similarity between the identified VOCs. Pearson's correlation coefficients (r) were calculated to determine whether the VOCs were associated with the microorganisms detected in cheese samples ($p \leq 0.05$). Data were processed in the Statistica v. 12.5 software (StatSoft Polska, Kraków, Poland).

RESULTS AND DISCUSSION

Chemical composition and microbiota of Swiss-Dutch-type cheese

The average water content of all cheeses was 42.65 g/100 g. Water content was the highest (43.46 g/100 g) in cheeses produced in 2012 and the lowest (42.06 g/100 g) in cheeses produced in 2013. There were no significant ($p>0.05$) differences among cheeses manufactured in different seasons or years. The fat content of the cheeses produced between 2012 and 2014 was comparable ($p>0.05$) and ranged from 25.67 g/100 g to 28.17 g/100 g. The average content of sodium chloride in cheeses produced in 2012 was 1.58 g/100 g and did not differ significantly from that noted in the cheeses produced in 2013 (1.72 g/100 g) and 2014 (1.51 g/100 g) (Table 2).

The counts of the determined bacterial groups are presented in Table 3. Starter bacteria (*Lactococcus, Leuconostoc*, and *Propionobacterium*) were detected in cheese samples in all analyzed seasons. The most prevalent bacterial group was *Lactococcus*, and its counts ranged from 5.45 to 7.14 log CFU/g of cheese. *Leuconostoc* and *Propionibacterium* counts were lower at 1.20−3.98 log CFU/g and 0.67−4.72 log CFU/g, respectively. These bacteria were more abundant in the cheese samples collected in all seasons of the first year of the experiment (Table 3). Cheese samples from all seasons contained also bacteria of the genera *Lactobacillus* (4.12 to 5.69 log CFU/g) and *Bacillus* (3.30 to 4.18 log CFU/g). The counts of the remaining bacterial groups (coliforms, *Enterococcus, Staphylococcus, Clostridium*) were considerably more varied across seasons, ranging from <1 log CFU/g (*Enterococcus* and *Staphylococcus* in the winter of 2012, *Clostridium* in the spring of 2013, coliforms in the spring of 2014)

TABLE 2. Chemical composition (g/100 g) of Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Chemical composition	2012		2013				2014	
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Water	43.92±0.48	43.00±0.40	41.72±0.37	41.59±0.31	42.97±0.67	41.95±0.72	43.29±0.43	42.78±0.33
Fat	26.50±0.50	27.67±0.29	26.00±0.50	26.17±0.58	27.50±0.50	27.33±0.29	25.67±0.29	28.17±0.29
Sodium chlorine	1.69±0.01	1.47±0.07	1.48±0.03	1.48±0.04	1.62±0.03	1.49±0.05	1.50±0.03	1.52±0.05

The presented values are means±standard deviation for $n=3$ (3 time-points) in each season.

TABLE 3. Microbial counts (log CFU/g) in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Genera/group of microorganisms	2012		2013				2014	
	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring
Lactococcus	6.51 ± 0.39^{ab}	7.01 ± 0.13^{a}	7.14 ± 0.14^{a}	6.79 ± 0.70^{ab}	5.51 ± 0.55^{ab}	6.28 ± 0.34^{ab}	5.45 ± 0.29^{a}	6.31 ± 0.39^{ab}
Leuconostoc	3.62 ± 0.49^{ab}	3.60 ± 0.19^{ab}	3.98 ± 0.36^{bc}	3.96 ± 0.66^{abd}	1.58 ± 0.65^{cd}	3.02 ± 0.40^{abcd}	1.20 ± 0.64^{c}	3.26 ± 0.73^{acd}
Propionibacterium	2.88 ± 0.54^{ab}	2.75 ± 0.85^{ab}	4.72 ± 0.25^{c}	3.70 ± 0.23^{bc}	1.47 ± 0.54^{a}	2.08 ± 0.52^{ab}	0.67 ± 0.50^{a}	2.86 ± 0.52^{ab}
Lactobacillus	5.08 ± 0.41^{abc}	5.64 ± 0.21^{c}	5.69 ± 0.23^{bc}	4.12 ± 0.52^{ab}	4.35 ± 0.45^{abc}	4.98 ± 0.13^{abc}	4.48 ± 0.17^{a}	4.79 ± 0.49^{abc}
Coliforms	1.31 ± 0.76^{ab}	3.00 ± 0.57^{abc}	3.15 ± 0.71^{ab}	3.71 ± 0.29^{bc}	3.18 ± 0.58^{ab}	3.13 ± 0.11^{ab}	1.50 ± 0.95^{abc}	$<1.00\pm0.00^{c}$
Enterococcus	2.77 ± 0.67^{a}	3.04 ± 0.80^{a}	$<1.00\pm0.00^{a}$	1.41 ± 0.76^{a}	2.25 ± 0.60^{a}	2.76 ± 0.70^{a}	2.13 ± 0.8^{a}	0.72 ± 0.50^{a}
Staphylococcus	1.00 ± 0.63^{a}	0.75 ± 0.62^{a}	$<1.00\pm0.00^{a}$	1.32 ± 0.74^{a}	2.69 ± 0.61^{a}	3.65 ± 0.37^{a}	0.95 ± 0.67^{a}	0.85 ± 0.74^{a}
Clostridium	2.22 ± 0.80^{ab}	4.02 ± 0.68^{a}	3.74 ± 0.83^{ab}	$<1.00\pm0.69^{b}$	2.77 ± 0.74^{ab}	3.70 ± 0.65^{a}	3.92 ± 0.67^{a}	3.03 ± 0.27^{a}
Bacillus	3.74 ± 0.20^{a}	3.69 ± 0.06^{a}	3.88 ± 0.18^{a}	3.54 ± 0.26^{a}	3.80 ± 0.19^{a}	3.89 ± 0.26^{a}	3.30 ± 0.19^{a}	4.18 ± 0.37^{a}

The presented values are means±standard deviation for $n=3$ in seasons. Mean values in rows with different superscript letters are significantly different ($p\leq0.05$). Limit of detection=1 log CFU/g.

to 4.02 log CFU/g (*Clostridium* in the autumn of 2012) (Table 3). Similar *Enterobacteriaceae* and *Staphylococcus* counts in cheese were reported by Frece *et al.* [2016].

Bacterial species were identified based on DGGE bands which were obtained with the use of the developed markers [Nalepa & Markiewicz, 2017] and given a score of 0 to 4 based on their brightness (Figure 1). PCR-DGGE is a semi-quantitative method where the quantity of target DNA corresponding to the number of cells from which it was isolated is positively correlated with the number of amplicons and the brightness of DGGE bands. The band brightness of starter cultures (*L. lactis, L. mesenteroides*, and *P. freudenreichii*) scored 0 to 3 points in the first year (summer 2012 – spring 2013), and 1 to 4 points in the second year (summer 2013 – spring 2014) of the experiment (Figure 1). The most prevalent nonstarter bacteria (lactic acid bacteria and propionic acid bacteria) were *L. brevis* and *L. fermentum*, but they showed in the cheese samples randomly. *P. thoenii* and *L. acidophilus* were found mainly in the samples collected between the summer of 2013 and the spring of 2014. *L. delbrueckii*, *L. helveticus* and *L. casei* were not observed in winter and spring samples, regardless of the experimental year. Among spore-forming bacteria, the most predominant microorganisms were *B. subtilis* and *C. tyrobutyricum*, where *B. subtilis* was more prevalent and was identified in all samples collected between the summer of 2013 and the spring of 2014. *E. coli* and *E. faecalis* were more prevalent in the samples collected between the summer of 2013 and the spring of 2014 than in the remaining months of the study (Figure 1).

In the group of the 24 analyzed species, 6 to 17 bacterial species were detected in the analyzed cheese samples (Table 4). A smaller number of bacterial species was identified in the first year: 11 in the summer of 2012, 11.3 in the autumn of 2012, 8.7 in the winter of 2013, and 10.5 in the spring of 2013 on average. In the second year of the study, the number of identified species was higher reaching 13 in the summer-autumn of 2013 and 10 in the winter of 2013 – spring of 2014 on average. Considerable variations in the number of species were observed between the months/seasons of both years. The number of bacterial species was higher in the samples collected in summer-autumn than in winter-spring periods. Starter bacteria were identified in all samples, excluding the sample from the autumn of 2012 where *L. lactis* was not identified (Figure 1). The most prevalent other bacteria were *L. brevis* and *B. subtilis* which were present in 17 samples (70.8%) (Table 4), followed by *C. tyrobutyricum* which was found in 15 samples (62.5%). *L. fermentum, E. coli*, and *S. thermophilus/S. xylosus* were detected in 14 samples (58.3%), *P. thoenii* and *L. acidophilus* were noted in 12 samples (50.0%), and *L. casei* and *E. faecalis* were found in 11 samples (45.8%).

DGGE band patterns were also used in 1D analysis and to determine the Shannon-Wiener diversity index (H').

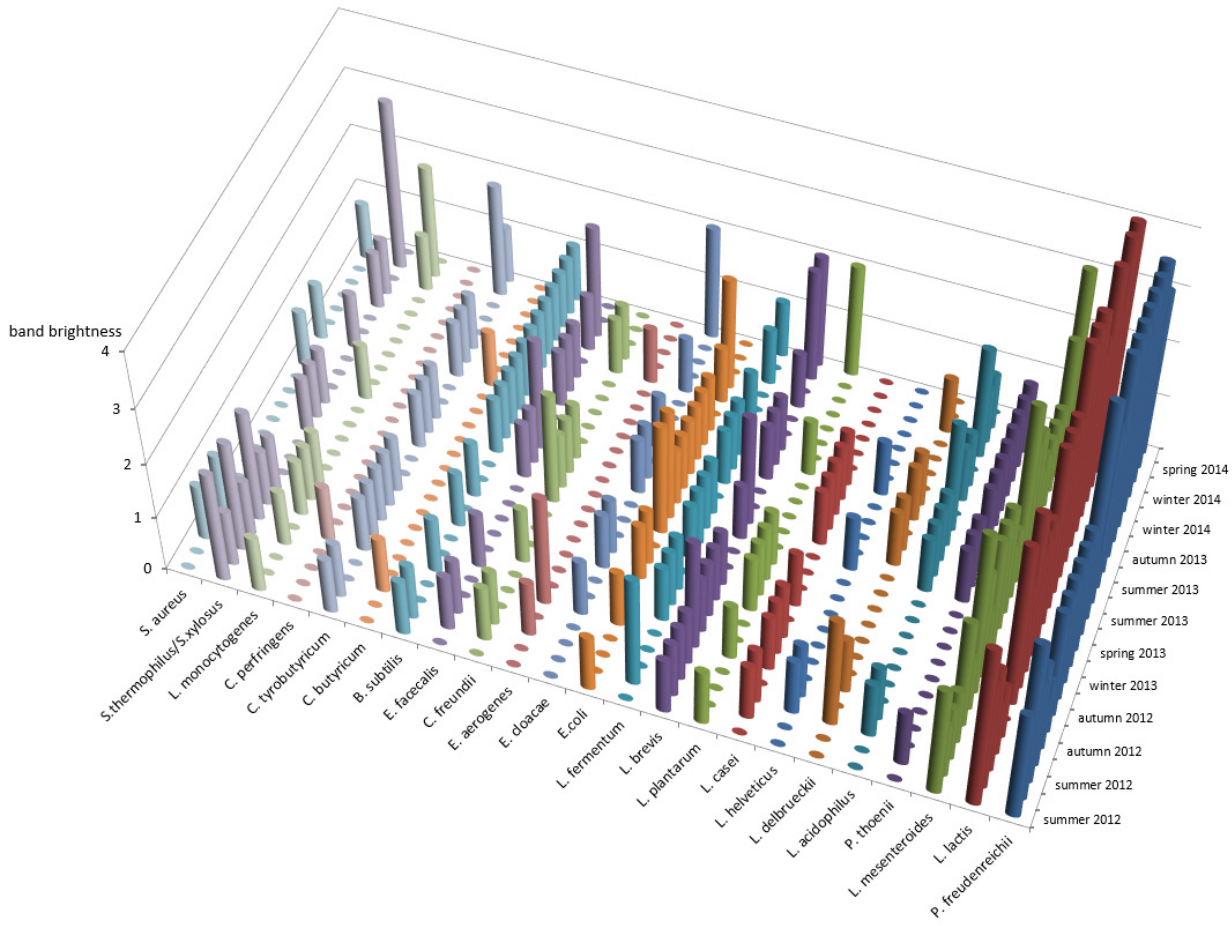

FIGURE 1. The identified microorganisms and relative band brightness on a scale of 0 to 4, determined in the PCR-DGGE assay of Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

The values of H' ranged from 1.6704 in cheeses with 6 bacterial species to 2.9779 in cheeses with 17 bacterial species (data not shown). The evaluated cheeses were characterized by high bacterial diversity and contained from 3 to 14 bacterial species in addition to starter cultures (*P. freudenreichii, L. lactis, L. mesenteroides*).

The sensory attributes of ripened cheeses, produced both traditionally and industrially, are determined by numerous factors, in particular by the qualitative and quantitative composition of microbiota, its physiological status and metabolic activity [Smit *et al.*, 2005]. For this reason, the microbiome of dairy raw materials and cheeses has to be monitored throughout the production process. Molecular biology methods, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) or next-generation sequencing (NGS), have been used by many authors to evaluate microbial communities in raw milk and cheeses [Alegría *et al.*, 2009; Duru *et al.*, 2018; Ercolini *et al.*, 2001; Gala *et al.*, 2008; Mangia *et al.*, 2016; Porcellato & Skeie, 2016; Randazzo *et al.*, 2006; Skelin *et al.*, 2012]. Most of these studies analyzed the microbiome composition of traditional cheeses produced in the Mediterranean region from unpasteurized milk and without starter cultures. Thermophilic species of lactic acid bacteria, such as *S. thermophilus, S. macedonicus, L. helveticus* and *L. delbrueckii*, are more frequently encountered in warmer regions of the southern Europe [Alegría *et al.*, 2009; Franciosi *et al.*, 2009]. In our study, *L. helveticus* and *L. delbrueckii* were identified only in the warmest seasons (summer–autumn) and were never observed in cold seasons (winter–spring), regardless of the year. Seasonal variations in the counts of mesophilic and thermophilic LAB were also reported in Perocino del Poro cheese [Caridi *et al.*, 2003] where mesophilic bacteria were more abundant in spring, and thermophilic bacteria – in summer. These results indicate that the manufacturing season could influence the growth of microbiota and, consequently, the quality of the final product. In other studies, ripened cheeses were found to contain mesophilic bacteria including *L. lactis, L. garvieae, L. mesenteroides, L. casei, L. paracasei, L. plantarum,* and *L. brevis*, but also enterococci (*E. faecalis, E. faecium,* and *E. hirae*), Gram-negative bacteria (*E. coli, Enterobacter* spp., and *Hafnia alvei*), and staphylococci (*S. saprophyticus* and *S. equorum*) [Abriouel *et al.*, 2008; Ercolini *et al.*, 2001; Flórez & Mayo, 2006; Marino *et al.*, 2003]. Randazzo *et al.* [2006] demonstrated that traditional cheeses made from raw milk without starter cultures were characterized by greater microbiological diversity than cheeses manufactured with starter cultures, where a predominance of starter culture species was reported. In our study,

TABLE 4. The number of bacterial species at different times of the year and the prevalence (%) of bacterial species in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Season	Number of identified species	Bacterial group	Species	Prevalence (%)
Summer 2012	10	Starter culture	*P. freudenreichii*	100.0
Summer 2012	12		*L. lactis*	95.8
Summer 2012	14		*L. mesenteroides*	100.0
Summer 2012	8		*P. thoenii*	50.0
Autumn 2012	15	Nonstarter bacteria, including nonstarter LAB	*L. acidophilus*	50.0
Autumn 2012	9		*L. delbrueckii*	29.2
Autumn 2012	10		*L. helveticus*	16.6
Winter 2013	11		*L. casei*	45.8
Winter 2013	9		*L. plantarum*	33.3
Winter 2013	6		*L. brevis*	70.8
Spring 2013	8		*L. fermentum*	58.3
Spring 2013	13	Fecal bacteria	*E. coli*	58.3
Summer 2013	16		*E. cloacae*	33.3
Summer 2013	14		*E. aerogenes*	12.5
Summer 2013	9		*C. freundii*	37.5
Autumn 2013	16		*E. faecalis*	45.8
Autumn 2013	17	Spore-forming bacteria	*B. subtilis*	70.8
Autumn 2013	10		*C. butyricum*	8.3
Winter 2014	9		*C. tyrobutyricum*	62.5
Winter 2014	10		*C. perfringens*	4.2
Winter 2014	11	Other pathogenic	*L. monocytogenes*	33.3
Spring 2014	10	bacteria	*S. aureus*	20.8
Spring 2014	11	Other non-pathogenic	*S. thermophilus*/	58.3
Spring 2014	9	bacteria	*S. xylosus*	

starter culture bacteria (*L. lactis, L. mesenteroides, P. freudenreichii*) were also most abundant in the analyzed cheeses. In DGGE profiles, the brightest bands corresponded to starter culture species, whereas the bands corresponding to other bacteria were less bright. The results of quantitative analyses revealed that starter culture bacteria were more prevalent than other bacteria (Figure 2 and Table 3). Duru *et al.* [2018] relied on next-generation sequencing (NGS) to demonstrate a predominance of *Lactococcus*, *Lactobacillus*, and *Propionibacterium* starter cultures which accounted for 80–82% of all reads in industrially manufactured Swiss-type cheese. In industrially produced Dutch-type cheese, starter bacteria (*Lactococcus*, *Leuconostoc*, and *Lactobacillus*) also accounted for more than 99% of the bacterial community in the NGS assay conducted by Porcellato & Skeie [2016]. However, the production of cheese from pasteurized milk with the addition of starter cultures does not guarantee the growth of starter bacteria only, but NSLAB may also grow and be active. Lindberg *et al.* [1996] and Jordan & Cogan [1993] identified *Lactobacillus paracasei*, *L. casei*, and *L. plantarum* nonstarter bacteria in Swedish and Norwegian cheeses produced with the addition of *Lactococcus* and *Leuconostoc* starter cultures. In our study, the most prevalent NSLAB were *L. brevis* and *L. fermentum*, whereas *L. casei*/*L. paracasei* and thermophilic *L. delbrueckii* and *L. helveticus* were identified only in the summer and autumn. The analyzed cheeses also frequently contained spore-forming bacteria *B. subtilis* and *C. tyrobutyricum*. Klijn *et al.* [1995] demonstrated that the late-blowing defect in cheese was caused mainly by *C. tyrobutyricum* and butyric acid, its main fermentation product, in quantities greater than 100 mg/kg. The natural environment and silage are the main sources of spore-forming bacteria that can lead to spoilage when transferred to raw milk and the cheese matrix.

The VOC content of Swiss-Dutch-type cheese

The content of selected volatile compounds (aldehydes, alcohols, esters, ketones, and fatty acids C_2-C_7) in the examined cheeses is presented in Table 5. Considerable differences

TABLE 5. The content of volatile organic compounds (VOCs) (mg/g) in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

Season	Aldehydes	Alcohols	Esters	Ketones	Fatty acids	Total
Summer 2012	0.00048	0.36485	–	0.13640	0.23643	0.73817
Summer 2012	0.00138	0.00842	–	0.17972	0.54277	0.73228
Summer 2012	0.00060	0.17595	0.000025	0.10961	1.79419	2.08038
Summer 2012	0.00021	0.10131	–	0.09144	1.42441	1.61738
Autumn 2012	0.00055	0.09510	–	0.23241	2.39689	2.72495
Autumn 2012	0.00069	0.12584	–	0.15029	3.06430	3.34111
Autumn 2012	0.00019	0.05991	–	0.07645	2.47629	2.61284
Winter 2013	0.04798	2.56398	0.09433	0.00348	74.81968	77.52943
Winter 2013	0.00025	2.37653	0.10095	0.00733	7.37763	9.86268
Winter 2013	0.05265	2.38913	0.21640	0.00375	74.23943	76.90135
Spring 2013	0.04455	2.92663	0.15565	0.00310	60.15818	63.28810
Spring 2013	0.05028	2.58768	0.21038	0.00458	120.03033	122.88323
Summer 2013	0.05385	2.42525	0.24041	0.00536	111.08645	113.81133
Summer 2013	0.05588	3.03558	0.24185	0.00860	127.93500	131.27690
Summer 2013	0.00045	4.65645	0.20115	0.00775	21.41735	26.28315
Autumn 2013	0.04420	–	0.00080	–	89.18433	89.22933
Autumn 2013	0.04820	2.37845	0.09713	0.00588	75.83090	78.36055
Autumn 2013	0.05385	2.39680	0.09740	0.00603	65.49263	68.04670
Winter 2014	0.04351	0.63736	0.09884	0.00464	95.75041	96.53476
Winter 2014	0.03669	1.78571	0.09324	0.00415	89.19380	91.11359
Winter 2014	0.03069	1.73941	0.10704	0.00345	84.79109	86.67168
Spring 2014	0.02041	1.32240	0.11075	0.00293	86.83456	88.29105
Spring 2014	0.02810	1.81126	0.17619	0.00141	84.25300	86.26996
Spring 2014	0.04028	1.22004	0.08508	0.01268	72.45116	73.80923

Aldehydes: acetaldehyde; Alcohols: methanol, ethanol; Esters: ethyl acetate, ethyl propionate, ethyl butyrate; Ketones: acetone, diacetyl, acetoin; Fatty acids: acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, caproic acid, heptanoic acid.

in VOC content were observed between seasons. In the summer-autumn of 2012, the total VOC content of the evaluated samples ranged from 0.73228 to 3.34111 mg/g of cheese and was substantially lower than in the winter of 2013–spring of 2014 when it ranged from 63.28810 to 131.27690 mg/g. Two exceptions were noted in the second year of the study (26.28315 mg/g in the summer of 2013 and 9.86267 mg/g in the winter of 2013) when VOC concentrations were lower than in the remaining samples from the corresponding period, but were still several times higher than in the summer-autumn of 2012 (Table 5). The predominant VOCs in all samples were fatty acids whose content ranged from 0.23643 to 127.93500 mg/g and was characterized by similar seasonal fluctuations as total VOC levels. In 2013, alcohol concentrations in the examined samples were determined at 2.37652 to 4.65645 mg/g and were significantly higher than in 2014 (0.63736–1.81126 mg/g) and 2012 (0.00841–0.36485 mg/g). Very low levels of total VOCs and fatty acids C_2–C_7 were noted in 2012. The contents of aldehydes and esters were also very low in 2012. Aldehyde levels reached 0.00059 mg/g in 2012 on average, and they were nearly 65-fold higher in the remaining seasons at 0.03834 mg/g on average. Even greater variations were observed in the ester content of cheeses. Ester contents were negligible in 2012, whereas in 2013–2014, they were determined in the range of 0.08507 to 0.24185 mg/g. An opposite trend was noted in ketone levels which were substantially higher in 2012 than in the remaining years. On average, ketone contents were determined at 0.1395 mg/g in 2012 and at 0.00500 mg/g in 2013–2014 (Table 5).

The unique flavor of different cheeses can be attributed to both starter and nonstarter bacteria. The sensory attributes of cheese, including flavor, aroma and consistency, are developed during complex interactions between bacteria. The synthesized VOCs play a very important role in this process [Pérès *et al.*, 2001; Mondello *et al.*, 2005; Januszkiewicz *et al.*, 2008]. Volatile organic compounds are produced *via* three

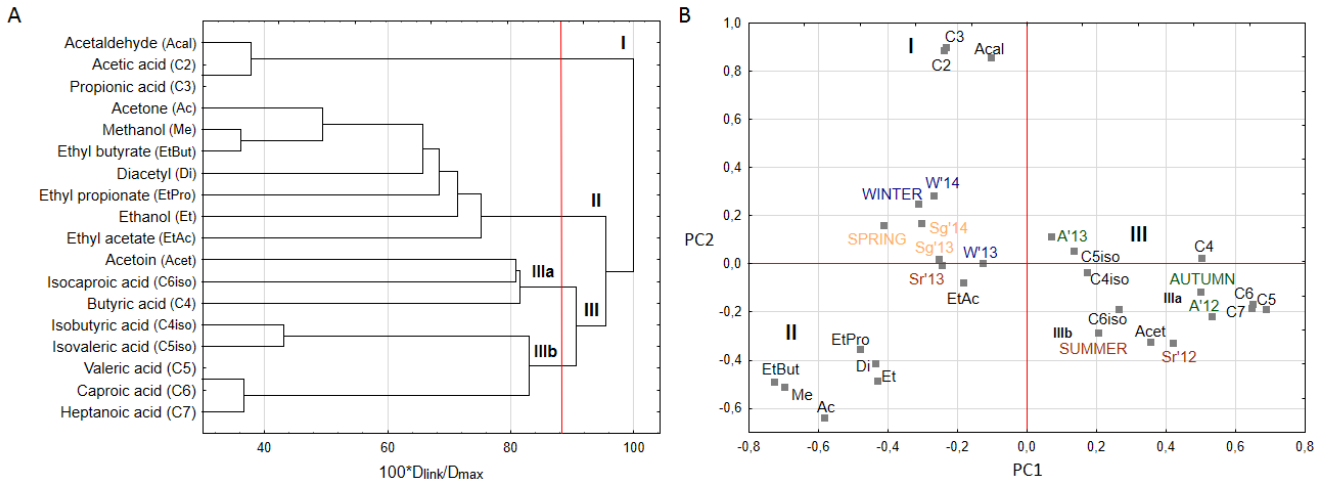

FIGURE 2. A – The dendrogram of a dissimilarity measure between the volatile compounds; B – The Principal Component Analysis *biplot* of seasonal variables and volatile compounds related to component 1 (*PC1*) and component 2 (*PC2*).

