

ITALIAN JOURNAL OF FOOD SCIENCE

*Rivista italiana
di scienza degli alimenti*



Volume XXXI
Number 4
2019



ITALIAN JOURNAL OF FOOD SCIENCE (RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aegis of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.A.I.)
Initially supported in part by the Italian Research Council (CNR) - Rome - Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage - Rome - Italy

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Impact Factor:

Impact Factor: 0.736 published in 2018 Journal of Citation Reports, Scopus CiteScore 2018: 0.92. IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; CurrentContents®/AB&ES; SciSearch® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); UDL-Edge Citations Index (Malaysia); EBSCO Publishing; Index Copernicus Journal Master List (PL).

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INFLUENCE OF TOMATO POWDER ON COMMINUTED MEAT PRODUCT QUALITY

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ABSTRACT

This study investigated the influence of tomato powder on the quality attributes (pH, colour, cooking loss, oxidative stability, sensory quality) of comminuted meat products. The addition of 2.0% and 2.5% of tomato powder significantly decreased lightness, increased redness and yellowness and delayed oxidation processes in the products. Tomato powder lowered the pH values of batters, but had no impact on pH or cooking losses of the products. Products enriched with tomato powder were more acceptable than the control sample. The results indicate the beneficial impact of tomato powder on the quality of comminuted meat products.

Keywords: functional foods, lipid oxidation, lycopene, meat product, sensory quality, tomato

1. INTRODUCTION

Tomatoes are valuable components of the human diet due to a high content of carotenoids, vitamins (C, K, E, B₁, B₂, B₆, B₉, PP, H) and mineral compounds (K, Na, P, Mg, Ca, Fe, Cu, Zn, Mn). Among the carotenoids, the most abundant is lycopene (FERNANDES *et al.*, 2016). Although fresh tomatoes are available on the market throughout the year, there are also many different processed tomato products offered to consumers, including ketchup, tomato puree (passata), concentrate, whole and cut canned tomatoes, sun-dried tomatoes and tomato powder. Although the processing of fruit and vegetables might reduce the concentration of valuable bioactive compounds, in the case of lycopene, thermal treatment of tomatoes increases lycopene concentration in the products from up to 4.2 mg/100 g in fresh tomatoes to up to 265 mg/100 g in tomato powder (SKIEPKO *et al.*, 2015). Tomato powder (TP) is produced from tomatoes subjected to drying and grinding, while lycopene itself might be obtained from waste material, such as tomato peel or seeds, or using chemical and microbial syntheses (HERNANDEZ-ALMANZA *et al.*, 2016).

A beneficial impact on human health has been attributed to lycopene consumption, due to its potential in alleviating chronic diseases, including cancer and coronary heart disease. Lycopene is recognized as a reactive oxygen species (ROS) scavenger and may therefore control ROS-mediated cell growth (HERNANDEZ-ALMANZA *et al.*, 2016; PALOZZA *et al.*, 2011). It has also been demonstrated that it induces cell-to-cell communication and improves the functioning of immune and hormone systems and may prevent osteoporosis (HERNANDEZ-ALMANZA *et al.*, 2016; SOŁTYSIAK and FOLWARCZNA, 2015). In order to induce a beneficial impact of lycopene on the human body, such as combating oxidative stress and averting chronic diseases, from 5 to 10 mg of lycopene should be consumed daily (RAO and SHEN, 2002). The main sources of lycopene in the diet in Poland are fresh and processed tomatoes, tropical fruit and watermelons. The average daily lycopene intake in Poland is estimated at 7 to 7.5 mg, which is similar to levels noted for inhabitants of California (6.6 mg) and Canada (6.4 mg) (SOŁTYSIAK and FOLWARCZNA, 2015). Although the intake of lycopene is higher than the minimum recommended level, an increase in its consumption (e.g. in meat products containing processed tomatoes) would be beneficial.

Incorporating lycopene into meat products has gained much interest among food scientists and in the meat industry. Numerous papers concerning the effect of lycopene addition on the quality of meat products have been published (CALVO *et al.*, 2008; EYLIER and OZTAN, 2011; GARCIA *et al.*, 2009; HAYES *et al.*, 2013). However, to the authors' knowledge there are few products with tomato powder available on the market. Although the influence of lycopene on meat product colour is well-documented, the sensory quality of the products depends strongly on the amount and the source of lycopene, the nature of the food matrix and its composition. The influence of lycopene on lipid oxidation in meat products during storage is not fully recognized due to various factors affecting the process, related to raw materials (composition, physical state, comminution degree), methods used in meat processing, storage conditions, the length of storage, etc. In a previous study by the authors, the effect of adding from 0.2 to 1.0% tomato powder to comminuted meat products stored under vacuum up to 14 days was investigated (MODZELEWSKA-KAPITUŁA, 2012). All products showed good sensory quality, but the addition of tomato powder did not retard lipid oxidation in meatloaves stored in vacuum packages. Since higher amounts of tomato powder were not used, the present study was undertaken to investigate the effect of adding 2.0% and 2.5% of tomato powder on the quality of comminuted meat products. Moreover, in contrast to the