Acal – acetaldehyde; Ketones: Ac – acetone, Di – diacetyl, Acet – acetoin; Alcohols: Me – methanol; Et – ethanol; Esters: EtAc – ethyl acetate, EtPro – ethyl propionate, EtBut – ethyl butyrate; Fatty acids: C_2 – acetic acid, C_3 – propionic acid, C_{4iso} – isobutyric acid, C_4 – butyric acid, C_{5iso} – isovaleric acid, C_5 – valeric acid, C_{6iso} – isocaproic acid, C_6 – caproic acid; C_7 – heptanoic acid.

major catabolic pathways that are involved in flavor development: fermentation (lactose and citrates), lipolysis (milk fat), and proteolysis (casein) [Gobbetti *et al.*, 2015]. During these processes, lactose is converted to diacetyl, acetoin, acetaldehyde, and acetic acid. Fatty acids are converted to ketones, secondary alcohols, esters, and lactones; and casein – to alcohols, aldehydes, acids, esters, and sulfur compounds [Smit *et al.*, 2005]. In this study, HS-GC revealed the presence of all VOC groups in Swiss-Dutch-type cheeses. The presence of alcohols, esters, ketones, short-chain and medium-chain fatty acids was also determined in Serbian Pirotski kačkavalj cheese [Milosavljević *et al.*, 2012] and French Cantal cheese [De Freitas *et al.*, 2007]. According to Pastink *et al.* [2008], considerable differences in the flavor-forming ability of LAB exist not only between species, but also between strains. The cited authors demonstrated that "wild" LAB strains produced much greater quantities of methyl aldehydes and methyl alcohols than industrial strains. In our study, cheese samples from all seasons of 2013 were abundant in alcohols, in particular methyl alcohol. The prevalence of NSLAB was particularly high in 2013, especially in the summer and autumn. In the cited study concerning Cantal cheese [De Freitas *et al.*, 2007], ethanol was the major VOC. Ethanol is a metabolite of complex processes involving yeast, *Leuconostoc, Lactococcus*, and thermophilic LAB. Like other alcohols synthesized in cheese, it is not directly responsible for the flavor of cheese, but it determines the production of ethyl esters which are flavor-forming compounds [Thierry *et al.*, 2006]. In our study, ester contents were also higher in cheese samples characterized by a higher alcohol content than in the samples where alcohol levels were low or where alcohol was not detected. Lactate fermentation, amino acid catabolism, and fat lipolysis are sources of free fatty acids in ripened cheese [McSweeney & Sousa, 2000]. Dolci *et al.* [2008] demonstrated moderate levels of lipolytic activity in Castelmango cheese containing short-chain fatty acids such as formic acid, acetic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid. The cited authors attributed their findings to the presence of enterococci, staphylococci, and yeast whose counts were high in all stages of the cheese production process. In another study, the presence of short-chain fatty acids was attributed not only to fecal streptococci, but to all secondary microorganisms [Fox *et al.*, 2004]. In our study, fatty acid contents were several dozen times higher in cheese samples characterized by a higher activity and/or prevalence of both starter and nonstarter bacteria, *i.e.* in the samples collected between the winter of 2013 and the spring of 2014.

Statistical analysis of VOC profiles

The seasonal variations in the VOC profiles of Swiss-Dutch-type cheeses produced between 2012 and 2014 were determined by principal component analysis (PCA) which was preceded by hierarchical cluster analysis (HCA) (Figure 2). An HCA dendrogram showed that VOCs were organized in three clusters: I, II and III, with the last one comprising two sub-clusters (IIIa and IIIb), which showed a dissimilarity >80 (Figure 2A) and was largely consistent with the results of the PCA (Figure 2B). However, the first (PC 1) and second (PC 2) principal component explained 46.63% of the total variation. Acetaldehyde (Acal), acetic acid (C_2), and propionic acid (C_3) were grouped together in cluster I, since they were variables with the highest negative loading in PC 2 (Figure 2 and Table 6). Cluster II was comprised primarily of acetone (Ac), methanol (Me), and ethyl butyrate (EtBut), since they were variables with moderate negative loading in PC 1 and PC 2. They were also accompanied by weak negative loadings of diacetyl (Di), ethanol (Et), ethyl propionate (EtPro), and ethyl acetate (EtAc). Cluster III was comprised primarily of valeric acid (C_5), caproic acid (C_6), and heptanoic acid (C_7), since they were variables with moderate positive loading in PC 1 and they accompanied by isobutyric acid (C_{4iso}), isovaleric acid (C_{5iso}) (cluster IIIa) as well as acetoin (Acet), isocaproic acid (C_{6iso}), and butyric acid (C_4) (cluster IIIb). As demonstrated by Figure 2, the resulting three

TABLE 6. The factor loadings after the varimax rotation of the volatile organic compounds (VOCs) data and the correlation coefficients (*r*) between VOCs and the microorganisms detected in Swiss-Dutch-type cheeses manufactured between 2012 and 2014.

	VOCs	PC1	PC2	Microorganisms (*r*)				
Cluster I	C_3	-0.23	-0.90***	*P. thoenii* (**0.70**[a])	*L. lactis* (**0.48**)	*B. subtilis* (**0.43**)	*L. acidophilus* (**0.43**)	*P. freudenreichii* (**0.42**)
	C_2	-0.24	-0.89***	*P. thoenii* (**0.65**)	*L. lactis* (**0.45**)	*P. freudenreichii* (0.39)	*B. subtilis* (0.39)	*L. acidophilus* (0.35)
	Acal	-0.10	-0.86***	*P. thoenii* (**0.52**)	*L. lactis* (**0.42**)	*P. freudenreichii* (0.37)	*B. subtilis* (0.39)	*L. acidophilus* (0.22)
Cluster II	Ac	-0.58**	-0.63**	*E. coli* (**0.46**)	*C. freundii* (0.35)	*L. fermentum* (0.24)	*L. mesenteroides* (0.08)	*L. helveticus* (0.03)
	Me	-0.70**	-0.50**	*E. coli* (**0.47**)	*L. lactis* (0.32)	*B. subtilis* (0.22)	*L. mesenteroides* (0.19)	*P. freudenreichii* (0.17)
	EtBut	-0.73**	-0.48*	*L. lactis* (0.35)	*E. coli* (0.30)	*P. freudenreichii* (0.29)	*B. subtilis* (0.28)	*P. thoenii* (0.17)
	Di	-0.44*	-0.41*	*E. cloacae* (**0.56**)	*L. plantarum* (**0.44**)	*B. subtilis* (0.35)	*L. lactis* (0.25)	*L. mesenteroides* (0.22)
	EtPro	-0.48*	-0.35*	*C. freundii* (0.34)	*E. coli* (0.25)	*P. thoenii* (0.24)	*L. acidophilus* (0.16)	*C. tyrobutyricum* (0.15)
	Et	-0.43*	-0.48*	*L. brevis* (0.35)	*P. freudenreichii* (0.26)	*L. delbrueckii* (0.25)	*E. cloacae* (0.23)	*L. casei* (0.23)
	EtAc	-0.18	-0.07	*E. faecalis* (**0.45**)	*P. thoenii* (0.35)	*L .monocytogenes* (0.28)	*E. cloacae* (0.26)	*B. subtilis* (0.26)
Cluster III IIIa	C_5	0.68**	-0.18	*L. casei* (**0.45**)	*C. perfringens* (0.29)	*E. aerogenes* (0.25)	*L. brevis* (0.24)	*L. delbrueckii* (0.20)
	C_6	0.64**	-0.16	*L. casei* (0.35)	*C. perfringens* (0.28)	*E. aerogenes* (0.28)	*L. delbrueckii* (0.30)	*L. brevis* (0.21)
	C_7	0.64**	-0.18	*L. casei* (**0.51**)	*C. perfringens* (0.33)	*E. aerogenes* (0.28)	*L. brevis* (0.27)	*L. delbrueckii* (0.16)
	C_{4iso}	0.17	-0.03	*L. casei* (0.29)	*E. cloacae* (0.17)	*C. tyrobutyricum* (0.16)	*L. plantarum* (0.16)	*L. brevis* (0.04)
	C_{5iso}	0.13	0.05	*L. casei* (0.21)	*E. cloacae* (0.19)	*L. plantarum* (0.19)	*C. tyrobutyricum* (0.06)	*E. coli* (0.03)
	Acet	0.35*	-0.32*	*C. perfringens* (**0.58**)	*E. aerogenes* (**0.53**)	*S. thermophilus/S. xylosus* (**0.50**)	*L. casei* (0.37)	*L. brevis* (0.32)
IIIb	C_{6iso}	0.26	-0.18	*S. thermophilus/S. xylosus* (0.29)	*C. tyrobutyricum* (0.25)	*L. fermentum* (0.22)	*B. subtilis* (0.16)	*L. brevis* (0.16)
	C_4	0.50*	0.02	*L. fermentum* (0.27)	*L. plantarum* (0.20)	*C. perfringens* (0.17)	*L .monocytogenes* (0.15)	*L. mesenteroides* (0.11)

* 0.30–0.50 (weak); **0.50–0.75 (moderate); ***>0.75 (strong loading).

[a] The r values in bold indicated significant ($p \leq 0.05$) differences.

Acal – acetaldehyde; Ketones: Ac – acetone, Di – diacetyl, Acet – acetoin; Alcohols: Me – methanol; Et – ethanol; Esters: EtAc – ethyl acetate, EtPro – ethyl propionate, EtBut – ethyl butyrate; Fatty acids: C_2 – acetic acid, C_3 – propionic acid, C_{4iso} – isobutyric acid, C_4 – butyric acid, C_{5iso} – isovaleric acid, C_5 – valeric acid, C_{6iso} – isocaproic acid, C_6 – caproic acid; C_7 – heptanoic acid.

clusters represented a collection of cheeses produced in different years and even seasons. The cheeses manufactured in 2012 were largely characterized by VOCs from cluster III, which were discriminated from the samples of 2013/2014 seasons. Moreover, cluster III contained sub-clusters a and b, which represented collections of samples from the autumn and summer, respectively. In turn, cheeses produced mainly in winter-spring 2013/14 were characterized either by VOCs from cluster I or VOCs from cluster II. Importantly, significant coefficients of correlation (r) were calculated for the relationships between C_3 *vs. P. thoenii* (0.70), *L. lactis* (0.48), *L. acidophilus* (0.43), *B. subtilis* (0.43), and *P. freudenreichii* (0.42); C_2 *vs*. *P. thoenii* (0.65) and *L. lactis* (0.45); as well as acetaldehyde (Acal) *vs*. *P. thoenii* (0.52) and *L. lactis* (0.42). The above indicates that these microorganisms contributed to the synthesis of acetaldehyde, C_2, and C_3 in cheese (Table 6). The prevailing *E. coli*, *E. cloacae*, and *E. faecalis* correlated with Ac/Me, Di, and EtAc from cluster II, respectively. Among VOCs from cluster III, C_5 and C_7 exhibited the highest correlation with *L. casei* and the Acet with *C. perfringens*, *E. aerogenes*, and *S. thermophilus/S. xylosus*, indicating that they could be considered as important contributors to these VOCs. An analysis of the correlations between VOCs and bacteria revealed that the sensory attributes of Swiss-Dutch-type cheeses were formed primarily by starter bacteria (*L. lactis, L. mesenteroides*, and *P. freudenreichii*), whereas nonstarter bacteria exerted strong flavor-forming effects after 52 days of ripening.

The two-year study yielded massive amounts of data (DGGE profiles, VOC profiles, bacterial counts) which were difficult to interpret. Advanced computational methods, such as HCA, PCA or partial least squares (PLS) regression, are increasingly often used in studies of this type. Milosavljević *et al.* [2012] relied on AHC and PCA to compare contents of VOC determined by different gas chromatography methods. Based on an analysis of GC data, they concluded that the results of AHC and PCA should be analyzed in combination to produce a more comprehensive VOC profile of ripened cheeses. In turn, Mauriello *et al.* [2003] were able to identify the region of origin of mozzarella cheese based on the results of PCA, whereas Duthoit *et al.* [2005] also relied on PCA and PLS regression analysis to determine changes in the sensory and microbiological properties of Salers cheese and reported correlations between sensory properties and bacterial cultures. Similarly to our study, they observed that nonstarter bacteria, such as *Enterobacteriaceae* and *Bacillus*, were responsible for the texture, flavor, and aroma of cheese.

A combination of culture-dependent and culture-independent methods supported more accurate analyses of the cheese microbiota and its role in the formation of volatile compounds, as well as the identification of bacterial species synthesizing compounds that affect the sensory quality of ripened cheese. Thus, the results of this study indicate that the diversity of bacterial communities and profiles of volatile compounds are highly dependent upon the seasonality of cheese production. For cheese manufacturers, the biodiversity of cheese batches, despite being carried out according to the same technological scheme, is a very important observation. Awareness of these dependencies may contribute to a better understanding of phenomena occurring during the cheese production process, giving the opportunity to prevent technological fluctuations and obtain the final product of excellent quality.

CONCLUSIONS

The study revealed differences in the composition of microbiota and the content of VOCs in Swiss-Dutch-type cheeses manufactured in different seasons of the year. Therefore, cheese samples were divided into two groups. The first group was composed of cheeses produced in winter-spring (clusters I and II). These samples were characterized by the prevalence of *P. thoenii, L. lactis, L. acidophilus*, and *B. subtilis* which were correlated with the contents of acetaldehyde and fatty acids C_2 and C_3, whereas *E. coli, E. cloacae*, and *L. plantarum* increased contents of methanol, acetone, and diacetyl. The second group was composed of cheeses produced in summer-autumn (cluster III). It was characterized by the prevalence of NSLAB (including *L. casei, L. fermentum*, and *L. brevis*) which increased the content of fatty acids C_4-C_7, as well as *C. perfringens, E. aerogenes*, and *S. thermophilus/S. xylosus* which probably increased the content of acetoin. The observations made in this study do not exhaust the possibilities of this approach, but indicate that the described methods can be effectively used to control the cheese production process and to monitor microbiological contamination.

ACKNOWLEDGEMENTS

We are grateful to Dr. Marta Mikš for calibration of the gas chromatograph for quantitative identification volatile compounds of microbiological origin in samples of milk and ripened chees.

RESEARCH FUNDING

This study was supported by the National Science Center, Poland (grant no. N N312 484140) and by University of Warmia and Mazury in Olsztyn (17.610.015–300).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Submitted: 13 February 2019. Revised: 15 May 2019. Accepted: 6 June 2019. Published on-line: 3 July 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 279–287
DOI: 10.31883/pjfns/109987
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Advantages and Disadvantages of Partial High Pressure Homogenisation of Milk in Relation to Full-Stream Homogenisation

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Key words: viscosity, milk, acidity, colloidal stability, high pressure homogenisation, fat globule size

This study determines the effect of full-stream and partial high pressure homogenisation (constant pressure of 100 MPa, different temperatures: 20°C, 40°C, 60°C) on selected distinguishing features of the colloidal and emulsion phases of milk. The pH values of milk decreased significantly ($p<0.05$) as a result of full-stream homogenisation, while they remained unaffected by partial homogenisation ($p\geq0.05$). Most of the changes in the titratable acidity and conductivity of milk triggered by full-stream homogenisation and partial homogenisation were not statistically significant ($p\geq0.05$). Partial high pressure homogenisation had a weaker effect on reducing rennet coagulation time and heat stability of milk than the process performed with the full-stream method. Full-stream and partial homogenisation resulted in a statistically significant ($p<0.05$) reduction in the thermal coagulation time by approx. 44% and 30%, respectively in comparison to the control sample. The values of thermal stability time of milk subjected to full-stream and partial high pressure homogenisation were different ($p<0.05$). The rennet coagulation time of milk did not differ depending on the homogenisation method applied ($p\geq0.05$). Fat globules size, described by parameters d_v10, d_v50, d_v90, d_{32}, and d_{43}, was statistically significant ($p<0.05$) lower upon full-stream homogenisation than upon partial homogenisation. The maximum decrease in d_{32} value was 6.5- and 2-fold as a result of full-stream and partial homogenisation, respectively. Microscopic images show that the low effectiveness of partial homogenisation was due to the flocculation of fat globules. Changes to the viscosity of milk as a result of full-stream homogenisation (a decrease) and partial homogenisation (an increase) were noted as well.

The advantage of partial high pressure homogenization over full-stream homogenization is the reduction of the influence of the process on the colloidal stability of milk, while the disadvantage is the low efficiency of the process. High pressure homogenization as an innovative method of preservation can be applied in the dairy industry. The desired features of emulsion and colloidal phases, depending on the product, are the result of selection of the method and temperature of the process.

INTRODUCTION

Milk and dairy products are dietary components with a comprehensive nutritional value, due to the large variety of valuable ingredients, their high bioavailability and biological activity. These include: proteins, bioactive peptides derived from proteins (fermented beverages) or only from casein (cheese), enzymes, vitamins, minerals, particularly calcium, and components of the emulsion phase, *i.a.* conjugated linoleic acid, short-chain and medium-chain fatty acids, polar lipids, and fat-soluble vitamins [Korhonen, 2009; Park, 2009].

The heat treatment is a commonly used method in the dairy industry. In an attempt to reduce undesirable changes in milk components, the alternative methods have become popular in the design of functional foods. Alternative methods of food preservation include, among others, mechanical methods based on the separation of microorganisms from milk using centrifugal force (bactofugation) or membranes (microfiltration) or the use of pulsed electric field, ultrasounds, pressurisation, and high pressure homogenisation [Butz & Tauscher 2002; Datta & Deeth, 2003; Devlieghere *et al.,* 2004]. The prerequisite for using the emerging technologies in the food production is their actions, which do not impair the nutritional value or organoleptic properties of food, and improve its shelf-life by limiting the development or by eliminating microorganisms. They have a potential to provide fresh-like products with the prolonged shelf-life and desired rheological characteristics, sensory properties, and nutritional value. Moreover, emerging technologies are potential methods for the fractionation of milk compounds [Kumar *et al.*, 2013] and for the production of novel functional dairy products as whey-based drinks [Pereira *et al.*, 2015], whey protein-based emulsions [Yan *et al.*, 2017], fermented dairy beverages [Masson *et al.,* 2011], and functional food supplements containing heat-sensitive components of milk [Aguayo *et al.*, 2017].

High pressure homogenisation (HPH) is a process carried out under dynamic pressure ranging from 100 to 400 MPa. When the process is carried out in a narrow pres-

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sure range of 300–400 MPa, it is usually referred to as ultra-high pressure homogenisation (UHPH) [Georget *et al.,* 2014]. The process, also referred to as dynamic high pressure treatment, may potentially be applied in the pharmaceutical, cosmetic, and food industries, including in the dairy sector [Dumay *et al.*, 2013]. In milk and cream of varying fat contents, the process is applied to disperse the emulsion phase in the continuous (dispersing) phase, *i.e.* plasma (milk components except the emulsion phase) and to obtain small-sized fat globules. An important consequence of high pressure homogenisation is its contribution to the reduction in the microbial count in milk to a degree dependent on process parameters (pressure, temperature) [Georget *et al.*, 2014; Hayes & Kelly, 2003a; Pedras *et al.*, 2012; Rodarte *et al.*, 2018]. An additional aspect of replacing pasteurisation with this process is the reduction in adverse environmental impact [Valsasina *et al.*, 2017]. High pressure homogenisation, as an emerging technology, may be applied in the production of dairy products to replace both pasteurisation and homogenisation. The desired effect of both obtaining the dispersion of milk fat and an improvement in microbiological quality of a product occurs while applying high pressure homogenisation at a cooling or room temperature, *i.e.* at a temperature lower than that during homogenisation under typical conditions, *i.e.* below 60°C [Pereda *et al.*, 2007; Thiebaud *et al.*, 2003]. Another effect of the application of high pressure homogenisation, which is of significance given the shelf-life of dairy products, is the inactivation of native milk enzymes such as alkaline phosphatase or, partially, peroxidase, as well as plasmin [Hayes & Kelly, 2003b; Pereda *et al.*, 2007]. Finally, high pressure homogenisation enables the modification of the remaining non-fat components of milk and its functional characteristics applied in the dairy industry [Hernández & Harte, 2008; Pedras *et al.*, 2014; Roach & Harte, 2008; Sandra & Dalgleish, 2005, 2007; Sørensen *et al.*, 2014].

In a process line for non-fermented milk beverages, either full-stream or partial homogenisation process may be applied. During full-stream homogenisation, milk with the previously standardized fat content is subjected to the process. In turn, during partial homogenisation, only the cream previously obtained from the centrifugation of raw milk (and not the obtained skimmed milk) is subjected to the process. The next stage is the standardisation of the fat content of the skimmed milk with homogenised cream [Innings, 2015]. The fact that the skimmed milk remains non-subjected to homogenisation is of particular importance due to the effect of homogenisation on plasma components and on the properties of milk [Kiełczewska *et al.*, 2008; Pereda *et al.*, 2009; Zamora *et al.*, 2012]. The application of partial homogenisation (during which only the previously obtained cream subsequently combined at a specific ratio with the skimmed milk, is subjected to the treatment) is also economically justified. In contrast to homogenisation performed in full-stream mode, it provides an opportunity to install homogenisers with significantly lower capacities than the capacity of the entire line on process lines. When designing a process line to account for partial homogenisation, preservation (thermal or mechanical through microfiltration or bactofugation) may be applied separately to skimmed or standardized milk as the last stage of the technological process. At the same time, there is little research on preserving natural milk structure in the technological process, and the results of excessive interference with the milk structure may not be neutral to the human body.

The aim of this study was to compare effects of full-stream and partial high pressure homogenisation on selected properties of milk, with particular emphasis on the distinguishing features of emulsion and colloidal phases.

MATERIAL AND METHODS

Research material

The research material was cow milk originating from the Bałdy Educational and Research Station of the University of Warmia and Mazury in Olsztyn, cooled to a temperature of ≤4°C, and preserved by the addition of a 2% sodium azide solution in an amount of 1 mL per 1 L in order to inhibit the growth of microorganisms. Bulk tank milk was sampled six times. The entire research was conducted in the Autumn 2017 and took 2 months.

Preparation of samples for homogenisation

Raw milk was centrifuged at a temperature of 45°C using a skimming centrifuge (Gea Westfalia Separator System GmbH, Oelde, Germany) to obtain skimmed milk and cream. Raw milk was standardized with skimmed milk to a fat content of 3.5% and earmarked for full-stream homogenisation. The cream was standardized to a fat content of 20% and earmarked for partial homogenisation and the subsequent standardisation of the skimmed milk to a fat content of 3.5%.

Homogenisation process

High pressure homogenisation of 800 mL of milk and 800 mL of cream was carried out at a constant pressure of 100 MPa and at various initial temperatures of 20°C, 40°C, and 60°C. Homogenisation was carried out using a Panda-PLUS 2000 homogeniser (Gea Niro Soavi, Parma, Italy).

The control sample was unhomogenised milk standardised to 3.5%.of fat content.

Acidity and conductivity measurements

The acidity of milk was assessed based on the measurements of its pH using an inoLab Level 1 pH meter (WTW, Weilheim, Germany) and its titratable acidity. Milk (100 mL) was titrated with 0.25 M NaOH using phenolphthalein as the indicator [AOAC, 1990; method 947.05]. The results were expressed as Soxhlet-Henkel degrees (°SH). The conductivity of milk was measured using an inoLab Cond Level 1 conductometer (WTW, Weilheim, Germany).

Colloidal stability of milk

An assessment was performed of heat stability of milk expressed as the thermal coagulation time at a temperature of 140°C in an oil bath (TewesBis, Barczewo, Poland) [Kruk *et al.*, 1979], and the rennet coagulation time [Petrovska *et al.,* 2017], using Chy-Max preparation (Chr. Hansen, Hørsholm, Denmark).

Fat globule size

An analysis of fat globule size measured with laser diffraction was carried out using a Mastersizer 3000 particle size analyser equipped with He-Ne laser (632.8 nm) with a Hydro EV dispersion unit (Malvern Instrument, Malvern, United Kingdom). The measurement was carried out using deionized water (Milli-Q, Millipore, Molsheim, France) and milk with refractive indexes 1.33 and 1.46, respectively.

The following parameters were determined:

- d_v10 – the diameter below which lies 10% of globules volume,
- d_v50 – median diameter, 50 % of the volume distribution is above, and 50 % is below observed diameter,
- d_v90 – the diameter below which lies 90% of globules volume,
- span as $(d_v90-d_v10)/d_v50$,
- a volume-surface mean diameter of fat globules (Sauter Mean Diameter) $d_{32}=\Sigma d_i^3 n_i/\Sigma d_i^2 n_i$,
- a mean diameter over volume (De Brouckere Mean Diameter) $d_{43}=\Sigma d_i^4 n_i/\Sigma d_i^3 n_i$,
- the specific surface area of fat globules in 1 g of milk fat, $ssa=6/d_{32}$,

where n_i is the number of fat globules with a diameter of d_i.

Microscopic analysis

The state of dispersion of milk fat in the milk and cream was assessed by the microscopic method using a Nikon Eclipse Ti-E confocal microscope (Nikon Instruments, Amsterdam, The Netherlands). Microscopic specimens of milk and cream samples were prepared according to the requirements of PN-A-86059:1975, using a solution of 2-naphthalenol,1-[2-[4-(2--phenyldiazenyl)phenyl]diazenyl] (Merck, Darmstadt, Germany) in ethanol. The observation and recording of microscopic images were performed at a magnification of 60.

Shear flow and viscosity

Shear stress and viscosity of milk were determined at a temperature of 20°C with a rotational rheometer (RheolabQC, Anton Paar GmbH, Graz, Austria) using a system of DG42/SS concentric cylinders. The samples were subjected to shear stress at a rate increasing within the range of 0–100/s. Given the considerable fluctuations of the analysed rheological values at a shear rate of <16, the manuscript provides measurement results at a shear rate within the range of 16–100/s.

Statistical analysis

Six independent experiments were conducted (n=6). Each laboratory analysis was performed in two replications. Test results were presented as mean values and their variation was expressed using standard deviations. The obtained data were compared using ANOVA and the least significant difference (LSD) test. A statistical analysis was carried out using software of StatSoft Inc. Statistica v. 10.0 (Tulsa, OK, USA).

RESULTS AND DISCUSSION

The pH values of milk decreased significantly ($p<0.05$) as a result of full-stream homogenisation (Table 1). The pH values of control sample and partially homogenised milk did not differ statistically ($p\geq0.05$). An increase (T=20 and 60°C) in the titratable acidity of milk as a result of full-stream homogenisation and a decrease (all temperatures) as a result of partial homogenisation were not statistically significant ($p\geq0.05$). In the case of titratable acidity, the values characterising milk subjected to full-stream and partial high pressure homogenisation differed significantly ($p<0.05$). Conductivity fluctuations were insignificant ($p\geq0.05$) and this milk characteristic was regarded as invariable upon performing homogenisation. In most cases, the differentiation of homogenisation temperature did not contribute to statistically significant changes ($p\geq0.05$) in the values of the analysed distinguishing features of acidity and conductivity (Table 1).

The literature data indicate a decrease in the pH value of milk as an effect of high pressure homogenisation [Hayes & Kelly, 2003a] or the lack of a significant effect of the process on this characteristic of milk [Kiełczewska *et al.*, 2003; Pedras *et al.*, 2014]. Conductivity of homogenised milk results from transformations of two phases, *i.e.* the emulsion phase, whose dispersion increase can contribute to the increase in impedance, and the soluble phase, related to the increase of its mineral salt content as a result of casein modification, which contributes to an increase in admittance [Banach *et al.*, 2008]. Changes in the electrical properties of milk as a result of the process are probably balanced out, which is reflected

TABLE 1. The effect of full-stream and partial homogenisation on selected physicochemical properties of milk.

Product			pH	Titratable acidity (°SH)	Conductivity (mS/cm)	Rennet coagulation time (min)	Thermal coagulation time (min)
Unhomogenised milk			6.73±0.04^{a}	6.70±0.11^{bc}	4.79±0.08^{a}	4.02±0.33^{a}	10.18±0.29^{a}
Homogenised milk	full-stream homogenisation	T=20°C	6.68±0.03^{b}	6.92±0.28^{ab}	4.82±0.12^{a}	3.27±0.34^{c}	5.73±0.24^{c}
		T=40°C	6.67±0.04^{b}	6.98±0.11^{a}	4.83±0.13^{a}	3.32±0.43^{c}	5.88±0.27^{c}
		T=60°C	6.68±0.04^{b}	6.72±0.18^{bc}	4.83±0.11^{a}	3.52±0.35^{bc}	5.70±0.26^{c}
	partial homogenisation	T=20°C	6.71±0.03^{ab}	6.51±0.18^{c}	4.78±0.11^{a}	3.62±0.17^{bcA}	7.02±0.18^{b}
		T=40°C	6.71±0.03^{ab}	6.53±0.21^{c}	4.77±0.16^{a}	3.70±0.24^{bc}	7.23±0.31^{b}
		T=60°C	6.72±0.02^{a}	6.51±0.37^{c}	4.75±0.11^{a}	3.78±0.16^{ab}	7.05±0.26^{b}

Results are expressed as mean value ± standard deviation. The values in the column marked with different letters differ significantly at $p<0.05$.

TABLE 2. The effect of full-stream and partial homogenisation on the distinguishing features of the dispersion of the emulsion phase of milk.

Product			d_v10 (μm)	d_v50 (μm)	d_v90 (μm)	span	d_{32} (μm)	d_{43} (μm)	ssa (m^2/g)
Unhomogenised milk			1.27±0.14^{a}	3.56±0.14^{b}	6.85±0.65^{c}	1.57±0.21^{e}	2.37±0.09^{a}	4.86±0.44^{c}	2.53±0.09^{g}
Homogenised milk	full-stream homogenisation	T=20°C	0.27±0.02^{c}	0.74±0.08^{e}	3.53±0.41^{d}	4.41±0.57^{a}	0.59±0.03^{e}	2.31±0.14^{d}	10.24±0.51^{c}
		T=40°C	0.23±0.02^{c}	0.48±0.07^{f}	2.35±0.52^{e}	4.38±0.63^{a}	0.43±0.04^{f}	2.13±0.14^{d}	13.94±1.21^{b}
		T=60°C	0.21±0.00^{c}	0.41±0.00^{f}	0.96±0.04^{f}	1.82±0.09^{de}	0.37±0.00^{g}	0.61±0.06^{e}	16.16±0.17^{a}
	partial homogenisation	T=20°C	0.53±0.06^{b}	4.91±0.12^{a}	11.77±1.29^{a}	2.29±0.21^{c}	1.86±0.06^{b}	8.62±0.80^{a}	3.22±0.11^{f}
		T=40°C	0.48±0.01^{b}	2.81±0.20^{c}	8.92±0.53^{b}	3.01±0.23^{b}	1.36±0.04^{c}	5.85±0.54^{b}	4.43±0.13^{e}
		T=60°C	0.47±0.00^{b}	1.56±0.04^{d}	3.82±0.35^{d}	2.15±0.19^{cd}	1.06±0.02^{d}	4.34±0.52^{c}	5.66±0.08^{d}

d_v10 – size where 10 % of the population is below; d_v50 – size where 50 % of the population is below/above; d_v90 – size where 90 % of the population is below; span – distribution width; d_{32} – Sauter Mean Diameter; d_{43} – De Brouckere Mean Diameter; ssa – the specific surface area of fat globules. Results are expressed as mean value ± standard deviation. The values in the column marked with different small letters differ significantly at $p<0.05$.

in the absence of any effect caused by the process on milk conductivity.

The application of high pressure homogenisation resulted in the lowering of the resistance of milk to the effects of coagulating factors, *i.e.* a high temperature or a proteolytic enzyme. This is reflected in the reduction in the thermal coagulation time and rennet coagulation time to a degree determined by the way the process was carried out, and by the temperature of milk intended for homogenisation (Table 1). The rennet coagulation time of milk was reduced as a result of full--stream homogenisation carried out at 20°C, 40°C, and 60°C by 19%, 17%, and 12%, respectively, as compared to the control sample. In turn, partial homogenisation contributed, though to a lesser extent, to changes in the rennet coagulation time of milk, which, in the case of temperatures of 20°C, 40°C, and 60°C amounted to 12%, 10%, and 9%, respectively (statistically insignificant changes at $\alpha=0.05$). A similar trend in changes depending on the homogenisation method was also observed for heat stability. Full-stream homogenisation carried out at temperatures of 20°C, 40°C, and 60°C resulted in a statistically significant ($p<0.05$) reduction in the thermal coagulation time by 44%, 42%, and 44%, respectively, while partial homogenisation carried out at analogous temperatures caused reductions by 31%, 29%, and 31%, respectively. The obtained results indicate that the way the process is carried out contributes to changes in the analysed properties of milk to a greater extent than the temperature of milk intended for homogenisation. In particular, this is reflected in statistically significant differences ($p<0.05$) between the values of heat stability of milk subjected to full-stream and partial high pressure homogenisation.

The literature data indicate a reduction in the rennet coagulation time of milk and a change to the properties of rennet curd [Zamora *et al.*, 2007], as well as a decrease in ethanol stability [Amador-Espejo *et al.*, 2014] and in heat stability [Kiełczewska *et al*., 2008] as a result of high pressure homogenisation. The following are responsible for the decrease in the resistance of homogenised milk to coagulation factors: the loss of dynamic balance of proteins due to the dissociation of a portion of certain fractions of micellar casein, including the fraction κ serving the protective function over casein micelles [Sandra & Dalgleish, 2005], the denaturation of whey proteins [Pereda *et al.*, 2009], and the adsorption of protein components of milk plasma on the interfacial surface [Zamora *et al.*, 2012].

Modification of the colloidal phase leads to a loss of balance of mineral salts of milk due to the dissociation of the colloidal calcium phosphate and phosphorus and an increase in the soluble phase of milk [Kiełczewska *et al.*, 2008; Zamora *et al.*, 2007]. Such destabilisation of the colloidal phase components, *i.e.* proteins and mineral salts is reflected in the change in the distinguishing features of technological suitability, *i.e.* heat stability and rennet coagulation time as well as the acidity and the conductivity of milk [Banach *et al.*, 2008; Kiełczewska *et al.*, 2003]. The research presented in this publication indicates that rennet coagulation time and heat stability of milk following partial homogenisation are closer to the values found for non-homogenised milk, and are longer than the corresponding values for the discussed features in milk subjected to full-stream homogenisation. Thus, it can be concluded that milk subjected to partial homogenisation has a longer shelf-life in the aspect of colloidal phase, preserving other production parameters in relation to milk subjected to full-stream homogenisation.

The effect of high pressure homogenisation on an increase in the sensitivity of full fat milk to coagulation factors is of particular significance during the preservation of dairy products using high temperatures and determines their storage stability. Carrying out high pressure homogenisation, due to the simultaneous action of preserving factors, *i.e.* pressure and temperature, enables the modification of milk technology and, when the parameters are optimised, provides the possibility of affecting the physicochemical properties of milk that are significant from a practical point of view. The application of partial homogenisation, which only involves homogenisation of cream, while skimmed milk, including all its components, remains unhomogenised, contributes to a lesser extent to changes in the discussed characteristics of milk. An additional issue is the impact of high pressure homogenisation on sensory properties of milk. Previous studies [Amador-Espejo *et al.*, 2014] did not indicate the influence of pressure homogenisation on the sensory characteristics of milk. However, research

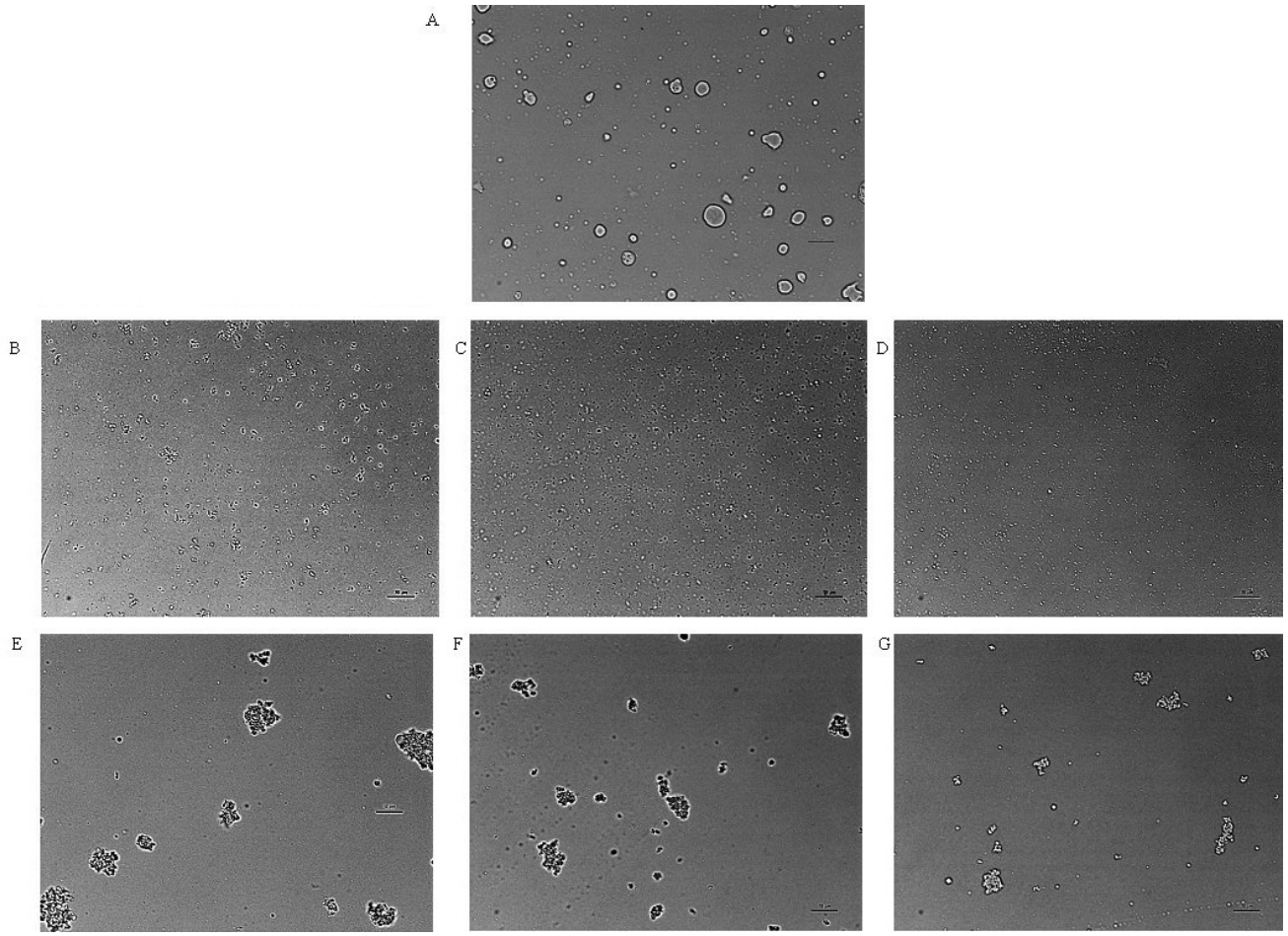

FIGURE 1. Microscopic images of unhomogenised milk (A) and milk homogenised under pressure of 100 MPa using the full-stream homogenisation at temperatures of 20°C (B), 40°C (C), and 60°C (D); and the partial homogenisation at temperatures of 20°C (E), 40°C (F), and 60°C (G).

on the impact of partial-stream homogenisation, in comparison with full-stream method, using quantitative and qualitative consumer methods and sensory analysis would be useful.

The main aim of homogenisation is to reduce and unify the sizes of fat globules. The effectiveness of the high pressure homogenisation process, *i.e.* the degree of reduction in (and unification of) the sizes of fat globules is determined by many factors, the most important of which include the composition of the emulsion (fat and non-fat dry matter contents), homogenisation parameters (pressure and temperature), the type and design of the homogeniser valve, the number of homogenisation steps, Thoma number [Kessler, 1981], and the way the process is carried out (full-stream and partial homogenisation).

The results of the performed measurements indicated the effect of full-stream and partial homogenisation on the reduction of all distinguishing features of fat globule sizes to range depending on temperature (Table 2). Many authors indicate the effect of increasing temperature of high pressure homogenisation on the reduction of fat globule sizes in milk [Hayes & Kelly, 2003a; Thiebaud *et al.,* 2003; Zamora *et al.*, 2012].

In the course of this study, higher values of the distinguishing features of dispersion of the emulsion phase were noted in the milk homogenised partially as compared to the milk homogenised by the full-stream method at analogous temperatures. Full-stream homogenisation contributed to a 4–4.4-fold decrease in the d_{32} value, while partial homogenisation only resulted in 1.3–2.2-fold changes in comparison to the control sample. A reduction in fat globule sizes is associated with an increase in their surface, which is appropriate to the way the process is carried out (homogenisation method, temperature). Statistically significant differences ($p<0.05$) were demonstrated in the values describing the size of fat globules and their surface area depending on both the way homogenisation was carried out and the temperature applied.

In the milk subjected to partial homogenisation, the occurrence of large-sized particles was found, characterised by high values of d_v90 and d_{43}, which are greater in size than values in the non-homogenised milk. This phenomenon can be explained by supplementing instrumental studies with an analysis of microscopic images of the emulsions under study, which provide information on the dispersion of fat globules, and primarily the occurrence and identification of the forms of emulsion instability, *e.g.* the flocculation of fat globules. Based on the analysis of microscopic images, it was found that particular fat globules in milk homogenised by both the full-stream and partial method had smaller sizes than those characterising fat globules in the unhomogenised milk

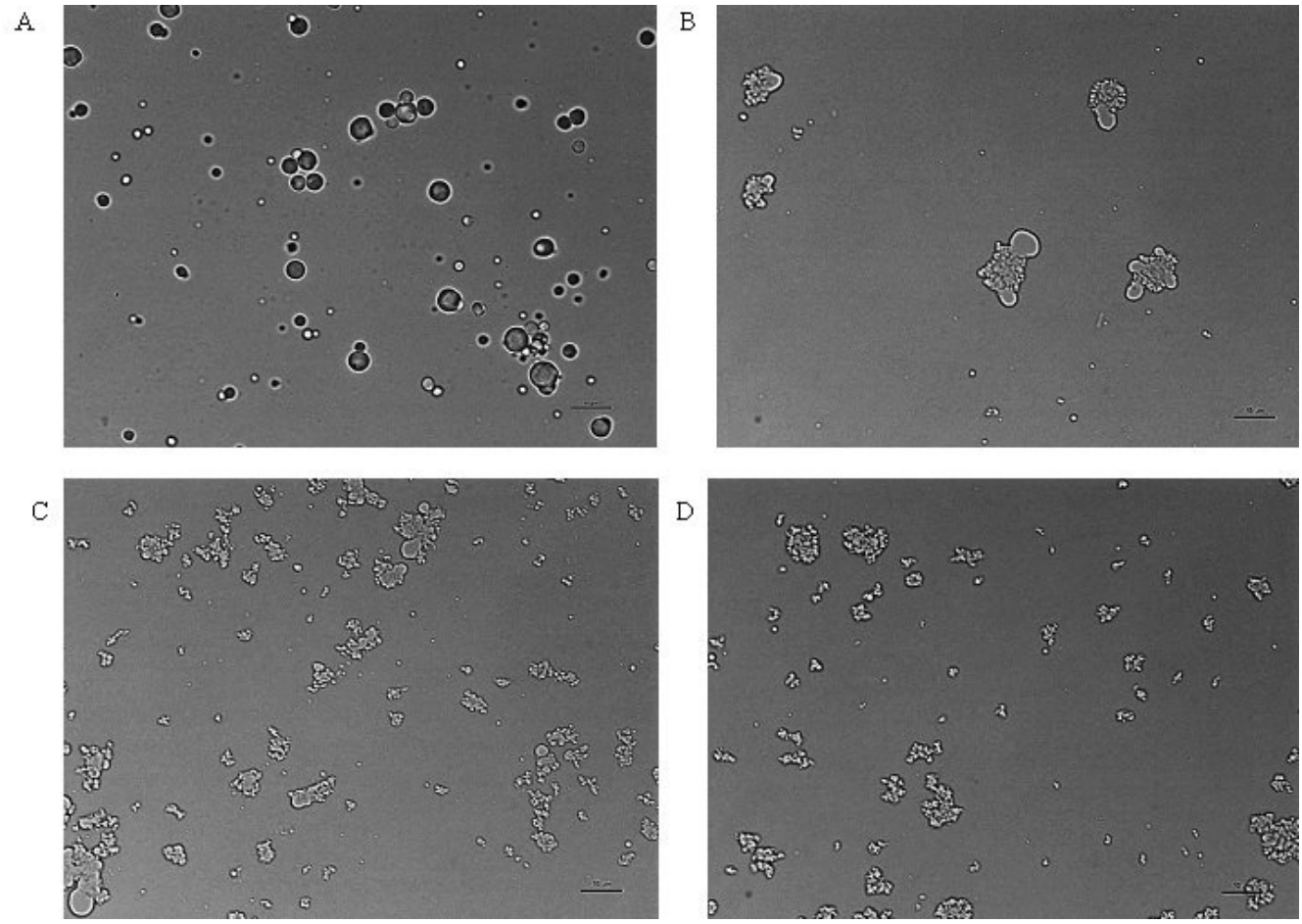

FIGURE 2. Microscopic images of unhomogenised cream (A) and cream homogenised under pressure of 100 MPa at a temperature of 20°C (B), 40°C (C), 60°C (D).

(Figure 1). This indicates a 4.7–6-fold reduction in the sizes of d_v10 of fat globules as a result of full-stream homogenisation and a 2.3–2.7-fold reduction as a result of partial homogenisation (Table 2). Therefore, in the milk subjected to partial homogenisation, a reduction in fat globule sizes also occurs; however, some of the globules are found in the form of agglomerates (Figure 1E-1G). This is also confirmed by microscopic observation of homogenised cream used to standardize milk (Figure 2).

During the determination of the size of particles with a device using laser diffraction, the measurements were applied to fat globules as well as to the agglomerates, which, during the measurement, were counted as separate particles. The fact that during the study, both individual fat globules and their agglomerates were measured in the measurement cell, is fully justified as in the potential finished product, both agglomerates and fat globules are subject to the formation of the cream layer. Therefore, partial homogenisation of milk should also apply one of the methods used for preventing the fat globule agglomeration process, *e.g.* two-stage homogenisation or an addition of an emulsifier.

In the shaping of the degree of dispersion of milk fat, it is the composition of the product being homogenised (mainly the fat content–to–milk plasma protein content ratio) that is crucial. The main determinant of the effectiveness of homogenisation is the fat content of the product and the number of surface-active plasma components, as they are involved in the reconstruction of an envelope on the newly-formed fat––milk plasma interfacial surface, *i.e.* on the surface of the fat globules being homogenised. The fat content–to–protein content ratio determines the size of fat globules as well as the associated surface of fat and the level of milk protein adsorption. In milk, the rate of protein adsorption on the surface of fat is higher than the rate of fat globule interactions, and, consequently, the dispersion of milk fat occurs (the so-called micronisation of the emulsion phase). With an increase in fat content, the rate of interactions between fat globules significantly exceeds the rate of protein adsorption on the fat/milk plasma interfacial surface and the effectiveness of the process decreases. This explains the increasing, stronger trend of fat globules for agglomeration at a high fat content and at high pressure, where the fat surface is too large for the envelopes to be completely reconstructed by the surface-active components of milk. It is considered that the main cause responsible for diminished effectiveness of homogenisation of dairy products with a high fat content, *e.g.* cream, is the flocculation, *i.e.* the formation of durable clusters of fat globules. This phenomenon is accompanied by a trend towards the formation of a cream layer on product surface. Such a state is referred to as viscolisation and the product is called viscolised. At elevated

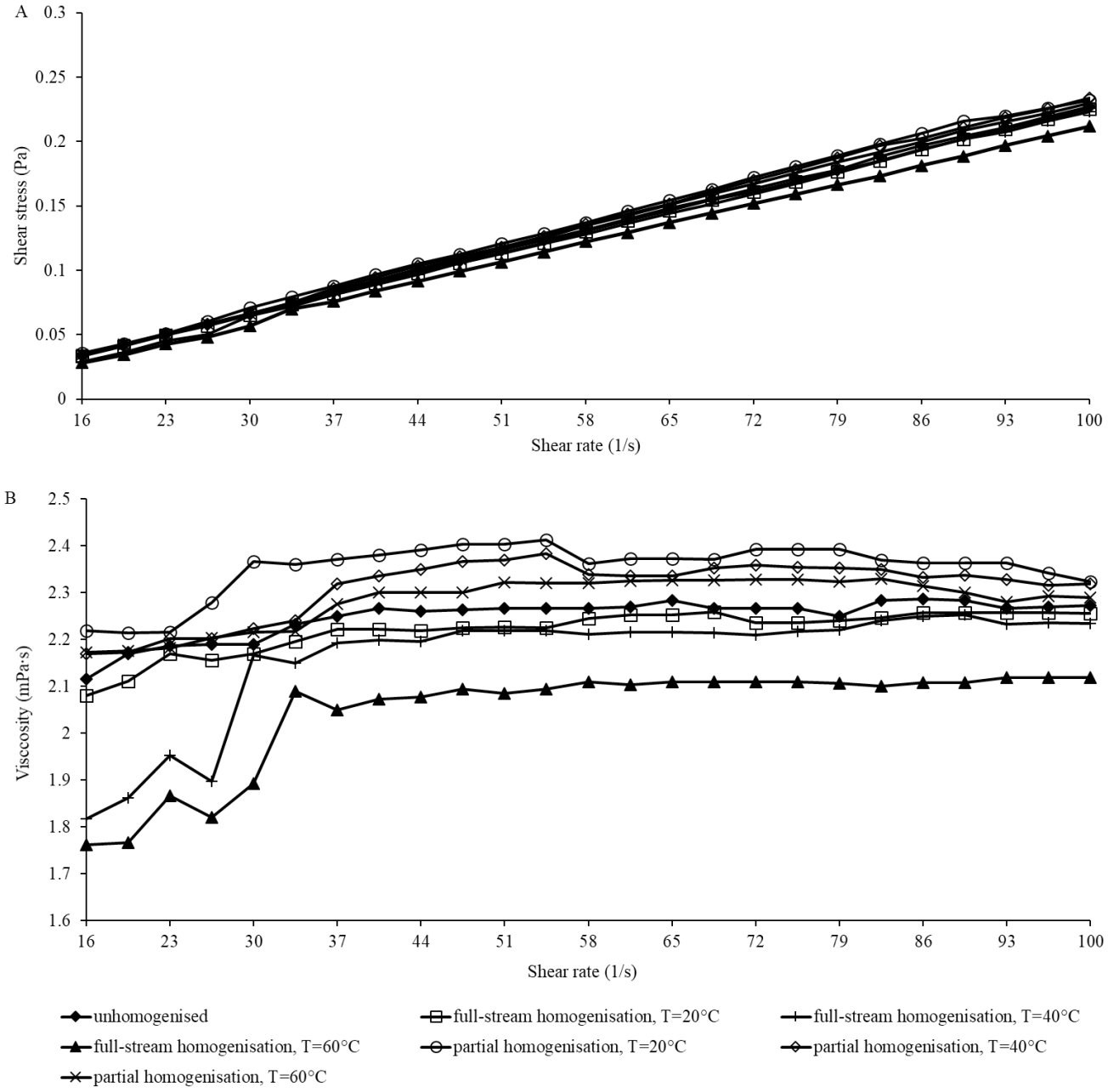

FIGURE 3. The effect of homogenisation on shear flow (A) and viscosity (B) of milk.

Legend: ♦ non-homogenised; ▲ full-stream homogenisation, T= 60ºC; + full-stream homogenisation, T=40ºC; □ full-stream homogenisation, T=20ºC; × partial homogenisation, T=60ºC; ◊ partial homogenisation, T=40ºC; ○ partial homogenisation, T=20ºC

pressure and temperature parameters, the coalescence of fat globules may occur, which can contribute to the greater variation in fat globule sizes [Kessler, 1981; Rodarte *et al.*, 2018].