previous study, the products were stored under aerobic conditions in a refrigerator for up to seven days, which resembles the conditions under which the products might be stored in households after opening a vacuum package in which products might be distributed and the influence of tomato powder on fat oxidation was studied. Therefore, the aim of the present study was to investigate the effect of an increased amount of tomato powder (up to 2.5%) on comminuted meat product quality, including pH values, thiobarbituric acid reactive substances (TBARS), sensory quality and colour.

2. MATERIAL AND METHODS

2.1. Materials and production

The meatloaves were produced from pork neck, which was ground twice through a size 5 mesh and mixed manually in a bowl with ice water (10%), bread crumbs (4%), fried onion (3%), an egg, salt (1.5%), pepper (0.1%), nutmeg (0.1%) and fresh garlic (0.07%). The amount of additives was calculated in respect to the mass of comminuted meat. Three treatments were produced: control – with no addition of tomato powder (TP) and with 2.0% and 2.5% of TP (ALTOMA 9010, Diana Naturals, Antrain, France, kindly provided by Kaczmarek-Komponenty, Mrowino, Poland). TP was added to a batter in the required amount and mixed manually to obtain an even distribution within the product. The batters (ca. 300 g) were placed in the individual aluminium forms and heated at 180°C in dry air to 72°C in a geometric centre. After thermal processing, products were cooled to 4°C±1°C and stored in darkness under aerobic conditions at 4°C±1°C until the 7th day after production. Three independent batches of meatloaves were produced on three different occasions.

According to the technical data sheet provided by the producer, the TP contained dehydrated tomato (*Lycopersicum esculentum* L., min. 99% dry matter) and anti-caking agent: silicon dioxide (E551). It was a powder (100% < 1 mm) showing a medium solubility in water and red colour. Total carotenoid content, expressed as lycopene equivalent (EC Dir 95/45: OD 472 nm E 1%, 1 cm = 3,450, hexane) > 850 mg/kg, fresh tomato ratio: 17:1 (w/w). Indicative nutritional data for 100 g of TP were as follows: carbohydrates from 45.0 g to 75.0 g, fat up to 1.0 g, proteins (N × 6.25) from 5 g to 15 g, minerals from 2.0 g to 4.0 g, energy from 200 kcal to 370 kcal.

2.2. Methods

The following analyses were conducted: sensory evaluation (at day 1, the next day after production), pH measurements (day 1, 3, 7), colour evaluation (day 1) and TBARS (day 1, 3, 7).

Cooking loss was calculated based on the differences in the mass of the products before and after thermal treatment.

Acidity (pH) values were determined in batters and products (day 1, 3, 7) in homogenates prepared with 10 g of a batter or a product and 10 g of distilled water (pH-meter HI 8314C, Hanna Instruments Polska, Olsztyn, Poland). Three homogenates were prepared for each sample.

The 2-thiobarbituric acid reactive substances (TBARS), as an indicator of fat oxidation in products, were determined according to the modified Salih method (PIKUL *et al.*, 1993;

described in detail in MODZELEWSKA-KAPITUŁA, 2012) at day 1, 3 and 7. Two replicates from each sample were prepared.

The colour (CIE Lab) of the meatloaves was determined at day 1, using Miniscan XE Plus (HunterLab, Reston, USA) in three different positions on the surface and the cross-section of the products.

Sensory evaluation was conducted by six panellists trained and experienced in sensory evaluation, using difference and preference tests (scoring and ranking methods, respectively). The following attributes of the products were scored on a 6-point scale: overall appearance (1 – irregular shape, surface extremely dry or wet; 6 – regular shape, surface dry and clean), colour on the cross-section (1 – extremely pale, atypical, uneven; 6 – uniform, desirable), consistency (1 – extremely greasy, crumbling; 6 – compact, elastic), taste (1 – bland or extremely intensive, atypical; 6 – perceptible, typical), aroma (1 – bland or extremely intensive, atypical; 6 – perceptible, typical). The panellists were then asked to rank the products according to their preferences from the most desirable (value of 1) to the least desirable (value of 3). The samples for evaluation were served sliced (5 mm thick) at a temperature of approx. 10°C, randomly presented on white plates, coded with two-digit, random numbers. Water at room temperature and bread were provided for cleansing the palate between samples. The evaluation was carried out at room temperature (approx. 20°C) under fluorescent lighting. In total, three sensory analysis sessions were performed, during which three meat product samples were assessed per session.