During the research, milk shear flow curves were determined, and a similar course of changes was found, both as regards a control sample and all milk samples under analysis, subjected to high pressure homogenisation, regardless of the method or temperature applied (Figure 3A). An effect was found of full-stream high pressure homogenisation that viscosity decreased, when the temperature increased (Figure 3B). This was confirmed by the results of studies by Amador-Espejo *et al.* [2014] and by Pereda *et al.* [2007], who demonstrated a reduction in viscosity as a result of high pressure homogenisation carried out at pressures of up to 200 MPa, with a trend of changes developing with an increase in the temperature. The application of partial homogenisation contributed to an increase in milk viscosity, with an increasing range of changes, with a reduction in the temperature of milk earmarked for homogenisation. The effect of homogenisation on milk viscosity was statistically significant at $p<0.05$. The causes of the increase in milk viscosity occurring as a result of partial homogenisation, should undoubtedly be sought in the flocculation of fat globules as well as the presence of large agglomerates, confirmed by the results of measurement of particle sizes, and documented by microscopic images. The slight decrease in the viscosity of partially

homogenised milk at higher shear rate values resulted probably from the breaking of fat globule agglomerates during the measurement.

CONCLUSIONS

Omission of the skimmed milk in the homogenisation process allows maintaining components of the colloidal and soluble phases at the least changed state. Milk properties that depend on milk plasma components (mainly proteins) are determined only through changes in the emulsion phase of cream, and are changed to the lowest extent in comparison to milk homogenised by a full-stream homogenisation. This mainly concerns rennet coagulation time and the heat stability of milk.

Striving to obtain the product with features most similar to those of raw milk, partial homogenisation seems to be the best technological solution. However, partial high pressure homogenisation is second to the process carried out by the full-stream homogenisation in terms of its effectiveness of dispersing fat globules in milk. Modification of the emulsion phase was reflected in the course of the viscosity curves subject to full-stream homogenisation (decrease) and partial homogenisation (increase). This drawback is caused by the fact that partial homogenisation results in the presence of post-homogenisation clusters in milk, which can, however, be split in the second stage of the process. This provides real opportunities for using partial homogenisation in combination with other alternative preservation methods which are non-invasive for milk components.

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, Project No. 010/RID/2018/19, amount of funding 12.000.000 PLN.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Submitted: 25 October 2018. Revised: 5 April and 30 May 2019. Accepted: 12 June 2019. Published on-line: 5 July 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 289–296
DOI: 10.31883/pjfns/110564
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Effects of Biopreservatives Combined with Modified Atmosphere Packaging on the Quality of Apples and Tomatoes

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Key words: bacteriocin-like substances, biopreservative, comprehensive storage, fresh fruit and vegetables, MAP

During the cultivation and harvesting of fruit and vegetables, a large number of microorganisms accumulate on their surface. Their active and excessive reproduction leads to spoilage of products. The purpose of the study was to assess the effect of combining various packaging technologies with different biopreservatives on the stability of physicochemical and microbiological characteristics of fresh vegetables and fruit during storage. Samples of fruit and vegetable products (apples, tomatoes) were subjected to the following procedures: packaging without treatment, treatment with a mixture of bacteriocin-like substances and packaging with or without modified atmosphere. Packaged samples were stored in a refrigerator at a temperature of 4°C for 25 days. The bacteriocin-like substances in combination with modified atmosphere reduced the contamination of samples by pathogenic microorganisms at least 4 times while maintaining the quality characteristics of the fruit during the storage period. A biopreservative in combination with modified atmosphere can be used to control microbial spoilage and to keep fruit and vegetables fresh after harvest.

INTRODUCTION

Fruit and vegetables are the best sources of nutrients. They contain natural ingredients such as carbohydrates, proteins, organic acids, vitamins, *etc*. [Watson & Preedy, 2016; Dyshlyuk *et al*., 2017]. Consumption of fruit and vegetables remains stable, despite their price increase. One of the reasons for the increase in production costs is waste (at least 20% of all fruit and vegetables produced spoil, not reaching the consumer) [FAO, 2011; De Laurentiis *et al*., 2018; Prosekov & Ivanova, 2018]. In the process of maturing, harvesting, transporting, and storing, the fresh vegetables and fruit remain unprotected from sources of contamination by pathogenic bacteria, spores of mold and fungi that remain active for a long time [Paskeviciute *et al.*, 2018]. Economic losses are also caused by microorganisms (bacteria, fungi, mold) that colonize fruit surface [Buzby & Hyman, 2012; Jaeger *et al.*, 2018]. Appropriate post-harvest treatments can minimize moisture loss, slow the respiration rate and save fruit for the consumer. The technologies for preserving the qualitative characteristics of fresh fruit and vegetable products include, first of all, ensuring certain climatic conditions (temperature, humidity, illumination), as well as washing, vacuum packaging, treatment with preservatives, irradiation with ultraviolet, use of a modified gas medium and their combination [Ma *et al*., 2017; Putnik *et al*., 2017].

The most promising is the complex use of modified atmosphere packaging with other technologies (temperature regime, biopreservation, UV irradiation, *etc*.), which determine a double barrier to the growth of microorganisms and ensure the safety and preservation of food products, including fresh fruit and vegetables [Allende & Artés, 2003; Alvarez *et al*., 2015; Hussein *et al.*, 2015; Oliveira *et al.,* 2015].

The work performed by Wang *et al*. [2015] presents technology of preserving cherries in the process of long-distance sea transportation at a temperature of 0°C. Colgecen & Aday [2015] preserved cherries in modified atmosphere packaging (MAP) with a chlorine dioxide dissolver for 5 weeks without visible damage to the fruit by rot and mold at a temperature of 4°C. Argyri *et al.* [2015] used packaging with modified gas medium for olives fermented with lactic acid bacteria; the probiotic characteristics of this product were retained within 6-month storage in refrigerator. There are enough studies that use bacteriocins of lactic acid bacteria or bacteriocin-like inhibitory substances (BLIS) produced by *Bacillus cereus* to preserve food products, including fruit and vegetable products [Settanni & Corsetti, 2008; Leroi *et al*., 2015;

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Leite *et al*., 2016; Cavicchioli *et al*., 2017; Ho *et al*., 2018]. The search for producers of bacteriocins of a broad spectrum of action and safe for humans is being continued [Nes *et al*., 2006; Molloy *et al*., 2011; Burke *et al*., 2013; O'Bryan *et al*., 2015]. However, there are no works in free access with the results of the joint use of biopreservatives and MAP.

In the present study, the effect of bacteriocin-like substances, a consortium of bacteria from the surface of fruit and vegetable products, in combination with various packaging technologies on the stability of the physicochemical and microbiological characteristics of fresh vegetables and fruit has been assessed under conditions simulating commercial use during storage at various temperatures.

MATERIALS AND METHODS

Materials and chemicals

Fresh ripe red tomatoes of Alcazar cultivar and yellow apples of Golden cultivar were purchased in the retail networks of the city of Kemerovo (Russian Federation) in August 2017. Fruit of medium size (apples 180±10 g, tomatoes 150±10 g) with no visible damage were selected.

Food gases (carbon dioxide CO_2, oxygen O_2, nitrogen N_2) were purchased from Linde Gas (St. Petersburg, Russia). The study used acetic acid and boric acid (LLC Component-Reactiv, Russia); hydrochloric acid, sodium acetate (LLC Component-Reactiv, Russia); isopropanol (LLC Plant Chemisol, Russia) and all other chemicals of analytical or higher grade.

Bacteriocin preparation

Cultures of microorganisms (*Bacillus pumilus, Bacillus safensis*) were isolated from fruit and vegetables [Zimina *et al*., 2016]. Triturated fruit were placed into a liquid nutrient broth medium (meat-peptone broth) and cultivated at 30±2°C, 37±2°C and 45±2°C for 1–5 days. When sufficient biomass was grown, inoculation was performed onto nutrient agar (meat-peptone agar) daily until separate colonies were formed. Genetic analysis of the strains was performed for identification *via* 16S RNA sequencing using the Sagner Method with "ABI Prism Big Dye Terminator Cycle Ready Reaction Kits Sequencing" (Appliedbiosistems, USA) and GSJunior 454 sequencer (Roche, Switzerland). Isolated microorganisms were stored in a freeze-dried state in ampoules at a temperature of 4±2°C for at least 24 months.

Lyophilized cultures of the isolated microorganisms were reconstituted by transferring the contents of ampoules to tubes containing sterile skimmed milk. Cultures were incubated at 37°C. 5.0% inoculum was added to MRS-broth and incubated at a temperature of 37°C for 16 h until the concentration of microorganisms reached 1.5×10^6 CFU/mL.

At the end of the cultivation, the biomass was separated from the nutrient medium by centrifugation at 8000×*g* for 20 min. Then, the supernatant was concentrated on hollow fibers which cut off substances with a molecular mass of more than 15 kDa; stirred with dry sodium chloride (0.5 M) at a stirring speed of 100 q/min for 20 min; centrifuged at 10,000×*g* for 15 min. Water was added to the precipitate at a volume of 0.1% of the supernatant washed with isopropyl alcohol (0.1% of the supernatant) for 30 min at a temperature of 0°C.

Evaporation of isopropanol was carried out with a rotary evaporator at a temperature of 60°C. The solution with water and activated carbon (0.5% w/v) was kept for 15 min, centrifuged at 10,000×*g* for 15 min; filtered through a membrane that cuts off molecules with a molecular weight of more than 10 kDa. Lyophilization of the obtained biopreservative compounds was carried out with the following parameters: duration 90 min, drying temperature 30°C, drying duration 6 h, drying layer thickness 2 mm.

Sample preparation

Apple and tomato fruit were stored at 4°C and at atmospheric pressure after purchase. Fresh fruit were washed with tap water, dried at room temperature and immersed in a 1% solution of biopreservative for 1 min. Each fruit was considered as an experimental unit and, after treatment with bacteriocin, was placed in a sealed packet with modified atmosphere. Three different combinations of fruit treatments were examined: in a package without treatment (control), in a package with biopreservative treatment (BIO), and in a package with a modified gas medium and with biopreservative treatment (BIO+MAP). In total, 288 fruit (144 apples and 144 tomatoes) were packed for each treatment method. The treated samples were stored at 4°C for 25 days. Sampling for microbiological analyses was performed on day 0, 5, 10, 15, 20, and 25 for measurements of the basic microbiological parameters (pathogenic bacteria and fungi growth), whereas sampling for the evaluation of quality indicators during storage was done on day 0 and 25. Sampling on day 0 was performed 2 h after packaging. Samples were aseptically removed from the package.

Packaging technologies

Fresh fruit were sorted at room temperature and packaged aseptically into 10 × 10 cm polypropylene bags (0.025 mm thickness) under the following conditions: MAP1 – 3% O_2 + 95% N_2 + 2% CO_2 (apples); MAP2 – 3% O_2 + 77% N_2 + 20% CO_2 (tomatoes). Bags containing fruit were sealed and checked for punctures or tightness of the seal by immersion into water.

Microbiological analysis

Washings from the surface of the fruit were done with a sterile cotton pad soaked in a peptone-salt solution (different parts of the fruit surface with a total area of 100 cm^3 were wiped). The peptone-salt solution was prepared as follows: under slow heating. The pad was placed in a test tube with 10 mL of the peptone-salt solution (8.5 g of sodium chloride and 1.0 g of peptone were dissolved in 1 L of distilled water with pH 7.0±0.1). The contents of the test tube were mixed thoroughly. The resulting suspension was considered the initial dilution.

The washings from the surface of fruit were put on the agar surface (VRBL and SABOURAUD). The total number of microorganisms after the treatment of the fruit was determined by inoculating washings from the surface of the fruit

on the agar in a Petri dish. Each Petri dish was filled with about 15 mL of 44–47°C VRBL-agar culture medium (Merck, Germany) to determine pathogenic microorganisms. After the medium in the Petri dishes had solidified, about 4 mL of 44–47°C used agar medium were poured to create a second layer. SABOURAUD-4% dextrose agar (Merck, Germany) was used to determine the presence of fungi.

After solidification, the prepared dishes (upside down) were incubated at a temperature of (37±1)°C for (24±2) h to detect pathogenic bacteria, and at a temperature of (25±1)°C for 5 days to detect fungi (additionally, growth media were left at room temperature for 1 to 2 days). Regardless of the presence or absence of growth signs including blackening, inoculation with a smear loop was performed from the culture liquid on the surface of one of the agarized selective diagnostic mediums: Chromocult Coliform Agar ES (Merck, Germany), OXFORD agar (Merck, Germany), or PALCAM-agar (FBUN SSC PMB Obolensk, Russia). After incubation, Petri dishes with 15 to 150 CFU of bacteria or yeast and 5 to 50 CFU of fungi were selected and counted. The sensitivity limit for the bacterial number was about 10^2 CFU/mL.

Species of fungi present on the surface of the fruit was determined by molecular genetic analysis. The nucleotide sequence of 16S RNA gene was determined through the 16S RNA fragment sequencing using the Sanger method [Sanger *et al*., 1977].

Quality analysis

To calculate the average weight of fruit in grams, 30 tomatoes and 30 apples were weighed, respectively, on the electronic scales (BK 600.1, MASSA-K, Russia) with an accuracy of 0.001 g.

Mass fraction of total soluble solids (TSS) was determined by placing 0.5 mL of a fruit homogenate, purified by centrifugation (4000×*g*, 10 min), on hand prism refractometer Atago PR1 (ATAGO CO., LTD, Tokyo, Japan), and the results are presented in ° Brix [Javanmardi & Kubota, 2006].

Titratable acidity was determined by titration of 10 mL of fruit homogenate to a pH value of 7.6 using 0.1 M sodium hydroxide and expressed in g/L of citric acid; measurement was performed with a digital pH-meter (pH-150M, Gomel'skiy Zavod Izmeritel'nykh Priborov, Russia).

The firmness of the fruit was evaluated by puncture strength (N) test with a digital penetrometer PCE-PTR 200 (PCE Instruments UK Ltd, Hampshire, UK). Fruit were compressed with a constant speed of 0.95 mm/s, the diameter of the puncture was 5 mm.

The color of vegetables was determined using Minolta Chroma Meter model CR-200B (Konica Minolta, Tokyo, Japan) at three different points located in the equatorial area [HunterLab, 2008]. The colorimeter has a beam diameter of 8 mm, three response detectors set at 0 viewing angle and a CIE standard illuminant C with diffuse illumination. This illuminant is accepted as having a spectral radiant power distribution closest to reflected diffuse daylight. Color changes were documented throughout the experiment. L values indicate lightness (black [L = 0] and white [L = 100]), a values indicate redness-greenness (red [a=100] and green [a=-100]), b values indicate yellowness-blueness (yellow [b=100] and blue [b=-100]). Chroma (C) ($C=\sqrt{(a^2+b^2)}$) measures color saturation or intensity and the hue angle ($h=1/\tan(b/a)$) determines the red, yellow, green, blue, purple, or intermediate colors between adjacent pairs of these basic colors. A lower hue value indicates a redder product [Ayala-Silva *et al*., 2005].

Statistical analysis

Each experiment was repeated three times and data were expressed as means ± standard deviation. Data processing was carried out by standard methods of mathematical statistics. Homogeneity of the sampling effects was checked using the Student's t-test. The data were subjected to the analysis of variance (ANOVA) using Statistica 10.0 (StatSoft Inc., 2007, USA). Differences between means were considered significant when the confidence interval is smaller than 5% ($P \leq 0.05$).

RESULTS AND DISCUSSION

At the preliminary stage of the study, the compositions of modified gas mixtures for fruit storage were determined, the gas ratio (O_2 : N_2 : CO_2) 3.0 : 95.0 : 2.0 and 3.0 : 77.0 : 2 0.0 in %, respectively, for apples and tomatoes. Similar combinations of gases of the modified gas mixture prevented the development of aerobic bacteria (*Salmonella, Clostridium Botulinum, Moraxella*, and *Acinobacter*), counteracted the development of mold, reduced the pH level in the medium of the packaged product, and did not deform or compress the product, which is important when packaging fresh-cut and minimally processed fruits and vegetables.

Samples of apples and tomatoes were examined for microbiological growth during storage, depending on the selected packaging technology (Tables 1–2). In all samples, regardless of the packaging technology, *E. coli* and other pathogenic bacteria (including *L. Monocytogenes*) were not detected during the entire study period. After 15 days of storage, apples and tomato fruit treated only with the biopreservative contained at least 40% less fungi than the untreated samples, while the fruit treated with the biopreservative in combination with MAP had no fungal growth. Apples, processed with the biopreservative and packaged with modified atmosphere, did not contain fungi after 25 days of storage. Fruit packaged without modified atmosphere contained at least 13% less fungi than samples without treatment. The content of fungi in the untreated tomato samples (control) exceeded the content of fungi in the treated samples packaged with modified atmosphere (over four times more), as well as in the treated fruit samples without MAP (on 84%).

Packaging of cherry tomatoes in polypropylene or polyethylene bags of low density in a modified gas mixture (5% O_2, 5% CO_2) increased their shelf life at a temperature of 5°C to 25 days. The combination of UV irradiation, low temperatures, and MAP (5.3% CO_2 + 5.5% O_2) allowed improving the antioxidant quality and microbiological safety, and extending the shelf life of cherry tomatoes [Choi *et al*., 2015]. Irradiation of strawberries and MAP (CO_2 – 10%: O_2 – 5%; N_2 – 85%) allowed increasing their storage period at a temperature of 4°C to 14 days with the preservation of external quality

TABLE 1. Changes in fungal growth of packaged apple samples within 25 days of storage.

Duration of storage (days)	*Penicillium expansum* (log CFU/g)	*Rhizopus stolonifer* (log CFU/g)	*Botrytis cinerea* (log CFU/g)
Control			
0	NF[a]	NF[a]	NF[a]
5	0.5±0.0[a]	1.0±0.1[a]	1.2±0.1[a]
10	2.2±0.1[a]	3.4±0.2[a]	3.0±0.3[a]
15	5.8±0.3[a]	6.5±0.3[a]	5.5±0.2[a]
20	7.1±0.4[a]	7.6±0.4[a]	6.4±0.3[a]
25	9.6±0.4[a]	10.0±0.4[a]	8.8±0.3[a]
BIO			
0	NF[b]	NF[b]	NF[b]
5	NF[b]	NF[b]	NF[b]
10	NF[b]	NF[b]	NF[b]
15	4.0±0.1[b]	3.5±0.1[b]	NF[b]
20	5.8±0.3[b]	4.9±0.3[b]	3.6±0.2[b]
25	8.5±0.3[b]	7.0±0.2[b]	4.5±0.1[b]
BIO+MAP1			
0	NF[c]	NF[c]	NF[c]
5	NF[c]	NF[c]	NF[c]
10	NF[c]	NF[c]	NF[c]
15	NF[c]	NF[c]	NF[c]
20	NF[c]	NF[c]	NF[c]
25	NF[c]	NF[c]	NF[c]

Control – samples not processed with biopreservative and packaged without modified gas mixture; BIO – samples treated with biopreservative; BIO + MAP1 – samples treated with biopreservative and packaged with a modified gas medium; NF – not found. The data are expressed as mean ± standard deviation (n=3). Values followed by different letters in a column are significantly different (P≤0.05) by LSD post-hoc test.

TABLE 2. Changes in fungal growth of packaged tomato samples within 25 days of storage.

Duration of storage (days)	*Penicillium expansum* (log CFU/g)	*Rhizopus stolonifer* (log CFU/g)	*Botrytis cinerea* (log CFU/g)
Control			
0	NF[a]	NF[a]	NF[a]
5	1.1±0.1[a]	0.8±0.0[a]	1.4±1.0[a]
10	2.5±0.2[a]	2.6±0.2[a]	2.2±0.1[a]
15	6.3±0.3[a]	7.0±0.3[a]	5.8±0.2[a]
20	8.7±0.5[a]	9.2±0.4[a]	7.5±0.3[a]
25	11.4±0.5[a]	12.3±0.5[a]	9.0±0.3[a]
BIO			
0	NF[b]	NF[b]	NF[b]
5	NF[b]	NF[b]	0.2±0.0[b]
10	1.8±0.1[b]	1.5±0.1[b]	1.7±0.1[b]
15	4.5±0.1[b]	3.0±0.1[b]	2.5±0.1[b]
20	5.0±0.2[b]	4.8±0.2[b]	3.8±0.2[b]
25	5.6±0.2[b]	6.0±0.2[b]	4.9±0.2[b]
BIO+MAP2			
0	NF[c]	NF[c]	NF[c]
5	NF[c]	NF[c]	NF[c]
10	NF[c]	NF[c]	NF[c]
15	NF[c]	NF[c]	NF[c]
20	NF[c]	NF[c]	NF[c]
25	1.2±0.1[c]	3.0±0.1[c]	1.8±0.1[c]

Control – samples not processed with biopreservative and packaged without modified gas mixture; BIO – samples treated with biopreservative; BIO + MAP2 – samples treated with biopreservative and packaged with a modified gas medium; NF – not found. The data are expressed as mean ± standard deviation (n=3). Values followed by different letters in a column are significantly different (P≤0.05) by LSD post-hoc test.

characteristics [Jouki & Khazaei, 2014; Fagundes *et al*., 2015]. The obtained data agree with those already published. Thus, we can state that combining the treatment of fresh vegetables and fruit with a biopreservative, based on bacteriocins of lactic acid or other bacteria, and MAP is safe for humans. In addition, MAP preserves the qualitative characteristics of fruit and vegetable products. The shelf life of these products can be prolonged as well, without using any chemicals.

The results of studying the qualitative characteristics (fruit weight, TSS, pH, firmness, and color) of fruit during storage are presented in Tables 3–4. The tissues structure of apples and tomatoes was not damaged during the experiment. The loss in fruit weight leads to the quality decrease of the product [Briassoulis *et al*., 2013]. Obviously, during storage, the weight of a fruit decreases, but its intensity is determined by the method of packaging and storage [Khorshidi *et al*., 2010]. When stored at room temperature, the reduction in fruit weight is always more pronounced than when stored in refrigeration units. Tomato and apple weight loss was less than 5% within 25 days of storage, depending on the method of packaging. According to Kader & Saltveit [2003], about 3–5% post-harvest weight loss of the product is due to the release of CO_2 through the fruit skin. Weight loss of more than 3–5% usually leads to the loss of freshness.

In the package without modified atmosphere, the weight loss was higher than in the package with modified atmosphere when storing tomatoes and apples, but did not exceed 5% within 25 days. It is possible that the presence of an antimicrobial coating and a modified gaseous medium led to a decrease in the moisture evaporation rate by reducing the permeability of the peel. However, weight loss of less than 3% after 25 days of storage is a good indicator for increasing the shelf life of tomatoes and apples in packages with modified atmosphere and an antimicrobial agent.

TABLE 3. Changes of quality indicators of packaged apple samples within 25 days of storage.

Duration of storage (days)	Indicators				
	Weight (g)	TSS (° Brix)	pH	Firmness (N)	Hue angle (h)
Control					
0	184.2±9.0[a]	12.9±0.6[a]	3.5±0.2[a]	73.6±3.6[a]	78.0±4.0[a]
5	182.5±9.1[a]	12.9±0.6[a]	3.5±0.2[a]	73.1±3.6[a]	78.0±4.0[a]
10	180.9±9.0[a]	13.0±0.7[a]	3.5±0.2[a]	73.0±3.6[a]	78.0±4.0[a]
15	178.2±8.9[a]	13.1±0.7[a]	3.5±0.2[a]	72.9±3.6[a]	78.0±4.0[a]
20	176.0±8.9[a]	13.3±0.7[a]	3.5±0.2[a]	72.8±3.6[a]	78.0±4.0[a]
25	175.4±8.9[a]	13.4±0.7[a]	3.4±0.2[a]	72.7±3.7[a]	78.0±4.0[a]
BIO					
0	184.2±9.0[a]	12.9±0.6[a]	3.5±0.2[a]	73.6±3.6[b]	78.0±4.0[a]
5	182.2±9.1[a]	13.0±0.7[a]	3.5±0.2[a]	73.5±3.6[b]	78.0±4.0[a]
10	181.5±9.1[a]	13.1±0.7[a]	3.5±0.2[a]	73.5±3.6[b]	78.0±4.0[a]
15	179.9±9.0[a]	13.1±0.7[a]	3.5±0.2[a]	73.4±3.6[b]	78.0±4.0[a]
20	176.1±8.8[a]	13.2±0.7[a]	3.5±0.2[a]	73.3±3.6[b]	78.0±4.0[a]
25	175.6±9.0[a]	13.5±0.6[a]	3.4±0.2[a]	73.3±3.6[b]	78.0±4.0[a]
BIO+MAP1					
0	184.2±9.0[a]	12.9±0.6[a]	3.5±0.2[a]	73.6±3.6[b]	78.0±4.0[a]
5	183.8±9.2[a]	13.0±0.7[a]	3.5±0.2[a]	73.6±3.6[b]	78.0±4.0[a]
10	181.3±9.1[a]	13.0±0.7[a]	3.5±0.2[a]	73.6±3.6[b]	78.0±4.0[a]
15	179.4±9.0[a]	13.2±0.7[a]	3.5±0.2[a]	73.5±3.6[b]	78.0±4.0[a]
20	179.0±9.0[a]	13.3±0.7[a]	3.5±0.2[a]	73.5±3.6[b]	78.0±4.0[a]
25	178.8±8.9[a]	13.5±0.6[a]	3.3±0.2[a]	73.4±3.7[b]	78.0±4.0[a]

Control – samples not processed with biopreservative and packaged without modified gas mixture; BIO – samples treated with biopreservative; BIO + MAP1 – samples treated with biopreservative and packaged with a modified gas medium. The data are expressed as mean ± standard deviation (n=3). Values followed by the same letter in a column do not differ significantly (P>0.05) by LSD post-hoc test.

During the storage period under consideration, TSS of tomato fruit decreased up to 4%, depending on the packaging method. Changes in the TSS content of apples were practically not observed (P=0.982). Wright & Kader [1997] and Erkan *et al*. [2004] reported that after nine months of storage there was a slight loss of TSS, explaining this either by the ratio of gases in MAP, or by the hydrolysis of polysaccharides to monosaccharides [Visser *et al*., 1968].

During the storage, the pH-value of tomatoes increased and the pH-value of apples decreased. In this case, the type of packaging did not affect the taste of fruit. It is known that taste sensations depend not only on the concentration of acids and sugars, but also on their ratio. Fruits can have a more acidic taste, not from a high content of organic acids, but from low sugar, and *vice versa*. It seems that after 25 days of storage, the content of sugars and the concentration of organic acids used for product's respiration was increased, leading to metabolic changes. The same results were obtained in the study by Tumwesigye *et al*. [2017]. The sugar content at the end of the shelf life decreased, and the pH value increased slightly over time as the concentration of organic acids was declining with maturity due to the utilization of malic acid as a substrate for respiration. The change was also attributed to the metabolic changes and water loss in cherry tomatoes. With those storage and package conditions, the fruit had fully met the necessary tomato market requirements.

Fruit firmness is one of the important quality parameters, both for apples and tomatoes. The loss of firmness in apples is noticeable with prolonged storage or with short-term storage of already overripe fruit, and is closely associated with a decrease in water content and metabolic changes [García *et al.*, 1998]. Firmness of tomatoes decreases more quickly than that of apples. After 25 days of storage, the firmness of tomatoes was almost halved, and the apples remained virtually unchanged (P=0.846).

There was a slight change in the intensity of the color of tomatoes, which can be explained by the ripening of these fruit (hydroxylation of carotenoids and synthesis of xanthophylls in them) [Gross, 1991]. Changes in the color of apples were not observed within 25 days of storage (P=0.999). A similar

TABLE 4. Changes of quality indicators of packaged tomato samples within 25 days of storage.

Duration of storage (days)	Indicators				
	Weight (g)	TSS (° Brix)	pH	Firmness (N)	Hue angle (h)
Control					
0	158.3±8.0[a]	5.7±0.3[a]	3.7±0.2[a]	2.6±0.1[a]	23.1±1.2[a]
5	156.7±7.8[a]	5.7±0.3[a]	3.7±0.2[a]	2.4±0.1[a]	23.6±1.2[a]
10	154.8±7.7[a]	5.6±0.3[a]	3.7±0.2[a]	2.2±0.1[a]	23.9±1.2[a]
15	153.0±7.7[a]	5.6±0.3[a]	3.7±0.2[a]	2.0±0.1[a]	24.8±1.2[a]
20	152.4±7.6[a]	5.5±0.3[a]	3.8±0.2[a]	1.7±0.1[a]	25.1±1.3[a]
25	151.3±7.8[a]	5.4±0.3[a]	3.8±0.2[a]	1.3±0.1[a]	25.3±1.3[a]
BIO					
0	158.3±8.0[ab]	5.7±0.3[a]	3.7±0.2[a]	2.6±0.1[a]	23.1±1.2[a]
5	155.9±7.8[ab]	5.7±0.3[a]	3.7±0.2[a]	2.5±0.1[a]	23.5±1.2[a]
10	155.0±7.7[ab]	5.7±0.3[a]	3.7±0.2[a]	2.1±0.1[a]	24.2±1.2[a]
15	153.6±7.7[ab]	5.6±0.3[a]	3.8±0.2[a]	1.6±0.1[a]	25.1±1.3[a]
20	152.5±7.6[ab]	5.6±0.3[a]	3.8±0.2[a]	1.4±0.1[a]	25.2±1.3[a]
25	151.7±7.8[ab]	5.5±0.3[a]	3.8±0.2[a]	1.3±0.1[a]	25.3±1.2[a]
BIO+MAP2					
0	158.3±8.0[b]	5.7±0.3[a]	3.7±0.2[a]	2.6±0.1[a]	23.1±1.2[a]
5	157.9±7.9[b]	5.7±0.3[a]	3.7±0.2[a]	2.0±0.1[a]	23.4±1.2[a]
10	157.5±7.8[b]	5.7±0.3[a]	3.7±0.2[a]	1.8±0.1[a]	23.8±1.2[a]
15	157.0±7.8[b]	5.6±0.3[a]	3.8±0.1[a]	1.7±0.1[a]	24.6±1.2[a]
20	156.4±7.8[b]	5.6±0.3[a]	3.8±0.1[a]	1.5±0.1[a]	25.0±1.2[a]
25	156.0±7.9[b]	5.5±0.3[a]	3.8±0.1[a]	1.4±0.1[a]	25.2±1.2[a]

Control – samples not processed with biopreservative and packaged without modified gas mixture; BIO – samples treated with biopreservative; BIO + MAP2 – samples treated with biopreservative and packaged with a modified gas medium. The data are expressed as mean ± standard deviation (n=3). Values followed by the same letter in a column do not differ significantly (P>0.05) by LSD post-hoc test.

result was reported for apples and pears [Bessemans *et al*., 2016; Mditshwa *et al*., 2017].

The three preservation methods had a significantly different effect on fungal growth. The ratio of the quality characteristic values for fruit without a biopreservative and with a preservative is comparable for both tomatoes and apples for 25 days of storage. The best results were observed in fruit for which a combined treatment method was used (BIO+MAP).

CONCLUSIONS

The need to ensure food security requires researchers to develop technologies for preserving food without deteriorating the quality characteristics and, preferably, at the lowest costs. The efficiency of the combined preservation technology (for apples and tomatoes) using a biopreservative and MAP was studied. The results of microbiological indicators of packaged fruit were obtained immediately after processing and packaging, and also after 5, 10, 15, 20, and 25 days of storage at a temperature of 4°C. Samples of fruit treated with biopreservatives and packaged with modified atmosphere produced the best microbiological results after 25 days of storage. Qualitative characteristics of packaged fruit remained within the permissible freshness for 25 days of storage. Undoubtedly, the physicochemical characteristics of the fruit we examined are determined by the variety, geographic and climatic conditions of growth, and even by the degree of maturity of the harvested fruit. Therefore, we assume that for other samples of tomatoes and apples, the differences can be more significant for the considered packaging and storage conditions. However, the technology of preserving vegetables and fruit with a biopreservative in combination with a MAP confirms its potential.

RESEARCH FUNDING

The work was carried out with partial financial support of the international financial initiative Eurotransbio [12467r/23886], Russian Foundation for Basic Research [18–016–00063], and Council of the President of the Russian Federation on grants [SP-1374.2018.4].

CONFLICTS OF INTEREST

Authors declare that there is no conflict of interest.

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Submitted: 18 February 2019. Revised: 25 April and 21 June 2019. Accepted: 9 July 2019. Published on-line: 21 August 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 297–306
DOI: 10.31883/pjfns/110734
http://journal.pan.olsztyn.pl

Original research article
Section: Food Technology

Influence of Pretreatments on Microwave Vacuum Drying Kinetics, Physicochemical Properties and Sensory Quality of Apple Slices

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Key words: apple slices, pretreatment, microwave vacuum drying, physicochemical properties, sensory quality

The objective of this study was to determine and compare the effect of five different pretreatments including sulfite treatment (ST), osmotic dehydration (OD), steam blanching (SB), steam blanching plus osmotic dehydration (SB+OD), and ultrasound treatment (UT), on the microwave vacuum drying (MVD) kinetics, the physicochemical properties, and sensory quality of the apple slices. The results showed that the pretreatments prior to MVD could reduce the drying time of apple slices by 25–45% as compared to the non-pretreated apple slices, and the drying time in the SB+OD apple slices sample was shortest. Whether pretreated or not, MVD process was controlled by diffusion and characterized by a two-stage falling-rate drying. As calculated according to the Fick's law of diffusion, the moisture diffusivities (D_{eff}) ranged from 1.64×10^{-8} to 3.46×10^{-8} m^2/s. Different pretreatment methods had a significant influence on the physicochemical properties and sensory qualities of the dried products ($p<0.05$). The SB+OD-pretreated apple slices showed the lowest shrinkage (59.5%) and the highest total sugar content (77.90 g/100 g dry matter). Besides, the OD-pretreated apple slices showed the highest density (0.953 g/cm^3) and the lowest titratable acidity (1.67 g/100 g dry matter). In addition, the ST-pretreated samples showed the highest titratable acidity (3.21 g/100 g dry matter) and vitamin C content (12.74 mg/100 g dry matter), while the sample pretreated by SB showed the highest total phenolics content (18.37 mg/g dry matter). Non-sulfite-pretreated samples were superior to the sulfite treatment or the control in flavor but inferior to the sulfite treatment in color. Results of preference sensory evaluation showed that the dried apple slices were preferred by the panelists in the following order: SB+OD>SB>UT>OD>ST>the control.

INTRODUCTION

Apple, the most important temperate fruit of the world, is regarded as one of the most important raw material in the food industry and is processed into a variety of products such as juice, jam, marmalade, and variously dried products [Beigi, 2016]. China is the largest country in the world, which cultivates and consumes apples. According to the Food and Agriculture Organization (FAO), the production amount of apple in China was more than 40 million tons in 2015, which accounted for about half of the global production [Xiao *et al*., 2018]. Currently, apples are mostly consumed fresh and the processing markets only account for 33% in China, which is extremely lower than above 70% in the developed countries; moreover, the intensively processed products are mainly the concentrated juice exported to the European and American markets [Bi *et al*., 2015]. There are some obvious problems such as simple processing production types, low processing rate as well as low value-added products in the postharvest processing of apples, which are restricting the healthy development of the apple industry in China.

Dried apples are rich in dietary fiber and bioactive components, and are convenient to handle, store, and utilize [Pasławska *et al*., 2017; Blanda *et al*., 2008]. They are a part of numerous prepared foods including snack preparations, integral breakfast foods, and so on [Vega-Gálvez *et al*., 2012]. In addition, drying fruits and vegetables into low water content provides new product formulas, such as fruits and vegetables crisps with unique texture and physical properties [Bi *et al*., 2016; Xiao *et al*., 2018; Velickova *et al*., 2014]. Furthermore, apple snacks play an important role in eating habits change, may substitute potato snacks and solve obesity problems among children or adults [Pasławska *et al*., 2017]. Therefore, apples dehydration process would be a promising path to deal with the problems as above mentioned.

The convective drying is still the most widely used drying method, which is applied to apples on the industrial production scale for its little capital, simple equipment and low energy input [Farias Aires *et al*., 2018; Velickova *et al*., 2014]. The main disadvantage of hot air drying is long drying time for exposure of the heat-sensitive materials to high temperature and aerobic environment, which may generate low quality dried products [Zhang *et al*., 2006]. Freeze drying (FD) under vacuum conditions is the best water removal method for all kinds of foods including apples, while it requires long drying time that would lead to high energy consumption and high capital investment. Therefore, FD is applied only with the high added-value products [Wang *et al*., 2018]. Microwave-vacuum drying (MVD) is increasingly used in food science research and in commercial food products drying

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[Zhang *et al*., 2006]. Numerous studies have confirmed that MVD yields high-quality dehydrated products that are similar to or better than those dried by FD [Nimmanpipug *et al*., 2013; Chong *et al*., 2013]. Besides, MVD possesses advantages including shortened drying time, improved energy efficiencies, and reduced energy consumption [Zhang *et al*., 2006].

Pretreatments prior to drying are often used to reduce the initial moisture content, accelerate the drying process, improve quality of dried products, modify the physicochemical properties of dried material, and influence the heat and mass transfer during drying [Doymaz *et al*., 2010]. Some common pretreatments include blanching, osmotic dehydration, sulfite treatment or a combination of the above treatments [Camel *et al*., 2019; Wang *et al*., 2018; Doymaz *et al*., 2010]. The sulfiting at low concentration is the most commonly used pretreatment method for apples and other fruits for its features of simple operation and well preserved color of products. Due to chemical residues in the final product, which may cause asthmatic reactions in some sensitive individuals and other health problems, there is an increasing demand to produce sulfur-free dried fruits [Igual *et al*., 2012; Krokida *et al.*, 2000]. Some novel pretreatments including ultrasound [Rodríguez *et al*., 2015; Nowacka *et al.*, 2012; Mothibe *et al*., 2014], microwave [Zielinska *et al*., 2018; Mothibe *et al*., 2014; Liu *et al*., 2015], vacuum [Blanda *et al*., 2008], have been developed to satisfy the growing health-conscious consumers. Ultrasonic wave has been used to enhance mass transfer since it can create microscopic channels in solid material by unique mechanical fluctuation and cavitation effect [Rodríguez *et al*., 2015; Nowacka *et al*., 2012]. It is reported that ultrasonic wave pretreatment could reduce drying time by 10–30% [Zhao *et al*., 2017; Horuz *et al*., 2017]. Ultrasound pretreatment of apples resulted in dried products with greater rehydration ratios and softer texture due to disruption of the cell walls and would be preferred when dried fruits of low calories are required because of its high sugar loss [Nowacka *et al*., 2012; Mothibe *et al*., 2014].

In recent years, both pretreatments and drying methods of the apples have been widely studied in order to obtain high quality dehydrated apple products. However, little was known about the effects of different pretreatments on microwave vacuum drying kinetics, physicochemical properties and sensory quality of the apple slices. This study was aimed to compare the effects of five types of pretreatments (sulfite treatment (ST), osmotic dehydration treatment (OD), steam blanching (SB), steam blanching followed by osmotic dehydration (SB plus OD), and ultrasound treatment (UT)) on the drying kinetics, physicochemical properties, and sensory quality in microwave vacuum drying (MVD) apple slices.

MATERIALS AND METHODS

Sample preparation

The fresh Red Fuji apples used in this study were purchased from a local market in Qingyang (Gansu, China) and stored in a refrigerator at 4∼8°C until the experiments were started.

Before each experiment, the apples were taken out and left to stand for 2 h in room to reach the environment temperature. Then, the apples were washed, peeled, cored, and cut into half circular shape of 10 mm thickness using a multifunctional apple peeler (DM-6; Deming, BeJing, China), for further use.

Pretreatments

Five pretreatments were applied to the apple slices before drying and an untreated sample was used as control. The pretreatments used were sulfite treatment (ST), osmotic dehydration (OD), steam blanching (SB), steam blanching plus osmotic dehydration (SB+OD), and ultrasound treatment (UT). All the pretreatment experiments were conducted in duplicate and details of each treatment were described as follows:

Control: A batch of 200 g fresh apple slices without any pretreatment were used for drying.

Sulfite treatment (ST): A batch of 200 g apple slices was dipped in 2% sodium bisulfite solution for 2 min, and then the surface water was removed using a filter paper prior to drying [Krokida *et al.*, 2000].

Osmotic dehydration (OD): A batch of 200 g apple slices were immersed in 60° Brix sucrose solution at 25°C for 180 min. The ratio of material to liquid was 1:8 (w/v) and a stirring was done with a glass rod every 30 min. After the osmotic dehydration was finished, the samples were taken out and the surface sucrose solution was removed using a filter paper prior to drying [Nimmanpipug *et al.*, 2013].

Steam blanching (SB): A batch of 200 g apples slices were leant on a stainless grid and blanched using 100°C steam for 180 s. After blanching treatment, they were cooled with air and the surface water was removed before drying.

Steam blanching plus osmotic dehydration (SB+OD): Apple slice samples of 200 g were blanching using 100°C steam for 180 s and then immersed in 60° Brix sucrose solution for 180 min. The other pretreated conditions were the same as the ones in the osmotic dehydration mentioned above. After the combined treatment, the samples were taken out and the surface sucrose solution was removed using a filter paper prior to drying.

Ultrasonic treatment (UT): According to a method described by Mothibe *et al*. [2014] with slight modifications, 200 g apple slices were immersed in distilled water and subjected to ultrasound waves for 10 min in an ultrasonic bath (KH-250DE; Kunshan Ultrasonic Instruments Ltd. Co., Kunshan, China). The water-to-fruit ratio was maintained at 4:1. The pretreatment was carried out at room temperature (25°C). The ultrasonic frequency was 25 kHz and the power was maintained at 200 W, and no mechanical stirring was used in the ultrasonic process. After ultrasonic treatment, the apple slices were blotted with filter paper and used for drying.

Microwave-vacuum drying (MVD)

Both the non-pretreated and pretreated apple slices were dried in a lab-scale microwave-vacuum dryer (ORW1.2S-5Z; Orient Microwave Co., Ltd, Nanjing, China). The samples to be dried were arranged in a single layer on a tray and the microwave-vacuum dryer was operated at 5 kPa (absolute pressure) and the power intensity for each drying test was 2.5 W/g (of original mass 200 g of fresh sample). The temperature

of the drying material to be dried was controlled at 50°C using an automatic on–off controller to avoid local charring. During the drying process, moisture loss was measured by periodically taking out using a digital balance (JH2102, Shanghai Precision & Scientific Instrument Co. Ltd., China) with 0.01 g precision. Each periodical interval was 5 min. After each weighing, the weight of the material and total drying time were recorded. The batch of weighed samples was abandoned and another batch of prepared original samples was dried until the next time interval. The samples were dehydrated until a moisture content of 0.76 kg water/kg dry matter (15%) was reached. Each drying experiment was conducted in triplicate. After the drying process was completed, the dried products were cooled down to room temperature and packed into a sealed polyethylene bag for further analyses.

Calculation of drying rate and moisture ratio

During MVD process, drying rate was determined as follows:

$$\text{Drying rate} = \frac{X_t - X_{t+\Delta t}}{\Delta t} \quad (1)$$

where: X_t, $X_{t+\Delta t}$ was moisture content (kg water/kg dry matter) at time t and $t+\Delta t$, respectively, Δt was time interval (min).

The change of moisture content in time was defined as a dimensionless parameter and expressed as the following equation:

$$MR = \frac{X - X_e}{X_0 - X_e} \quad (2)$$

where: MR was the dimensionless moisture ratio, X_0, X and X_e stood for the initial moisture content, the moisture content at time t and the equilibrium moisture content, respectively. The parameter t was the drying time (min). All results were expressed per dry basis (kg water/kg dry matter). The MR can be simplified to $\frac{X}{X_0}$ instead of $\frac{X - X_e}{X_0 - X_e}$ because of the value of dynamic equilibrium moisture content X_e which was very small in comparison with X and X_0 [Kaya *et al*., 2007].

Calculation of moisture diffusivity

Drying process was proven to occur mostly in the falling rate period, and moisture transfer during drying was controlled by internal diffusion [Kaya *et al*., 2007]. The Fick's second diffusion law (Equation 3) had been widely used to describe the drying process in the falling rate period for most biological materials [Vega-Gálvez *et al*., 2012; Schoessler *et al*., 2012].

$$\frac{\partial X}{\partial t} = D_{eff} \frac{\partial^2 X}{\partial x^2} \quad (3)$$

where: D_{eff} was the effective moisture diffusion coefficient (m^2/s), which was a mass diffusion property of the product, and x was the diffusion path (m).

Assuming unidirectional and constant moisture diffusion, negligible shrinkage and temperature change during drying, analytical solutions of Equation 4 for an infinite slab geometry were expressed as Equation 4 [Crank, 1975]:

$$\frac{X - X_e}{X_0 - X_e} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \cdot \exp\left(-\frac{(2n+1)^2 \pi^2 \cdot D_{eff}}{4L^2} \cdot t\right) \quad (4)$$

where: L was the thickness of the apple slices (m).

For long drying periods, the first term of the series solution in Equation 4 can be used as stated in Equation 5.

$$MR = \frac{8}{\pi^2} \exp\left(\frac{\pi^2 \cdot D_{eff}}{4L^2} \cdot t\right) \quad (5)$$

Equation 5 can be simplified to a straight-line equation as follows:

$$\ln MR = \ln\left(\frac{8}{\pi^2}\right) - \frac{\pi^2 \cdot D_{eff}}{4L^2} \cdot t \quad (6)$$

Plotting experimental drying data in terms of ln(MR) *versus* time, a straight line can be obtained, D_{eff} were calculated according to the slop of the straight line.

Moisture content determination

Moisture was determined by oven drying (101-A; Kewei, Beijing, China) at 105°C to the constant weight [AOAC, 1995].

Total sugar (TS) content determination

Total sugar in fresh and dried apples was analyzed with the Fehling's method [GB/T 5009.7–2016]. Appropriate amount of ground sample (2.5–5.0 g) was put into a 250 mL volumetric flask. Next, 50 mL water, 5 mL zinc acetate solution (219 g/L), and 5 mL potassium ferrocyanide solution (106 g/L) were added to the sample, and then water was added to 250 mL. The mixture was mixed well, stood for 30 min, filtrated, and the clear filtrate was collected. The filtrate (50 mL) was absorbed and transferred to a 100 mL volumetric flask and 5 mL 50% HCl were added. They were kept in a water bath at 70°C for 15 min and then neutralized with a sodium hydroxide solution (200 g/L). The prepared solution was titrated with a calibrated Fehling g's solution. The assay was performed in triplicate. The results were expressed as g of glucose per 100 g of dry matter of sample.

Titratable acidity (TA) determination

Titratable acidity in fresh and dried apples was determined according to a titrimetric method [GB/T 12456–2008]. An appropriate amount of the sample (about 5 g) was put in a mortar and the boiled water of the same weight as the sample was added, then the sample was grounded and transferred to a 250 mL volumetric flask with the boiled water having the temperature of 80°C. The total amount of the sample and the water in the flask was controlled to about 150 mL. After boiling for 30 min in a hot bath, the sample was cooled to room

temperature, dilluted to 250 mL, and then filtered. The filtrate (20 mL) was absorbed and transferred to a 250 mL Erlenmeyer flask. Distilled water (40 mL) and 1% phenolphthalein indicator (0.2 mL) were added to the flask, then titration was done with 0.01 M or 0.05 M NaOH. The assay was repeated twice. The results were expressed as g of malic acid equivalents per 100 g of dry matter of sample.

Total phenolics content (TPC) determination

A 3 g ground sample was extracted subsequently by a methanol:water solution acidified with HCl (50:50 v/v, pH 2, 25 mL/g sample) and an acetone:water solution (70:30 v/v, 25 mL/g sample) with continuous stirring at room temperature for 60 min. The extracts were filtered through a Whatman No.1 filter paper, then the supernatants were combined and centrifuged at 3000×*g* for 15 min (TDL-40B; Anting, Shanghai, China) [Ovando-Martinez *et al*., 2009]. The total phenolics content was determined with the Folin-Ciocalteau reagent [Singleton *et al*., 1999]. The obtained supernatant (0.5 mL, diluted if necessary) was mixed with 0.5 mL of the Folin-Ciocalteu reagent in a 25 mL glass-stopped tube. After 3 min, 10 mL of a sodium carbonate solution (75 g/L) were added and mixed. Additional distilled water (14 mL) was added up to make a final volume of 25 mL, and then the sample was mixed thoroughly by inverting the tubes several times. After 1 h, the absorbance at 750 nm was recorded with a spectrophotometer (751-UV–VIS; Shanghai analytical Instrument Factory, Shanghai, China). Gallic acid (Sigma Chemical Co., St. Louis, MO, USA) (0–50 mg/L) was used as a standard for the calibration curve. The tests were performed in triplicate. The total phenolics content was expressed as mg GAE per g of dry matter.

Vitamin C (V_C) content determination

L-ascorbic acid content was determined by the 2,6-dichlorophenol-indophenol (Merck KGaA, Darmstadt, Germany) titrimetric method [GB/T 5009.86–2016]. A 5 g ground sample was transferred to a 100 mL volumetric flask with the oxalic acid solution (20 g/L) and diluted to the scale. After shaking well and filtering, 10 mL filtrate was accurately absorbed and transferred to a 50 mL Erlenmeyer flask. Titration was done with the calibrated 2, 6-dichloroindophenol solution until the solution became pink and did not fade after 15 seconds, taking 10 mL oxalic acid solution as the control. All assays were done in triplicate; the vitamin C content was expressed as mg/100 g dry matter.

Color analysis

A chroma meter CR-400 (Onica Minolta, Tokyo, Japan) calibrated with a white standard board was used to determine the color of sample surface. Readings were expressed in the CIE1976 *L*a*b**scale, where *L** meant lightness, with 100 being very white and 0 being dark; the *a** value represented green (-60) to red (+60), and *b** value represented blueness (-60) to yellowness (+60). The hue angle (h^o), the chroma (*C**), and the total color difference (ΔE) were calculated according to the values of *L**, *a** and *b** as follows:

$$h^\circ = \text{arctg}(b^*/a^*)(a^*>0, b^*>0)$$
$$\text{or } h^\circ = 180^\circ + \text{arctg}(b^*/a^*)\ (a^*<0, b^*>0) \quad (7)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (8)$$

$$\Delta E = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2} \quad (9)$$

where: L^*_0, a^*_0 and b^*_0 were initial values of fresh apple slices; *L**, *a**, and *b** were dried sample values. The h^o revealed the color nuance; the values were defined as follows: red-purple: 0^o, yellow: 90^o, bluish-green: 180^o, and blue: 270^o. The C* represented chromaticity and denoted the purity or the saturation of the color. The measurements were carried out with 5 slices for each treatment.

Rehydration property analysis

Rehydration property analysis was performed according to the method reported by Vega-Gálvez *et al.* [2012] with small modification. Two dried apple slices were rehydrated by soaking in 25°C distilled water. The samples were withdrawn from the flask at 30 min interval, drained on the filter paper and weighed. This step was repeated until the difference between two consecutive recordings was smaller than 0.001 g. For all experiments, the solid-liquid ratio was kept at 1:50. The rehydration ratio (RR_t) was defined as the ratio of the mass of rehydrated samples to the mass of the sample and calculated as follows:

$$RR_t = \frac{W_t}{W_d} \quad (10)$$

where: W_d and W_t were the masses (g) of samples before rehydration and time (*t),* respectively. Each experiment was conducted in triplicate and the results were expressed as the mean values.

Shrinkage and density analysis

The volume of both raw and dried slices was measured by the displacement method using toluene [Schoessler *et al*., 2012].

Five dried apple slices were randomly selected from each sample lot and weighed (W_1) with the accuracy of 0.0001 g. The slices were put into an empty 150 mL flask, filled up with toluene and weighed again (W_2). The corresponding volume of dried samples (V_{ds}) was calculated as follows [Beigi, 2016]:

$$V_{ds} = 150 - \frac{W_2 - W_1}{\rho_{\text{toluene}}} \quad (11)$$

The density of toluene (ρ_{toluene}) is 0.87 g/mL at 20°C. After determination of the volume of dried slices, the shrinkage of the samples was calculated as follows:

$$S\% = (1 - \frac{V_{ds}}{V_{fs}}) \times 100 \quad (12)$$

where: *S* – shrinkage (%), V_{ds} – the final volume of dried apple slice (mL), and V_{fs} – the volume of apple slice before drying (mL).

The density (ρ) of the dried material was calculated as follows:

$$\rho=\frac{m}{V} \quad (13)$$

where: m (kg) was the mass of dried slices, and V (L) was the volume of dried slices. Five analyses were carried out for each test.

Sensory evaluation

A descriptive quantitative method was used to determine the sensory profile of the non-pretreated and pretreated dried apple slices. In this analysis, in order to understand the sensory attributes of the dried apple slices, which include color, texture, aroma, sweetness and sourness, a 15-number panel was trained with the fresh apple. The samples were evaluated in groups of 4 per session. They were coded with three digits and presented randomly. A categorized 20-point scale, anchored with "nothing" for number 1 and "very intense" for number 20, was used to measure attributed intensity [González-Herrera, *et al*., 2016]. In order to evaluate the preference of the non-pretreated and pretreated dried apple slices, the 15 panelists who frequently consume such products were asked to rate their liking of overall acceptability within a balanced nine-point hedonic rating where 9 denoted "like very much", 5 neither like nor dislike, and 1 indicated "dislike very much" [Velickova *et al*., 2014]. Three samples for each treatment were provided to each panelist; each sample consisted of five slices. All the prepared samples were coded with three-digit random numbers and presented in random order to the panelists.

Statistical analysis

Differences among mean values were estimated within an analysis of variance (ANOVA) with the application of Duncan's tests by SPSS 17.0.1. Mean values were considered significantly different when $p \leq 0.05$.

RESULTS AND DISCUSSIONS

Drying characteristics

The original moisture content in the fresh apple sample was determined to be 89.8% (Table 1). The initial moisture contents of the apple slices submitted to ST, OD, SB, SB+OD, and UT were found to be 87.9%, 63.5%, 88.6%, 68.8%, and 88.7%, respectively. As compared with the fresh sample, both OD and SB+OD pretreatments resulted in a significantly lower moisture content of the apple slices due to osmotic dehydration in sugar liquid ($p<0.05$).

Figure 1 revealed the variation of moisture content of the non-pretreated and pretreated apple slices with drying time during MVD. As shown in Figure 1, the moisture content decreased sharply at the initial drying stage and subsequently reduced slowly as the drying proceeded. In a relative short duration of less than 60 min, all the apple samples were dehydrated to the desired moisture content of 0.17 kg water/kg dry matter (d.m.) in the MVD. The total drying time was 45, 37, 40, 33, and 36 min for ST, OD, SB, SB+OD, and UT apple slices, respectively. The pretreatment had a significant influence on the total drying time and the various pretreatments used could reduce the drying time by 25–45%. The SB+OD apple slices had the shortest drying time, which indicated that SB+OD could reduce the drying time by 45% as compared with the one (about 60 min) of the no-pretreated apple slices. The decrement in the total drying time was associated with the reduction in the original moisture content and the change in water transfer in the samples to be dried. Nowacka *et al.* [2012] reported that the drying time of apple slices dried by hot air after ultrasonic pretreatment was 31% shorter than that of untreated apple slices due to the appearance of microscopic channels within the fruit tissue structure. Dandamrongrak *et al*. [2002] found that the freezing and the combined blanching with freezing pretreatment were the most effective methods of improving the drying rate when drying banana because heating as well as freezing leads to cell disruption.

Drying rate, a function of drying time and moisture content, was a fundamental parameter that was computed from the drying data by estimating geometric derivation occurring in each consecutive time interval, and was expressed as kg water/(kg dry matter min). Figure 2 depicts the variation of drying rate with moisture content in the apple slices submitted to different treatments. The entire drying process in all the samples underwent a transient warm-up period at the beginning, followed by a falling rate period for a long

TABLE 1. Contents of moisture (%), total sugar (TS), titratable acidity (TA), vitamin C (V_C), and total phenolics (TPC) in microwave-vacuum dried (MVD) apple slices subjected to different pretreatments.

Treatment	Moisture (%)	TS (g/100 g dry matter)	TA (g/100 g dry matter)	V_C (mg/100 g dry matter)	TPC (mg/g dry matter)
Fresh	89.8±0.20^{a}	65.84±1.05^{c}	3.52±0.05^{a}	19.24±0.05^{a}	17.65±0.05^{a}
Untreated	15.3±0.11^{b}	63.85±2.16^{d}	3.21±0.16^{b}	8.56±0.16^{d}	9.90±0.06^{e}
ST	14.2±0.06^{c}	61.02±0.18^{e}	3.10±0.18^{bc}	12.74±0.18^{b}	12.98±0.07^{c}
OD	15.8±0.15^{b}	71.74±1.45^{b}	1.67±0.05^{f}	10.56±0.45^{c}	10.54±0.09^{de}
SB	13.7±0.15^{d}	62.89±3.25^{de}	2.93±0.25^{c}	6.89±0.25^{e}	18.37±0.08^{a}
SB+OD	14.2±0.21^{c}	77.90±2.25^{a}	1.72±0.15^{e}	5.91±0.45^{f}	14.21±0.10^{b}
UT	15.7±0.04^{b}	51.45±3.55^{f}	1.93±0.55^{d}	6.45±0.55^{e}	11.90±0.11^{d}

Each value is expressed as mean value ± standard derivation (n=3). Different letters in the same column indicate significant differences at $p<0.05$. ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment.

time. The drying rates in the falling rate stages of the drying process in the non-pretreated, ST, SB, and UT apple slices were quite high for high moisture content in the apple slices. It showed that drying rate decreased continuously as the moisture content decreased. There was no discernible constant rate period of drying in all samples, which suggested that the internal moisture diffusion played a decisive role in the drying process of apple slices and determined the drying characteristics of raw materials.

Effective moisture diffusivity

The graph of experimental values of $ln(MR)$ of the apple slices against drying time was plotted, and the effective moisture diffusivity (D_{eff}) of the apple slices was calculated according to Equation 6 and presented in Figure 3. The D_{eff} values in MVD for ST, OD, SB, SB+OD, and UT were in the range of 1.64–3.46×10^{-8} m^2/s, respectively. It was observed that the moisture diffusivity in the ST samples was significantly higher than in the control samples ($p<0.05$), while the moisture diffusivities in the other pretreated samples were significantly lower than in the control samples ($p<0.05$). The lowest value was found in the SB+OD samples. The values of D_{eff} obtained from this study were in range of 10^{-11} to 10^{-6} m^2/s for food materials drying [Beigi, 2016]. The differences in the values may be caused by the differences in the initial moisture content, moisture state, and the cell disruption induced by different pretreatments.

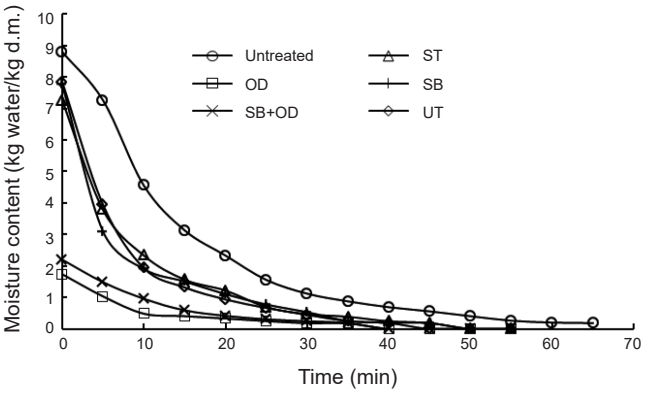

FIGURE 1. Microwave vacuum drying curves of apple slices as affected by different pretreatments. Note: ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching plus osmotic dehydration; UT: ultrasound treatment.

Chemical characterization

The contents of chemical and bioactive components in the fresh, the non-pretreated, and pretreated dried apple slices were determined, and respective results were listed in Table 1. As shown in Table 1, the contents of TS, TA, V_C, and TPC in fresh apple sample were 65.84 g/100 g d.m., 3.52 g/100 g d.m., 19.24 mg/100 g d.m., and 17.65 mg/g d.m., respectively. Pre-drying pretreatments used in this research strongly affected contents of the chemical and bioactive components in the dried products.

TS content in the dried samples ranged from 51.45 g/100 g d.m. to 77.90 g/100 g d.m. As expected, as compared with the fresh sample, both OD and SB+OD pretreatment led to an increase of 8.96–18.3% in TS content in the dried apple slices due to sugar uptake during OD, while lower TS content was observed in the non-pretreated, SB, UT, and ST pretreated dried apple slices. The highest TS content was observed in OD and SB+OD treatment (71.74 g/100 g d.m. and 77.90 g/100 g d.m.) while UT samples had the lowest TS content (51.45 g/100 g d.m.) because of sugar loss during ultrasound treatment process. Mothibe *et al*. [2014] found that ultrasound pretreatment resulted in a 24.7% loss of sugars of the apple cubes.

TA of the dried samples was in the range of 1.67 to 3.21 g/100 g d.m. and the significant differences in the dried apple slices were also observed ($p<0.05$). As compared with the fresh sample, MVD resulted in a decrease of 8.8–52.6% in TA content in all the dried products. The TA contents in the OD and SB+OD samples were lower than in the no-pretreated and the other pretreated dried apple slices. The lowest value was observed in OD samples while the highest value was observed in ST samples. Pasławska *et al*. [2017] and Kowalska *et al*. [2018] reported similar phenomenon of TA value decrease after fruit immersion in a sugar solution.

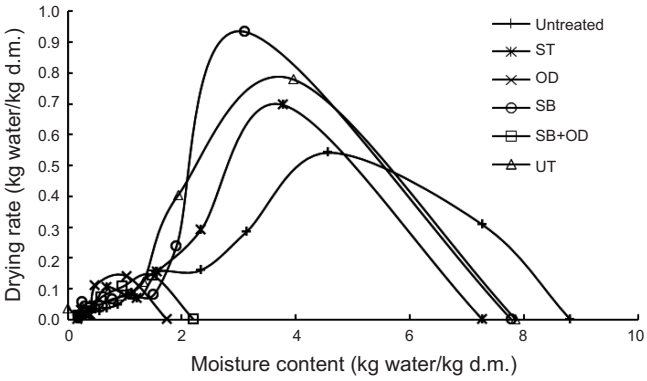

FIGURE 2. Microwave vacuum drying rate curves of apple slices as affected by different pretreatments. Note: ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment.

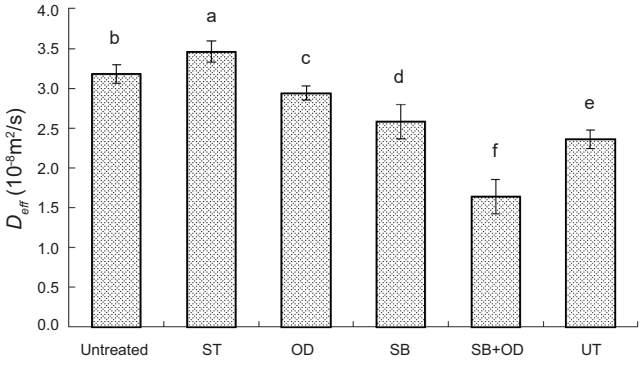

FIGURE 3. Effective moisture diffusion coefficient of dehydrated apple slices as affected by different pretreatments. Note: ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching plus osmotic dehydration; UT: ultrasound treatment. Different letters indicate a significant difference ($p\leq0.05$).

The changes in TA may be attributed to the elution of organic acids from tissue during OD process [Kowalska *et al*., 2018].

Ascorbic acid and phenolics are important nutritional or bioactive compounds in apples, and these chemicals are well-known antioxidants that play an important role in promoting health [Loncaric *et al*., 2014; Blanda *et al*., 2008]. As expected, drying treatment had an adverse effect on the content of V_C and TPC in all the dried samples. As compared with the fresh apples, MVD resulted in a decrease of 33.8–69.3% in ascorbic acid content in all dried products. The ascorbic acid content in the OD and ST samples was significantly higher than in the non-pretreated sample and the opposite tendency was observed in the SB, SB+OD, and UT samples. The loss in V_C was attributed to the thermal disruption and leakage of water-soluble V_C during pretreatment [Wang *et al*., 2018; Horuz *et al*., 2017]. However, it was reported that the presence of sugar would stabilize vitamin C in fruits [Pasławska *et al*., 2017].

With regard to the total phenolics, MVD resulted in a significant decrease (19.5–43.9%) in the total phenolics content in all the other dried products as compared with the fresh sample ($p<0.05$) except for a slight increase of 4% in the SB--pretreated samples. Moreover, the total phenolics content in the pretreated dried products was significantly or slightly higher than in the non-pretreated samples depending on which pretreated method was used. The highest TPC value was observed in the SB-treated samples (18.37 mg/g d.m.) followed by the SB+OD samples (14.21 mg/g d.m.), then followed by the ST samples (12.98 mg/g d.m.). The TPCs of OD and UT samples were comparable and were the lowest one (about 11 mg/g d.m.) due to the increment in dry matter induced by the leaching process. Processing including soaking, osmosis, heating and drying, may lead to the loss of phenolics in the food systems due to the leaching process, thermal degradation and the dilution effect [Mejia-Meza *et al*., 2010; Khandelwal *et al*., 2010]. At the same time, the amount of non-extractable phenolics was almost double of the extractable phenolics in the plant, and these non-extractable phenolics may release from the tissue in acid and high temperature environment as well as upon long-term ultrasonic treatment, which could cause an increase of phenolics content in the dried samples [Faller & Fialho, 2010; Horuz *et al*., 2017]. Additionally, sugar addition before processing was found to reduce phenolics loss in frozen and freeze-dried apple purée [Loncaric *et al*., 2014]. Obviously, the differences in the total phenolics content of the dried samples are ascribed to the multiple factors as mentioned above.

Color

Color is an important quality attribute of dried foods, which is the first parameter customers use to judge the quality of dried products [Chong *et al*., 2013]. Desirable dried products are the closest to the fresh fruit in color. The color parameters including L^*, a^*, b^*, C^*, ΔE and h^o of both the fresh and the dried apple samples were determined and the results were listed in Table 2. Both drying and pretreatments applied in this research greatly changed the original color of the flesh in fresh apples. As compared with the fresh samples, dehydration resulted in a significant decrease in the L^* and h^o values and a significant increase in a^*, b^*, and C^* values in the non-pretreated and pretreated dried apple slices ($p<0.05$). The ΔE values of all the dried samples were in the range from 2.82 to 27.64, and the non-pretreated sample had the greatest ΔE value, followed by the UT and OD samples; the lowest ΔE value was observed in the ST dried products. Obviously, the pretreatment prior to drying inhibited these changes in color to a different extent. It is noteworthy that the individual value of color parameter for the ST apples slices was closer to that of the fresh samples, followed by SB and SB+OD samples. The most remarkable change in color was observed in the dried product submitted to OD and UT, indicating that the severe browning occurred in both of them. The browning in fruits may commonly attribute to the oxidation of phenolics and to caramelization [Rodríguez *et al*., 2015; Nimmanpipug *et al*., 2013]. Various methods including lowering pH, applying citric acid as a metal chelator, and avoiding exposure to oxygen have been studied for inhibiting PPO or slowing the browning reaction, and the sulfur dioxide treatment is still the most effective method and has been widely used in the food industry [Krokida *et al.*, 2000; Igual *et al*., 2012].

The h^o of the fresh sample was about 92.08°, while those of all the dried samples were in the range of 68°–89°, which

TABLE 2. Effect of different pretreatments on the color parameters of microwave-vacuum dried (MVD) apple slices.

Treatment	L*	a*	b*	ΔE	C*	h^o
Fresh	70.84 ± 0.06^{a}	-0.56 ± 0.12^{g}	15.08 ± 0.15^{d}	–	15.09 ± 0.15^{e}	92.08 ± 0.13^{a}
Untreated	45.34 ± 1.09^{f}	8.22 ± 0.18^{a}	21.12 ± 0.35^{a}	27.64 ± 0.16^{a}	22.66 ± 0.34^{a}	68.77 ± 0.12^{g}
ST	68.34 ± 1.08^{b}	0.23 ± 0.15^{f}	16.12 ± 0.25^{c}	2.82 ± 0.14^{f}	16.12 ± 0.15^{d}	89.23 ± 0.05^{b}
OD	52.91 ± 1.21^{e}	4.46 ± 0.19^{b}	15.58 ± 0.18^{d}	18.63 ± 0.21^{c}	16.21 ± 0.25^{d}	74.06 ± 0.35^{f}
SB	57.84 ± 0.6^{d}	2.49 ± 0.12^{d}	18.07 ± 0.15^{b}	13.68 ± 0.12^{d}	18.24 ± 0.31^{c}	82.20 ± 0.25^{d}
SB+OD	60.56 ± 1.01^{c}	1.71 ± 0.11^{e}	17.57 ± 0.35^{bc}	10.82 ± 0.05^{e}	17.65 ± 0.25^{cd}	84.48 ± 0.15^{c}
UT	53.56 ± 1.01^{e}	3.71 ± 0.21^{c}	20.17 ± 0.29^{a}	21.79 ± 0.11^{b}	20.51 ± 0.09^{b}	79.63 ± 0.23^{e}

Each value is expressed as mean value ± standard derivation (n=3). Different letters in the same column indicate significant differences at $p<0.05$. ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment. L^* : white 100 to 0 dark; a^*: green (-60) to red (+60) ; b^*: blueness (-60) to yellowness (+60); h^o :hue angle; C^*: chroma; ΔE: total color difference.

represented an early yellow nuance. The ST products provided the highest h^o value, followed by the SB+OD and SB products. The lowest h^o value was observed in the non-pretreated dried products.

Rehydration characteristics

Rehydration is a determination of damage to the material caused by drying process. Additionally, most dehydrated products need to be rehydrated before usage; a rapid and complete rehydration is needed when immersion in water is desirable. Figure 4 presents the variation of the rehydration ratio *versus* time for the non-pretreated and pretreated dried apple slices during rehydration. The rehydrated rate sharply increased with rehydration time in the initial rehydration stage, then gradually decreased to zero; the high rate of the water uptake at the beginning stage can be explained by the capillaries and cavities near the material surface, which are rapidly filled up with water [Horuz *et al*., 2017].

The rehydration rate of dehydrated apple slices treated by SB was the fastest, followed by UT, ST, SB+OD, and OD

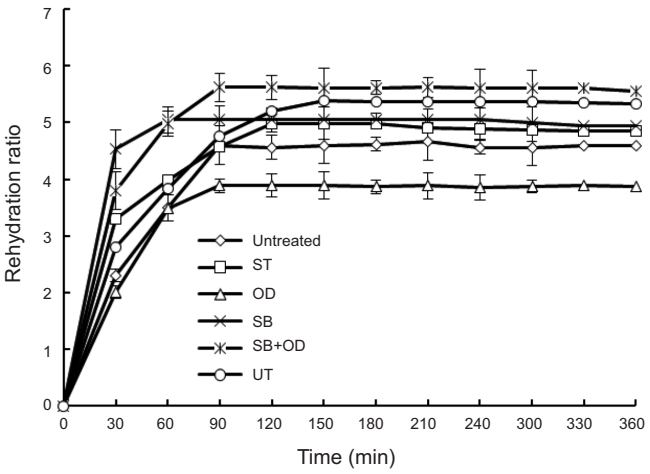

FIGURE 4. Effect of different pretreatments on the rehydration ratio of dried apple slices. Note: ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment.

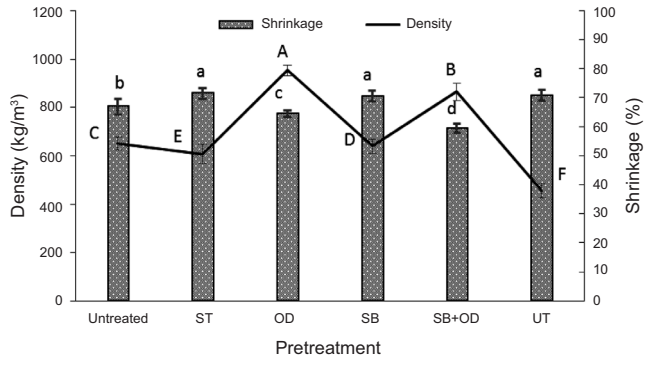

FIGURE 5. Effect of different pretreatments on shrinkage and density of dried apple slices. Note: ST: sulfite treatment; OD: osmotic dehydration treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment. Different letters indicate a significant difference ($p \leq 0.05$) in the same legend.

samples, while the rehydration rate of untreated dried products was the slowest. The stabilized rehydration ratio for SB, OD, SB+OD, untreated groups, ST and UT was 5.05, 3.89, 5.62 and 4.6, 4.98 and 5.38, respectively. The apple slices pretreated by SB+OD had the highest rehydration ratio. Ultrasound and thermal treatments destroyed the structure of the material to be dried, and formed many micro-channels on the surface of the material, thus resulting in faster rehydration rate but lower rehydration ratio during drying [Vega-Gálvez *et al*., 2012; Horuz *et al*., 2017].

Shrinkage and density

Figure 5 presents the shrinkage and apparent density of the non-pretreated and pretreated dried apple slices. Compared with the non-pretreated samples, the three pretreatments including ST, SB and UT increased the shrinkage of the samples while the other two pretreatments, *i.e.* OD and SB+OD, contributed to shrinkage decrease. There were no significant differences among the ST, SB and UT in shrinkage percent ($p > 0.05$), and their shrinkage was about 70%, which was significantly higher than the one in the non-pretreated samples ($p < 0.05$). The dried samples pretreated by OD had the lowest shrinkage percent (59.5%) followed by the SB+OD pretreated samples, which was significantly lower than the one in the non-pretreated samples ($p < 0.05$). It was reported that shrinkage was associated with the moisture content of the samples to be dried during MVD. When moisture was removed from tissues, a pressure imbalance was created between the inside and outside of the tissue, which may generate compressive stress and lead to shrinkage [Nahimana & Zhang, 2011].

The apparent density of the non-pretreated and pretreated dried apple slices was found out in the range of 455––953 kg/m^3 and the pretreatment methods had a significant influence on the apparent density of the dried samples ($p < 0.05$). The apparent density of the non-pretreated dried sample was 652 kg/m^3, which was significantly higher than in the dried samples pretreated by UT, ST and SB, but lower than in the dried samples pretreated by OD and SB+OD ($p < 0.05$). The dried samples pretreated by OD and SB+OD obtained a relatively higher apparent density while the dried samples pretreated by UT had the lowest value of this parameter due to their lower shrinkage and sugar loss [Nowacka *et al*., 2012; Mothibe *et al*., 2014].

Sensory evaluation

The results of sensory evaluation for the non-pretreated and pretreated dried apple slices are listed in Table 3. It revealed that the pretreatment had a significant influence on sweetness, texture, aroma, sourness, color, and over acceptability of apple slices ($p < 0.05$). The dried apple slices pretreated by ST had the best color, but the poorest aroma. Both OD and UT pretreatments were the best with regard to maintaining the original aroma of the fresh apple in the dried apple slices which also had a softer texture. The dried samples submitted to SB+OD and OD had a harder texture, were more sweet, and had weaker sourness than the other samples. As for the overall acceptability, the five selected pretreatments could improve the sensory quality of the dried apple slices

TABLE 3. Effect of different pretreatments on sensory qualities of microwave-vacuum dried (MVD) apple slices.

Treatment	Sweetness	Texture	Aroma	Sourness	Color	Overall acceptability
Untreated	10.8±0.9^{c}	15.4±0.8^{c}	16.3±1.2^{a}	14.7±0.7^{a}	8.3±0.7^{e}	5.6±0.9^{e}
ST	10.3±0.9^{c}	14.3±0.7^{d}	12.7±0.6^{c}	14.6±0.4^{a}	19.3±1.1^{a}	6.1±1.7^{d}
OD	18.3±1.1^{a}	19.1±0.9^{a}	15.7±0.7^{a}	5.3±0.7^{d}	10.9±0.3^{d}	5.5±0.8^{e}
SB	12.2±0.6^{b}	14.9±0.7^{cd}	14.2±0.8^{b}	12.9±1.5^{b}	13.2±0.6^{c}	8.1±1.2^{a}
SB+OD	19.0±0.7^{a}	16.9±1.1^{b}	14.5±0.4^{b}	4.9±0.7^{d}	16.5±0.7^{b}	7.6±2.5^{b}
UT	7.9±0.8^{d}	11.2±0.7^{e}	15.8±0.7^{a}	6.8±0.9^{c}	13.9±0.8^{c}	7.1±2.7^{c}

The results are mean ± standard deviation (n=3).Values with different superscript letters in the same column are significantly different ($p<0.05$).ST: sulfite treatment; OD: osmotic treatment; SB: steam blanching; SB+OD: steam blanching followed by osmotic dehydration; UT: ultrasound treatment.

in comparison with the non-pretreated samples to a different extent. The overall acceptability score of the dried apple products submitted to different pretreatments was decreased in the following order: SB> SB+OD> UT> ST>OD.

CONCLUSION

Based on the results of the present investigation, we concluded that the pretreatments applied had a profound influence on the microwave vacuum kinetics and the quality of the dehydrated apple products. Five selected pretreatments could shorten the microwave vacuum drying time of the apple slices, and the effective moisture diffusivity (D_{eff}) ranged from 1.64×10^{-8} to 3.46×10^{-8} m^2/s under different pretreatment conditions. As compared with the non-pretreated dried apple slices, different pretreatments significantly changed contents of the chemical and bioactive components and improved sensory quality of the dried apple products in a specific way. With the enhancement of consumers' health awareness, non-sulfur pretreatments including SB, OD, UT as well as their combinations are recommended in producing the dried apples. The selection of a reasonable pretreatment method prior to drying depends on the different specific demand and the investment cost of the industry. For example, the advantages of the sulfur-related pretreatment include a relatively low production cost and a cheap product price; while the ultrasound pretreatment would be preferred when dried fruits of low calories are required since it results in a high sugar loss.

FUNDING

The authors gratefully acknowledged the financial supports provided by the National Natural Science Foundation of China (No. 31460398) and the Scientific Research Starting Foundation for Doctor of Longdong University(No: 2014XYBY11).

CONFLICT OF INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Submitted: 24 March 2019. Revised: 1 July 2019. Accepted 16 July 2019. Published on-line: 21 August 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 307–317
DOI: 10.31883/pjfns/111405
http://journal.pan.olsztyn.pl

Original research article
Section: Food Quality and Functionality

Fruit Low-Alcoholic Beverages with High Contents of Iridoids and Phenolics from Apple and Cornelian cherry (*Cornus mas* L.) Fermented with *Saccharomyces bayanus*

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Key words: phenolics, iridoids, antioxidants, alcoholic beverages, natural products, Cornelian cherry

In this study, we produced novel, natural and fermented apple-Cornelian cherry beverages rich in natural antioxidants. These products were examined for their physicochemical parameters, and antioxidative properties as well as subjected to the quantitative and qualitative identification of iridoids and phenolics. The highest concentration of total phenolics determined with the Folin-Ciocialteu method (964.28 mg GAE/L) and the strongest antioxidative properties measured with the DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and FRAP tests (7.90, 11.04, and 12.86 mmol TE/L) were determined in the beverages with the addition of juice from red-fruit Cornelian cherry. The most numerous group of compounds in the analyzed beverages were iridoids, with loganic acid (LA) found to predominate (424 mg/L). Results obtained demonstrate that the addition of juice from Cornelian cherry fruits during the production of fermented apple beverages causes a significant increase in their antioxidative properties, modifies their phenolics profile, and allows enriching them with iridoids.

INTRODUCTION

Latest research indicates the global alcohol consumption to decline in recent years. However, this decrease has been found to result from the fact that more aware consumers search for novel, special, and valuable products characterized by interesting and diversified flavors [Podstawski *et al*., 2017; Muggah & McSweeney, 2017]. For this reason, products that have exceptional sensory attributes and also valuable composition seem to respond to these needs. Fermented fruit beverages represent an interesting group of products considering their sensory diversity and their multiple health-promoting compounds which exhibit various biological activities like, *e.g*.: phenolics, vitamins, organic acids or carotenoids [Cusano *et al*., 2018; Escudero-López *et al*., 2018].

In turn, apples are commonly available fruits, whose global production accounts for *ca*. 77.3 million tons annually (data for 2017/2018), [USDA Foreign Agricultural Service, 2017]. Apart from the production of juices, one of the significant sectors of apple processing includes the manufacture of ciders, *i.e.* natural fermented alcoholic beverages produced as a result of apple must fermentation, containing from 1.2 to 8.5%v/v of ethanol and exhibiting antioxidative and antimicrobial activities [Verdu *et al*., 2013]. Apples, which are the basic ingredients in cider production, contain a wide spectrum of polyphenolic compounds and dietary fiber; in addition they exert a bacteriostatic effect and prevent digestive system disorders [Condezo-Hoyos *et al*., 2014]. Considering their exceptional values and their availability, in this study we produced novel alcoholic beverages with the addition of Cornelian cherry fruits characterized by a very high content of natural antioxidants compared to other fruits.

Cornelian cherry (*Cornus mas* L.) is a fruit growing wild in Europe and Asia. It may be consumed fresh or may be processed to produce liqueurs, juices, jams, concentrates or alcoholic beverages [Kucharska *et al*., 2007; Kucharska, 2012; Sokół-Łętowska *et al*., 2014; Bozdogan, 2017]. Numerous investigations have proved Cornelian cherry fruits and products made of them to be characterized by an exceptionally rich phenolics composition [De Biaggi *et al*., 2018], and by other health-promoting properties like *e.g.* hypocholesterolemic [Hosseinpour *et al*., 2017] or anti-inflammatory ones [Moldovan *et al*., 2016]. Regular consumption of small amounts of natural fermented beverages rich in antioxidants may supplement an everyday fruit-vegetable diet. This study was aimed at developing a production technology for apple-Cornelian cherry low-alcoholic beverage with strong antioxidative

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properties and high concentrations of iridoids and phenolics, and to identify the best Cornelian cherry variety and the best production method of these beverages.

MATERIALS AND METHODS

Standards and reagents

Dimethyl sulfoxide (DMSO), 2,4,6-tri(2-pyridyl)-s--triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman--2-carboxylic acid (Trolox), acetonitrile, 1,1-diphenyl-2-picryl--hydrazyl radical (DPPH$^{\bullet}$), $FeCl_3$, sulfuric acid, formic acid, and sodium hydroxide were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetic acid was obtained from Chempur (Piekary Śląskie, Poland). Acetonitrile was acquired from POCh (Gliwice, Poland), whereas acetic acid from Chempur (Piekary Śląskie, Poland). Loganic acid (LA), loganin (L), sweroside (S), ellagic acid (EA), 5-*O*-caffeoylquinic acid (5-CQA, chlorogenic acid), *p*-coumaric acid (*p*-CuA), quercetin 3-*O*-glucoside (Q glc), kaempferol 3-*O*-glucoside (Kf glc), cyanidin 3-*O*-glucoside (Cy glc), (+)-catechin (C), procyanidin dimer B1, procyanidin dimer B2, (–)-epicatechin, and phloretin 2'-*O*-glucoside (Ph 2'-glc) were purchased from Extrasynthese (Lyon Nord, France). All reagents were of analytical grade. The Folin-Ciocialteu phenol reagent was purchased from Chempur (Piekary Śląskie, Poland).

Biological material

Apple juice and apple-Cornelian cherry juices were fermented with wine yeast *Saccharomyces bayanus* – Safspirit fruit, purchased from the Fermentis company (Lesaffre, Marcq-en-Barœul, France). Dry yeast were rehydrated in bi-distilled water at a temperature of 25°C for 30 min. Fermentation worts were inoculated with the activated yeast cells in a dose of 0.5 g of dry matter per liter (g d.m./L).

Raw material

Raw materials used in the study included: all-year round available on the Polish market, freshly pressed, naturally cloudy and pasteurized apple juice made of three apple varieties: Gloster, Champion, and Jonaprince, produced by the SOKPOL company (Myszków, Poland); as well as three varieties of Cornelian cherry: 'Podolski' var. with red fruits, 'Yantarnyi' var. with yellow fruits, and 'Koralovyi' var. with coral fruits. All fruits were harvested in the Arboretum in Bolestraszyce, near Przemyśl, Poland. The plant materials were authenticated by Prof. Jakub Dolatowski (Arboretum and Institute of Physiography in Bolestraszyce, Przemyśl, Poland), and the adequate voucher specimens ('Yantarnyi' – BDPA 14131; 'Koralovyi' – BDPA 14136; 'Podolski' – BDPA 10462) have been deposited at the Herbariums of Arboretum in Bolestraszyce, Poland. Fruits were harvested in September 2016, and immediately frozen at −20°C. Before fermentation, the fruits were pressed through a Zodiak laboratory hydraulic press from SRSE company (Warsaw, Poland) to obtain Cornelian-cherry juices.

Preparation of samples and fermentation process

Two methods differing in the course of the technological process were used in the study to produce apple-Cornelian cherry alcoholic beverages. The first method (M-1) consisted in the preparation of worts containing 90% of apple juice (630 mL) and 10% of juice from Cornelian cherry varieties differing in fruit color (70 mL). The prepared apple-Cornelian cherry worts were inoculated with yeast and fermented in 1-liter glass laboratory flasks at a temperature of 22°C for 7 days. Afterwards, the samples were decanted from above the yeast precipitate and left for aging at a temperature of 4°C for two weeks. In the second method (M-2), worts containing 100% of apple juice (700 mL) were fermented under the same conditions as in method M-1. Next, the samples were decanted from above the yeast precipitate and 10% of the resultant sample (sample after fermentation but before aging) was replaced by juice made of fruits of a given variety of Cornelian cherry, and the sample was left for aging under conditions as described in method M-1. A control sample (A) was prepared as well, in which 100% of apple juice was used for both the fermentation and aging processes which were conducted under the same technological conditions as in method M-1. All research variants were fermented in triplicate. Description of symbols used throughout the manuscript are presented in Table 1.

TABLE 1. Symbols and description of worts, beverage production methods, as well as worts and beverages manufactured in the study.

Symbol	Description	
A	wort	wort containing 100% of apple juice
AY		wort from apple juice with the addition of juice from yellow fruits of Cornelian cherry
AC		wort from apple juice with the addition of juice from coral fruits of Cornelian cherry
AR		wort from apple juice with the addition of juice from red fruits of Cornelian cherry
M-1	production method	production method in which 10% of Cornelian cherry juice was added to the wort before the primary fermentation process
M-2		production method in which 10% of post-fermentation liquid was replaced by Cornelian cherry juice
A-1	products manufactured with method M-1	fermented beverage containing 100% of apple juice
AY-1		fermented apple-Cornelian cherry beverage with juice from yellow fruits of Cornelian cherry manufactured with method M-1
AC-1		fermented apple-Cornelian cherry beverage with juice from coral fruits of Cornelian cherry manufactured with method M-1
AR-1		fermented apple-Cornelian cherry beverage with juice from red fruits of Cornelian cherry manufactured with method M-1
AY-2	products manufactured with method M-2	fermented apple-Cornelian cherry beverage with juice from yellow fruits of Cornelian cherry manufactured with method M-2
AC-2		fermented apple-Cornelian cherry beverage with juice from coral fruits of Cornelian cherry manufactured with method M-2
AR-2		fermented apple-Cornelian cherry beverage with juice from red fruits of Cornelian cherry manufactured with method M-2

Fermentation dynamics and pH value

Fermentation dynamics was determined based on carbon dioxide loss during the fermentation process. The mass loss of fermented samples was monitored every 24 h by weighing the containers on a WTB 2000 laboratory scale (RADWAG, Radom, Poland). The pH value of all samples was measured using an MP 220 pH-meter (Mettler Toledo, Greifensee, Switzerland).

Carbohydrates, ethanol, and glycerol concentrations

The concentrations of sucrose, glucose, fructose, ethanol, and glycerol were determined with the method of high performance liquid chromatography (HPLC) [Kawa-Rygielska *et al*., 2018]. The samples with 100% of apple juice were diluted with redistilled water in a water to sample ratio of 1:2 (v/v), whereas samples with Cornelian cherry were diluted in a ratio of 1:5. The samples were analyzed using a Prominence liquid chromatography system (Shimadzu Corp., Kyoto, Japan) equipped in a Rezed ROA-Organic Acid H+ column (300 × 4.6 mm) from Phenomenex (Torrance, CA, USA). The following parameters of measurements were applied: injection volume 20 μL, elution temperature 60°C, flow rate 0.6 mL/min, mobile phase 0.005 M H_2SO_4, and thermostat refractometric detector at 50°C. Concentrations of glucose, ethanol, and glycerol were determined based on a five-point calibration curve integrated in Chromax 10.0 software by Pol-Lab (Wilkowice, Poland).

Determination of phenolics concebtration and antioxidative activity

Total phenolics concentration

The total phenolics content was determined using the spectrophotometric method, with the Folin-Ciocalteu reagent test (F-C) [Prior *et al*., 2005]. The analyzed sample was mixed with the F-C reagent, and after 20 min Na_2CO_3 20% and distilled water were added. After 60 min of incubation, absorbance was measured at 765 nm. The results are expressed in mg of gallic acid equivalents (GAE) per liter of beverage (mg GAE/L), as the average of three replicates.

Antioxidative activity based on the DPPH$^\bullet$ test

The antioxidative properties of the analyzed samples were measured with the DPPH$^\bullet$ test [Yen & Chen, 1995]. The sample and the distilled water were pipetted into the cuvette and then the DPPH$^\bullet$ reagent was added. The samples were incubated for 10 min and next the absorbance was measured at 517 nm. The results are presented as the content of Trolox equivalents (TE) per liter of the sample (mmol TE/L), as the average of three replicates.

Antiradical activity based on the ABTS$^{\bullet+}$ test

The antioxidative activity of the analyzed beverages was also determined based on the ABTS$^{\bullet+}$ assay [Re *et al*., 1999]. A sample with the ABTS$^{\bullet+}$ solution was mixed in a cuvette. After 6 min of incubation, the absorbance was measured at 734 nm. The results are expressed as the average of three replicates, as the content of Trolox equivalents (TE) in a liter of the sample (mmol TE/L).

Antioxidative activity based on the FRAP test

Antioxidative activity of the analyzed samples was also measured with the spectrophotometric method, based on the FRAP test [Benzie & Strain, 1996]. After mixing the sample with distilled water, the FRAP reagent was added. The prepared samples were incubated for 10 min and then the absorbance was measured at 593 nm. Results are expressed as the average of three measurements, as the content of Trolox equivalents (TE) per liter of the sample (mmol TE/L).

Absorbance measurements in the above tests were made using a DU 650 spectrophotometer from the Beckman Coulter company (Atlanta, GA, USA).

Quantification of bioactive compounds with HPLC-PDA

Detailed description of the quantification procedure was described in works by Kucharska *et al*. [2017] and Kawa-Rygielska *et al*. [2018]. It was performed using a Dionex (Germering, Germany) system equipped with an Ultimate 3000 model diode array detector, LPG-3400A quaternary pump, EWPS-3000SI autosampler, TCC-3000SD thermostated column compartment, and controlled by the Chromeleon v.6.8 software (Thermo Scientific Dionex, Sunnyvale, CA, USA). A Cadenza Imtakt C5-C18 column (75×4.6 mm, 5 m) was used (Imtakt, Kyoto, Japan). The mobile phase was composed of solvent A (4.5% aq. formic acid, v/v) and solvent B (100% acetonitrile). The elution system was as follows: 0–1 min 5% B in C, 20 min 25% B in A, 21 min 100% B, 26 min 100% B, and 27 min 5% B in A. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 μL. The column was operated at 30°C. Iridoids were detected at 245 nm, phenolic acid at 320, ellagic acid at 254 nm, anthocyanins at 520 nm, flavanols at 280 nm, dihydrochalcones at 280 nm, and flavonols at 360 nm. Loganic acid and cornuside were expressed as loganic acid, loganin, sweroside; and secoxyloganin as loganin; caffeoylquinic acids as 5-*O*-caffeoylquinic acid' *p*-coumaroylquinic acid as *p*-coumaric acid; ellagic acid as ellagic acid; anthocyanin derivatives as cyanidin 3-*O*-glucoside; (–)-epicatechin and procyanidin dimers as (–)-epicatechin; (+)-catechin as (+)-catechin; phloretin derivatives as phloretin 2'-*O*-glucoside; quercetin derivatives as quercetin 3-*O*-glucoside; and kaempferol 3-*O*-galactoside as kaempferol 3-*O*-glucoside. Results provided in the manuscript represent a mean value of three replications and are expressed as mg per liter of the sample. Results obtained were controlled and analyzed by Chromeleon v.6.8 software (Thermo Scientific Dionex, Sunnyvale, CA, USA). Iridoids and phenolics were identified by their HPLC retention times, UV-Vis spectra, the HPLC profile, and by comparison with the known standards and literature data.

Statistical analysis

Analysis of selected data was conducted with Statistica 13.5 software (StatSoft, Tulsa, OK, USA) based on one-way analysis of variance (ANOVA test), at a significance level of $\alpha=0.05$. Differences between mean values were computed with the Duncan test (p-value <0.05). Standard deviations are provided in tables.

RESULTS AND DISCUSSION

Fermentation dynamics control

Figure 1 depicts the dynamics of the alcoholic fermentation of the analyzed worts. The fermentation dynamics of all worts were similar in the first 24 h. After 48 h, weaker dynamics was demonstrated for the sample with the addition of juice made of yellow fruits of Cornelian cherry (AY), compared to all other variants. On the third day, the greatest amount of CO_2 was emitted from the wort made of apple juice (A), and this CO_2 loss was by 20% greater than in the other samples. After 96 h, the highest fermentation dynamics was again determined in the wort produced from apple juice (A) and in the sample with the addition of yellow-fruit juice (AY). On the same day, the loss of CO_2 was by 10% lower in the variant with the addition of coral fruit juice (AC) and by 20% lower in the wort with the addition of red fruit juice (AR). In the last three days of the fermentation process, its dynamics decreased in the control sample (A) and in the variant with the addition of yellow-fruit juice. Since that moment till the end of the process, the average loss of carbon dioxide reached 12%, whereas in the other worts (AC and AR), CO_2 loss accounted for *ca.* 21%. The control of the dynamics of alcoholic fermentation in the study of fermentation processes is important because it allows for the proper course of the process [Kawa-Rygielska *et al*., 2018]. The use of various additives in specified amounts may modify parameters of the dynamics of alcoholic fermentation of fermented beverages [Roldán *et al*., 2011].

Carbohydrates, ethanol, glycerol concentrations, and pH value

Table 2 presents concentrations of sucrose, glucose, fructose, ethanol, and glycerol as well as pH values of the worts and fermented apple-Cornelian cherry beverages. The highest concentration of carbohydrates in the analyzed worts (80 g/L) was determined in the variants with the addition of juices from coral and red fruits of Cornelian cherry (AC and AR), whereas in the control sample its concentration was lower by 50 g/L. Sugars concentration in worts depends on the composition of a fruit juice, whereas sugars content in apples depends on both their variety and color of their fruits, with the red varieties having a higher sugar content [Kumar *et al*., 2018]. The worts with 10% addition of Cornelian cherry juice had a higher concentration of sugars because they represent 60% of dry matter in these fruits [Kucharska, 2012].

The fermented apple beverage (A-1) contained trace amounts of sugars, while no sucrose and glucose were detected in the fermented apple-Cornelian cherry beverages produced with the first method (M-1), irrespective of Cornelian cherry variety (AY-1, AC-1, AR-1). In the beverages produced with the second method (M-2), glucose and fructose concentration was higher (20.0 g/L), because prior to the aging process, 10% of fermented apple juice was replaced with fresh juice pressed from Cornelian cherry fruits. The addition of this fresh juice increased the concentration of sugars which were not consumed by wine yeast because the fermented beverages were decanted from above the yeast precipitate before aging, and the aging process was conducted at a temperature of 4°C at which these yeast are inactive. The sensation of sweetness is one of the most important traits of fermented beverages in the sensory assessment made by consumers who usually prefer slightly sweeter flavors [de Jesus Filho *et al*., 2018]. In the case of apple-Cornelian cherry alcoholic beverages produced in this study with the second method (M-2), the level of sweetness was not high despite the higher sugars

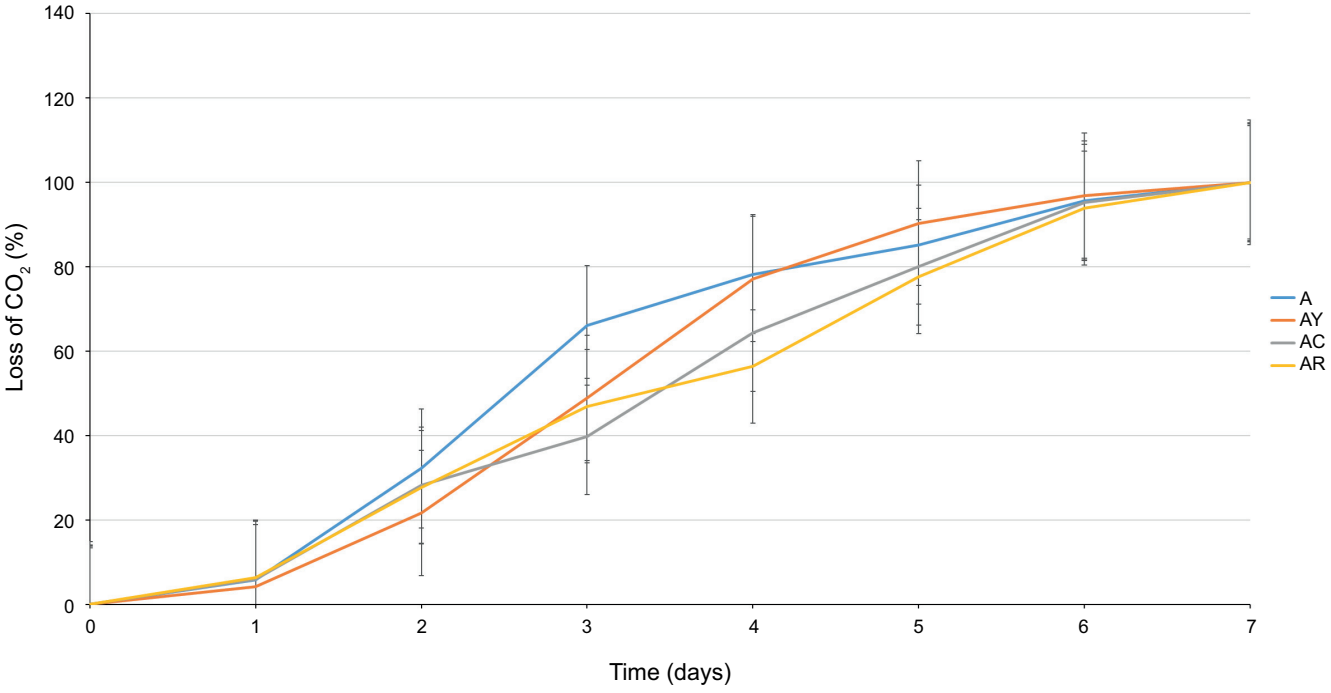

FIGURE 1. Fermentation dynamics expressed as the percentage loss of carbon dioxide.

A – apple wort, AY – apple wort with the addition of juice from yellow fruits of Cornelian cherry, AC – apple wort with the addition of juice from coral fruits of Cornelian cherry, AR – apple wort with the addition of juice from red fruits of Cornelian cherry.

TABLE 2. Concentrations of sucrose, glucose, fructose, ethanol, and glycerol (g/L) and the pH value of worts and beverages.

Variety of beverage	Stage of the process	Sucrose	Glucose	Fructose	Ethanol	Glycerol	pH
A	W[1]	1.68±0.02[c]	19.8±1.29[c]	12.7±0.02[b]	nd	nd	3.51±0.01[a3]
AY		6.93±0.07[a]	24.2±0.88[b]	49.1±0.46[a]	nd	nd	3.18±0.01[b]
AC		5.49±0.02[b]	32.3±0.49[a]	48.6±0.02[a]	nd	nd	3.25±0.01[b]
AR		6.45±0.15[a]	29.2±1.09[a]	48.3±0.01[a]	nd	nd	3.20±0.01[b]
A-1	M-1	nd[2]	0.35±0.02[e]	2.52±0.00[e]	49.1±4.88[a]	4.46±0.45[b]	3.19±0.00[b]
AY-1		nd	nd	1.44±0.01[f]	51.4±1.15[a]	5.48±0.85[a]	3.00±0.00[c]
AC-1		nd	nd	2.25±0.00[ef]	42.9±0.91[c]	4.73±0.00[b]	2.98±0.01[c]
AR-1		nd	nd	2.70±0.00[e]	47.4±0.52[b]	4.80±0.00[b]	3.02±0.04[c]
AY-2	M-2	nd	15.8±2.13[c]	10.8±0.16[cd]	42.9±1.41[c]	4.69±0.01[b]	2.93±0.01[c]
AC-2		nd	13.1±0.41[d]	11.6±0.12[c]	36.6±0.81[d]	3.34±0.08[c]	2.89±0.00[c]
AR-2		nd	13.4±0.78[d]	10.2±0.00[d]	39.0±7.79[c]	3.49±0.70[c]	2.90±0.01[c]

[1] W, wort before fermentation; M-1, beverages produced with the first method; M-2, beverages produced with the second method; [2] nd, not detected; [3] Values are expressed as the mean (n = 3) ± standard deviation. Mean values with different letters (a, b, c, etc.) within the same column are statistically different (p-value < 0.05). Symbols: A, AY, AC, AR, A-1, AY-1, AC-1, AR-1, AY-2, AC-2, and AR-2 as explained in Table 1.

concentration than in the beverages made with the first method (M-1), because Cornelian cherry fruits are sour and bitter [Kucharska, 2012]. In addition, excessive consumption of products rich in simple sugars, including sweet beverages, may contribute to the development of many diseases [Crovetto *et al*., 2018]. For this reason, the products made with the first method (M-1) and being free of sucrose and glucose might contribute to a well-balanced diet.

Ethanol concentration of alcoholic beverages ranged from 36.60 g/L (4.6% v/v) in these produced with the addition of coral-fruit Cornelian cherry juice using the second method (AC-2) to 51.39 g/L (6.5% v/v) in these produced with the addition of yellow-fruit juice using the first method (AR-1). An equally high concentration of ethanol was determined in the control sample (A-1), which was correlated with results of their fermentation dynamics analysis. Results indicate that the first method (M-1) allows producing beverages with a higher ethanol concentration (by 7.7 g/L on average) compared to the second method (M-2). Girschik *et al*. [2017] produced ciders with a similar ethanol content – 6.1% v/v on average, while Gonzalez Flores *et al*. [2017] obtained ciders with a higher ethanol content (*ca*. 1% v/v) [Gonzalez Flores *et al*., 2017]. The level of alcohol in fermented fruit beverages depends on many factors which affect the fermentation process, including *e.g*.: variety and ripeness of fruits [Gonzalez Flores *et al*., 2017] or amount of fruits used in the production process [Caldeira *et al*., 2018]. It has been proved that the consumption of small amounts of low-alcohol orange beverages may have a beneficial effect on a human body [Hornero-Méndez *et al*., 2018; Escudero-López *et al*., 2018].

Glycerol levels in the produced apple-Cornelian cherry alcoholic beverages ranged from 3.34 to 5.48 g/L. The highest glycerol concentration was determined in the beverage with the addition of yellow Cornelian cherry juice and the lowest one – in products manufactured with the second method with the addition of coral-fruit (AC-2) and red-fruit (AR-2) juices. These levels of glycerol were comparable to the results reported for ciders by Suarez Valles *et al*. [2008], but slightly lower than these found in apple wines analyzed by Satora *et al*. [2016], *i.e.* 10.5–12.8 g/L. Glycerol is one of the basic by-products of yeast fermentation and has a significant effect on the sensory perception of the manufactured alcoholic beverages by affecting the fullness of their flavor, their sweetness sensations, and their viscosity [Gawel *et al*., 2007]. The produced worts and finished products were additionally analyzed for their acidity level based on their pH values. Among the analyzed worts, the highest pH value was measured in the variant without Cornelian cherry juice addition (A), whereas pH of the other worts was slightly lower. The mean pH value of all alcoholic apple-Cornelian cherry beverages was about 2.95, whereas the pH value of the product containing 100% of apple juice (A-1) was 0.2 higher. The lower pH values of the worts and finished beverages with the addition of juice from Cornelian cherry fruits are due to the naturally high acidity of these fruits not exceeding pH 3 [Kucharska *et al*., 2011]. The pH values of the apple-Cornelian cherry alcoholic beverages produced in our study are slightly lower compared to the values reported for the other fermented apple beverages, *i.e.* 3.5–3.7 [Girschik *et al*., 2017], 4.2–4.6 [Venkatachalam *et al*., 2018], and 3.1–3.4 [Gonzalez Flores *et al*., 2017].

Total concentration of phenolics and antioxidative properties

Results obtained in this study (Table 3) demonstrate that the addition of Cornelian cherry juice to worts caused a significant increase in their total phenolics content compared to the control wort containing 100% of apple juice (A). The highest total concentration of phenolics was determined in the worts with the addition of red-fruit juice (AR) and cor-

TABLE 3. Concentration of total polyphenols and antioxidative activity of worts and beverages.

Variety of beverage	Stage of the process	Total polyphenols content (mg GAE/L)	DPPH• (mmol TE/L)	ABTS•+ (mmol TE/L)	FRAP (mmol TE/L)
A	W[1]	683±0.70[e2]	3.99±0.04[d]	5.75±0.19[d]	7.69±0.35[gh]
AY		921±6.62b[c]	6.93±0.14[b]	11.0±0.97[ab]	8.40±0.47[e]
AC		1176±1.98[a]	7.98±0.32[a]	11.5±0.69[ab]	13.0±0.05[b]
AR		1163±1.62[a]	8.42±0.19[a]	12.2±0.63[a]	13.5±0.28[a]
A-1	M-1	482±2.25[f]	2.78±0.06[e]	4.93±0.24[d]	7.20±0.14[h]
AY-1		718±5.99[e]	5.46±0.22[c]	8.81±0.63[c]	10.0±0.30[e]
AC-1		825±5.97[d]	7.08±0.46[b]	10.1±0.44[abc]	11.9±0.17[cd]
AR-1		915±1.59[bc]	7.78±0.03[a]	10.5±0.86[abc]	12.5±0.13[bc]
AY-2	M-2	855±2.26[cd]	5.90±0.26[c]	9.75±2.95[bc]	11.5±0.22[d]
AC-2		901±4.06[bc]	7.13±0.60[b]	10.3±0.20[abc]	12.2±0.10[c]
AR-2		964±2.69[b]	7.90±0.07[a]	11.0±0.97[ab]	12.9±0.23[b]

[1] W, wort; M-1, beverages produced with the first method; M-2, beverages produced with the second method; [2] Values are expressed as the mean (n = 3) ± standard deviation. Mean values with different letters: a, b, c etc. are statistically different ($p < 0.05$). Symbols: A, AY, AC, AR, A-1, AY-1, AC-1, AR-1, AY-2, AC-2, and AR-2 as explained in Table 1.

al-fruit juice (AC) and it was by *ca.* 490 mg GAE/L higher than in the control wort (A). The total phenolics content in the wort with the addition of juice made of yellow-fruit variety of Cornelian cherry (AY) was higher by 240 mg GAE/L than in the control wort. The antioxidative activity of the worts measured with the DPPH•, ABTS•+, and FRAP tests was the highest in the worts with the addition of red-fruit juice (AR) and coral-fruit juice (AC) and slightly lower in the wort with the addition of yellow-fruit juice (AY). The addition of Cornelian cherry fruit juice to worts significantly enhanced their antioxidative properties compared to the control wort (A), whose antioxidative capability was by even 6.4 mmol TE/L lower than that of the wort with red-fruit juice addition (AR) – according to results of the ABTS•+ test.

The analysis of the total phenolics content in the fermented fruit beverages demonstrated that the addition of juice from Cornelian cherry fruits caused *ca*. twofold increase in the concentration of these compounds compared to the 100% apple beverage (A). In addition, the second method of production, in which Cornelian cherry juice was added to wort before the beginning of the aging process (M-2), enabled manufacturing beverages with higher concentrations of phenolics compared to the first method (M-1) in which juice was added to wort before the fermentation process. The highest concentration of phenolics was determined in the samples with the addition of red-fruit juice (AR-1, AR-2), however, it was by *ca*. 50 mg GAE/L higher in the beverages produced with the second method (AR-2). The antioxidative activity determined in the finished products with the DPPH• test was the highest in the beverages with the addition of red-fruit juice (AR-1, AR-2), and slightly lower in the samples with coral-fruit juice (AC-1, AC-2) and yellow-fruit juice (AY-1, AY-2). The statistical analysis of results achieved with the DPPH• method showed no significant differences in the antioxidative capabilities of products manufactured with methods M-1 and M-2. According to the DPPH• assay, the antioxidative activity of the control sample (A-1) was nearly two- and three times lower than in the samples with 10% addition of Cornelian cherry juice. Similar results were obtained in the ABTS•+ assay which also demonstrated that the antioxidative capability of the control sample (A-1) was almost twofold lower than in the variants with Cornelian cherry juice addition. The fermented apple-Cornelian cherry beverages produced in our study had similar antioxidative properties. The strongest antioxidative properties assayed with the ABTS•+ test (like in the case of DPPH• test and determination of total phenolics content) were exhibited by the sample with the addition of juice from red fruits of Cornelian cherry (AR-1, AR-2). The antioxidative capabilities were stronger in the beverages produced with the second method. The above-discussed data confirm results obtained in the Fe^{3+} reducing capability test (FRAP), in which the control sample had a weaker antioxidative capability (by 4.6 mmol TE/L beverage on average) than the beverages produced with the addition Cornelian cherry juice. Irrespective of the production method, the highest antioxidative capability measured with the FRAP test was demonstrated for the beverages with the addition of juice from red and coral fruits of Cornelian cherry (AR-1, AR-2, AC-1, AC-2). Despite a lack of significant differences in the antioxidative activity between beverages with coral-fruit and red-fruit juice, measured with the FRAP assay, results obtained allow concluding that – like in the case of the other analytical methods – slightly stronger antioxidative capabilities were determined in the beverages produced with the second method (AC-2, AR-2) compared to these produced with the first method (AC-1, AR-1). This is confirmed by results achieved for the variant with yellow-fruit juice addition, in which the beverage manufactured with the second method (AY-2) exhibited significantly stronger antioxidative activity (by *ca.* 1.5 mmol TE/L) compared to the beverage manufactured with the first

method (AY-1). The significant increase in the concentration of phenolics and antioxidative activity demonstrated in the beverages produced with the addition of juice from Cornelian cherry fruits results from the confirmed high content of natural antioxidants in these fruits [Kucharska, 2012; De Biaggi *et al*., 2018]. Studies have proved that various food products made of Cornelian cherry fruits or with their addition have a higher nutritional value [Bozdogan *et al*., 2017; Kawa-Rygielska *et al*., 2018]. This also applies to alcoholic beverages made of Cornelian cherry fruits, including fruit distillates or liqueurs [Kucharska, 2012; Sokół-Łętowska *et al*., 2014; Kawa-Rygielska *et al*., 2019]. Similar results were reported for fruit meads made with the addition of juices from different varieties of Cornelian cherry where the highest concentration of total phenolics and the strongest antioxidative capabilities measured with the same tests were determined for the mead produced with red-fruit juice [Adamenko *et al*., 2018]. Fruit wines analyzed by Ortiz *et al*. [2013] were characterized by a slightly higher concentration of total phenolics, reaching on average 999 mg GAE/L in apple-blackberry wines and 608 mg GAE/L in apple wines, but by a lower antioxidative activity measured with the DPPH$^{\bullet}$ test, accounting for 6.2 and 2.8 mmol TE/L, respectively. Substantially weaker antioxidative capability was demonstrated by Alberti *et al*. [2016] for apple ciders, *i.e.* barely 0.4–0.8 mmol TE/L cider according to the FRAP test. Polish apple ciders assayed with the ABTS$^{\bullet+}$ method by Kowalczyk *et al*. [2015] also exhibited a lower antioxidative activity, which reached 3.0 mmol TE/L on average.

Identification of iridoids and phenolics

Table 4 lists compounds identified in the produced worts and fermented apple-Cornelian cherry beverages which included four iridoids: loganic acid (LA), sweroside (S), loganin (L), and cornusides (Co and Co1); seven phenolic acids: 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA), 4-*O*-caffeoylquinic acid (4-CQA), caffeoylquinic acid derivatives (CQA1 and CQA2), *p*-coumaroylquinic acid (*p*-CoQA), and ellagic acid (EA); five anthocyanins: delphinidin 3-*O*-galactoside (Df 3-gal), cyanidin 3-*O*-galactoside (Cy 3-gal), cyanidin 3-*O*-robinobioside (Cy 3-rob), pelargonidin 3-*O*-galactoside (Pg 3-gal), and pelargonidin 3-*O*-robinobioside (Pg 3-rob); four flavanols: procyanidin dimer B1 (Dimer B1), (+)-catechin (Cat), procyanidin dimer B2 (Dimer B2), and (–)-epicatechin (epiC); four dihydrochalcones: 3-hydroxyphloretin 2'-*O*-xyloglucoside (3-OH-Ph 2'-xylglc), 3-hydroxyphloretin 2'-*O*-glucoside (3-OH-Ph 2'-glc), phloretin 2'-*O*-xyloglucoside (Ph 2'-xylglc), and phloretin 2'-*O*-glucoside (Ph 2'-glc) as well as four flavonols: quercetin 3-*O*-galactoside (Q 3-gal), quercetin 3-*O*-glucuronide (Q 3-glr) + quercetin 3-*O*-glucoside (Q 3-glc), kaempferol 3-*O*-galactoside (Kf 3-gal), and quercetin 3-*O*-xyloside (Q 3-xyl). 3-CQA, *p*-CoQA, dihydrochalcones, and quercetin 3-*O*-xyloside were detected only in the worts and beverages containing 100% of apple juice, which is due to the fact that these compounds are typical components of apples but do not occur in Cornelian cherry fruits [Alberti *et al*., 2016; Laaksonen *et al*., 2017]. Whereas compounds typical of the Cornelian cherry fruits, such as iridoids, ellagic acid, anthocyanins, quercetin 3-*O*-glucuronide, and kaempferol 3-*O*-galactoside, were identified only in worts and beverages containing juice pressed from these fruits (Table 3). In the apple-Cornelian cherry beverages, of all the identified compounds, the most abundant were iridoids, among which LA was the predominating compound (87–91%). The highest concentrations of these compounds were determined in worts and in beverages produced with the addition of coral-fruit juice (AC, AC-1, AC-2). 5-CQA was found to predominate among phenolic acids. Its highest concentration (93.5 mg/L on average) was assayed in the samples containing 100% of apple juice (A, A-1). In contrast, no EA was identified in these variants, but it was detected in all samples containing juice from Cornelian cherry fruits. Anthocyanins were not detected in worts and in the beverages made of apple juice (A, A-1) nor in the beverages containing juice from yellow fruits of Cornelian cherry (AY, AY-1, AY-2). The samples with coral-fruit juice (AC, AC-1, AC-2) contained only Cy 3-gal and Pg 3-gal, whose concentrations were several times lower than in worts and in the beverages with the addition of red-fruit juice (AR, AR-1, AR-2). In the latter samples, Cy 3-gal and Pg 3-gal were identified as the predominating anthocyanins and their contents constituted 73–76% of all detected anthocyanins. Among flavanols, Dimer B2, and epiCat were identified in all produced worts and fermented beverages, whereas Dimer B1 and Cat were detected only in the variants containing 100% of apple juice (A, A1). All worts and beverages contained a complete pool of identified dihydrochalcones. Flavonoles represented the smallest group of the identified phenolics; Kf 3-gal was identified only in the samples containing juice from red fruits of Cornelian cherry (AR, AR-1, AR-2), whereas concentration of Q 3-xyl was the same in all samples. Furthermore, Q 3-gal and Q 3-glr + Q 3-glc were detected in all analyzed variants. Their lowest concentrations were determined in the products without Cornelian cherry juice (A, A-1), whereas the highest concentrations were determined in the samples containing juice from coral and red fruits of Cornelian cherry. Our study demonstrated that the production method of fermented apple-Cornelian cherry beverages had no significant effect on concentrations of iridoids, phenolic acids, dihydrochalcones, and flavonols in the finished products. An opposite observation was made for anthocyanins and flavanols, whose higher concentrations were determined in the products manufactured with the second method in which Cornelian cherry juice was added after the effervescent fermentation (M-2). This points to greater degradation of anthocyanins and flavanols – being less stable compounds – during ethanolic fermentation compared to the other compounds. The composition of phenolics in the control variants without Cornelian cherry juice addition (A, A-1) is similar to the polyphenolic profiles reported by other authors [Venkatachalam *et al*., 2018;, Alberti *et al*., 2016; Laaksonen *et al*., 2017], who analyzed this group of compounds in apple ciders and also identified phenolics, flavonols, flavanols, and dihydrochalcones, however failed to identify any representatives of iridoids, which were detected in the fermented apple-Cornelian cherry beverages produced in our study. In turn, Marks *et al*. [2007] who investigated phenolics composition of 23 English ciders, demonstrated the presence of compounds from four groups of phenolics,

TABLE 4. Concentrations of iridoids and phenolics (mg/L) in worts and beverages.

No	Compound	Wort before fermentation				Beverages produced with the first method				Beverages produced with the second method		
		A	AY	AC	AR	A-1	AY-1	AC-1	AR-1	AY-2	AC-2	AR-2
						Iridoids						
1	LA[1]	nd[3]	464±4.53[b4]	521±4.53[a]	327±4.33[d]	nd	437±6.31[c]	513±4.38[a]	322±4.35[d]	429±7.07[c]	516±7.35[a]	325±3.95[d]
2	S + L	nd	22.4±3.45[a]	23.2±2.96[a]	16.5±3.11[b]	nd	19.7±0.86[ab]	23.0±4.24[a]	16.2±1.63[b]	19.7±1.41[ab]	23.6±1.56[a]	16.4±2.90[b]
3	SecoL	nd	3.24±0.28[ab]	4.03±0.17[a]	3.56±0.42[ab]	nd	2.85±0.57[b]	3.45±0.28[ab]	2.93±0.18[b]	3.15±0.43[b]	3.65±0.14[b]	3.36±0.33[ab]
4	Co	nd	35.6±3.21[a]	35.2±1.67[a]	27.7±1.83[bc]	nd	22.5±2.10[d]	24.4±1.84[cd]	19.9±1.98[d]	31.0±1.41[ab]	34.9±2.34[a]	27.7±2.11[bc]
5	Co1	nd	tr[2]	2.19±0.08[a]	0.40±0.04[c]	nd	tr	1.44±0.06[b]	0.38±0.09[c]	tr	1.49±0.11[b]	0.36±0.03[c]
						Phenolic acids						
1	3-CQA	0.57±0.07[def]	0.55±0.05[def]	0.63±0.03[cde]	0.81±0.04[a]	0.54±0.04[def]	0.49±0.06[f]	0.65±0.01[cd]	0.80±0.04[a]	0.52±0.03[ef]	0.68±0.06[bc]	0.78±0.06[ab]
2	5-CQA	96.8±4.53[a]	81.1±4.30[b]	77.9±2.98[bc]	81.9±2.40[b]	90.3±2.86[a]	70.0±3.13[d]	74.9±3.53[bcd]	78.9±2.02[bc]	72.0±3.21[cd]	76.2±2.88[bcd]	79.8±3.12[bc]
3	4-CQA	6.95±0.67[bc]	8.38±0.92[a]	9.04±0.27[a]	6.66±0.28[c]	6.63±0.71[c]	6.33±0.45[c]	8.14±0.35[ab]	5.77±0.60[c]	6.33±0.69[c]	8.73±0.52[a]	6.32±0.28[c]
4	CQA 1	1.78±0.33[a]	1.55±0.11[ab]	1.41±0.25[ab]	1.35±0.18[ab]	1.49±0.36[ab]	1.26±0.06[b]	1.36±0.13[ab]	1.27±0.12[b]	1.23±0.08[b]	1.19±0.23[b]	1.13±0.13[b]
5	CQA 2	0.51±0.04[c]	0.51±0.03[c]	0.47±0.03[c]	0.47±0.03[c]	0.88±0.04[a]	0.53±0.08[c]	0.54±0.03[c]	0.55±0.01[c]	0.65±0.06[b]	0.65±0.03[b]	0.69±0.04[b]
6	p-CuQA	6.94±0.59[bcd]	7.43±0.16[abc]	7.05±0.38[bcd]	8.15±0.57[a]	6.22±0.45[de]	5.89±0.45[e]	6.50±0.34[cde]	7.77±0.37[ab]	6.05±0.23[de]	6.83±0.58[bcde]	7.79±0.31[ab]
7	EA	nd	0.10±0.02[e]	0.18±0.02[cd]	0.20±0.04[bc]	nd	0.13±0.03[de]	0.25±0.03[b]	0.22±0.02[bc]	0.10±0.01[e]	0.37±0.03[a]	0.18±0.01[cd]
						Anthocyanins						
1	Df 3-gal	nd	nd	nd	0.69±0.01[a]	nd	nd	nd	0.26±0.03[c]	nd	nd	0.58±0.02[b]
2	Cy 3-gal	nd	nd	0.47±0.03[b]	10.2±0.47[b]	nd	nd	0.20±0.01[b]	4.63±0.33[b]	nd	0.39±0.03[b]	8.86±0.28[a]
3	Cy 3-rob	nd	nd	nd	4.81±0.20[a]	nd	nd	nd	2.94±0.18[b]	nd	nd	4.41±0.12[a]
4	Pg 3-gal	nd	nd	3.37±0.33[d]	20.0±0.47[a]	nd	nd	1.78±0.17[e]	10.9±0.36[c]	nd	2.85±0.19[d]	17.8±0.13[b]
5	Pg 3-rob	nd	nd	nd	3.77±0.21[a]	nd	nd	nd	2.59±0.23[b]	nd	nd	3.49±0.22[a]
						Flavanols						
1	Dimer B1	4.46±0.37[a]	nd	nd	nd	4.32±0.42[a]	nd	nd	nd	nd	nd	nd
2	Cat	1.89±0.31[a]	nd	nd	nd	1.90±0.12[a]	nd	nd	nd	nd	nd	nd
3	Dimer B2	18.9±0.86[b]	19.3±0.57[b]	19.6±0.55[b]	22.3±0.94[a]	14.2±0.25[d]	14.2±0.51[d]	16.3±0.40[c]	19.0±0.42[b]	16.3±0.31[c]	18.9±1.00[b]	20.3±0.76[b]
4	epiCat	20.2±0.85[a]	14.8±0.98[de]	18.2±0.93[ab]	18.9±1.38[ab]	18.6±0.62[ab]	16.7±1.02[bcd]	15.7±0.87[cd]	13.3±0.61[e]	18.8±0.90[ab]	16.8±0.93[bcd]	17.8±0.99[bc]

Dihydrochalcones											
1	3-OH-Ph 2'-xylglc	12.9±1.30^{a}	11.2±1.03^{bc}	10.7±0.46^{bc}	10.9±0.40^{bc}	11.4±0.88^{ab}	9.8±0.74^{bc}	9.8±0.82^{bc}	10.1±0.44^{bc}	9.55±0.73^{c}	10.3±0.33^{bc}	9.96±0.42^{bc}
2	3-OH-Ph 2'-glc	6.82±0.53^{a}	5.27±0.37^{b}	4.99±0.49^{b}	5.51±0.64^{a}	6.81±0.25^{b}	5.18±0.33^{b}	5.01±0.46^{b}	5.69±0.40^{b}	5.16±0.41^{b}	5.28±0.41^{b}	5.73±0.34^{b}
3	Ph 2'-xylglc	9.85±0.85^{a}	8.05±0.79^{bc}	7.86±0.81^{c}	8.29±0.42^{abc}	9.00±0.63^{ab}	6.75±0.62^{bc}	7.10±0.77^{c}	7.46±0.52^{bc}	6.80±0.47^{c}	7.60±0.64^{bc}	7.71±0.82^{bc}
4	Ph 2'-glc	11.6±0.78^{a}	9.97±0.35^{b}	9.78±0.23^{b}	9.80±0.30^{b}	11.6±0.77^{a}	9.04±0.27^{b}	9.55±0.19^{b}	9.68±0.24^{b}	9.38±0.20^{b}	9.64±0.25^{b}	9.79±0.22^{b}
Flavonols												
1	Q 3-gal	0.80±0.07^{d}	1.06±0.12^{bcd}	1.46±0.19^{a}	1.32±0.18^{ab}	0.81±0.02^{d}	0.91±0.09^{d}	1.33±0.16^{ab}	1.26±0.11^{abc}	1.01±0.06^{cd}	1.37±0.07^{a}	1.37±0.10^{a}
2	Q 3-glcr + Q 3-glc	0.30±0.04^{d}	5.30±0.41^{b}	6.93±0.25^{a}	4.38±0.39^{c}	0.28±0.06^{d}	4.50±0.40^{c}	6.48±0.42^{a}	4.02±0.28^{c}	4.72±0.24^{bc}	6.84±0.29^{a}	4.34±0.28^{c}
3	Kf 3-gal	nd	nd	nd	3.32±0.06^{a}	nd	nd	nd	2.91±0.12^{a}	nd	nd	3.21±0.24^{a}
4	Q 3-xyl	1.38±0.11^{a}	1.22±0.25^{a}	1.26±0.16^{a}	1.19±0.13^{a}	1.32±0.08^{a}	1.05±0.14^{a}	1.16±0.08^{a}	1.12±0.06^{a}	1.11±0.12^{a}	1.24±0.18^{a}	1.18±0.08^{a}

[1]LA, loganic acid; S, sweroside; L, loganin; Co, cornuside; 3-CQA, 3-*O*-caffeoylquinic acid (neochlorogenic acid); 5-CQA, 5-*O*-caffeoylquinic acid (chlorogenic acid); 4-CQA, 4-*O*-caffeoylquinic acid (cryptochlorogenic acid); CQA, caffeoylquinic acid; *p*-CoQA, *p*-coumaroylquinic acid; EA, ellagic acid, Df 3-gal, delphinidin 3-*O*-galactoside; Cy 3-gal, cyanidin 3-*O*-galactoside; Cy 3-rob, cyanidin 3-*O*-robinobioside; Pg 3-gal, pelargonidin 3-*O*-galactoside; Pg 3-rob, pelargonidin 3-*O*-robinobioside; Dimer B1, procyanidin dimer B1; C, (+)-catechin; Dimer B2, procyanidin dimer B2; epiC, (−)-epicatechin; 3-OH-Ph 2'-xylglc, 3-hydroxyphloretin 2'-*O*-xyloglucoside; 3-OH-Ph 2'-glc, 3-hydroxyphloretin 2'-*O*-glucoside; Ph 2'-xylglc, phloretin 2'-*O*-xyloglucoside; Ph 2'-glc, phloretin 2'-*O*-glucoside; Q 3-gal, quercetin 3-*O*-galactoside; Q 3-glcr, quercetin 3-*O*-glucuronide; Q 3-glc, quercetin 3-*O*-glucoside; Kf 3-gal, kaempferol 3-*O*- galactoside; Q 3-xyl, quercetin 3-*O*-xyloside; [2]tr, traces: detected but not quantified; [3]nd, not detected; [4]Values are expressed as the mean (n = 3) ± standard deviation. Mean values with different letters: a, b, c etc. are statistically different ($p < 0.05$); Symbols: A-1, AY-1, AC-1, AR-1, AY-2, AC-2, and AR-2 as explained in Table 1.

i.e.: flavan-3-ols, hydroxycinnamates, flavonols, and dihydrochalcones. Analysis of the phenolics composition of the ciders indicated that it depended on the variety of apples and on the apple processing method. The English ciders were also characterized by a richer composition of phenolics compared to the earlier investigated apple juices [Marks *et al*., 2007].

CONCLUSIONS

Results achieved in this study prove that the produced apple-Cornelian cherry alcoholic beverages had strong antioxidative properties and high concentrations of natural antioxidants. The 10% addition of juice from red-fruit Cornelian cherry enabled producing beverages with the highest concentration of these compounds and, consequently, with the highest antioxidative capabilities. Apart from such phenolics as phenolic acids, anthocyanins or flavonols, they contained many compounds from the group of iridoids, in particular in the products with red and coral-fruit Cornelian cherry. Production method affected both concentrations of the mentioned compounds and antioxidative properties of the beverages. Juice addition after the primary fermentation caused better effects than its addition before this process. The novel natural fermented beverages produced in this study may be found interesting products on the market of alcoholic beverages and may also complete an everyday diet in phenolics. In the future, it is necessary to perform consumer sensory analysis for a deeper understanding of the scientific significance of the new products, as well as the possibility of introducing these beverages to the market.

RESEARCH FUNDING

Publication supported by the Wroclaw Centre of Biotechnology, Leading National Research Centre programme (KNOW) for the years 2014–2018.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Submitted: 18 April 2019. Revised: 30 may and 22 July 2019. Accepted: 30 July 2019. Published on-line: 21 August 2019.

Pol. J. Food Nutr. Sci., 2019, Vol. 69, No. 3, pp. 319–321
http://journal.pan.olsztyn.pl

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