2.3. Statistical analysis

The results were analysed using Statistica 12 (StatSoft. Inc., Tulsa, USA) at a significance level $p < 0.05$. The influence of tomato powder addition on colour, cooking loss and batter pH were evaluated using one-way variance analysis, as well as the influence of storage time on pH and TBARS. The results of sensory evaluations were analysed using a non-parametric Kruskal-Wallis test, whereas the results of a preference test were analysed using χ^2 Pearson's test.

3. RESULTS AND DISCUSSION

The influence of TP addition on pH and cooking loss of products is presented in Table 1. The addition of TP significantly lowered the pH values of the batters, which was caused by acidic pH of tomatoes. TP had no impact on the pH of final products or cooking losses. The pH values of products with and without TP addition did not change during 7- day storage in a refrigerator ($P < 0.05$).

Reduction in pH values as a result of tomato products, such as dried tomato peel, sun-dried tomatoes and tomato paste, when added to meat products was reported also by other authors (CONDOGAN, 2002; GARCÍA *et al.*, 2009; ØSTERLIE and LERFALL, 2005). Reduced pH values of meat batters might increase cooking losses due to reduced water holding capacity of meat proteins (HUFF-LONERGAN and LONERGAN, 2005). In the present study, the decrease in pH values was apparently too low to exert an adverse effect on cooking loss.

Table 1. The influence of tomato powder (TP) on pH and cooking loss of comminuted meat products.

Attribute	Product		
	0% TP	2.0% TP	2.5% TP
pH: batter	6.1±0.1 ^a	5.9±0.1 ^b	5.7±0.1 ^b
pH: product day 1	6.8±0.2 ^{aA}	6.6±0.2 ^{aA}	6.5±0.2 ^{aA}
pH: product day 3	6.8±0.2 ^{aA}	6.6±0.2 ^{aA}	6.5±0.2 ^{aA}
pH: product day 7	6.8±0.1 ^{aA}	6.6±0.1 ^{aA}	6.5±0.1 ^{aA}
Cooking loss (%)	8.0±0.1 ^a	8.5±0.1 ^a	8.9±0.1 ^a

^{a,b} means in rows with different letters differ significantly at $p < 0.05$; ^A means in columns with the same letter do not differ significantly at $p < 0.05$.

The colour of the surface and the cross-section of the products containing TP and those without the ingredient differed significantly and, moreover, the amount of TP affected the intensity of the cross-section colour (Table 2). The surface of the control samples (0% TP) had higher L^* and lower a^* and b^* values than TP-containing products (2.0% TP and 2.5% TP). A cross-section of the control sample also had higher L^* values than both TP-containing samples. The values of a^* and b^* increased with the increase in TP addition, which indicates that when more TP was added to the batter, the cross-section colour of the products became more intense and the proportion of red and yellow hues was higher and, thus, the colour turned toward orange. These results resemble those presented by GARCÍA *et al.* (2009), EYILER and OZTAN (2011), HAYES *et al.* (2013).

Table 2. The influence of tomato powder (TP) on the colour and sensory attributes of comminuted meat products.

Attribute	Product		
	0% TP	2.0% TP	2.5% TP
Colour: surface			
L^*	43.5±5.3 ^a	23.6±1.9 ^b	35.2±4.5 ^b
a^*	7.4±1.5 ^b	15.8±0.8 ^a	15.7±1.5 ^a
b^*	19.6±3.4 ^b	23.4±1.7 ^a	22.2±2.5 ^a
Colour: cross-section			
L^*	59.6±5.4 ^a	53.5±3.8 ^b	53.5±2.8 ^b
a^*	5.4±0.8 ^c	13.8±2.3 ^b	16.7±1.3 ^a
b^*	17.8±0.7 ^c	23.6±1.7 ^b	26.7±0.7 ^a
Sensory quality			
Overall appearance	3.9±1.1 ^a	4.4±1.1 ^a	4.4±0.9 ^a
Colour of the cross-section	4.0±1.2 ^a	4.3±1.0 ^a	3.8±1.0 ^a
Consistency	4.6±0.9 ^a	4.5±1.1 ^a	4.3±1.0 ^a
Taste	4.4±1.1 ^a	4.8±1.2 ^a	4.7±1.2 ^a
Aroma	4.5±1.2 ^a	4.3±0.9 ^a	4.2±1.0 ^a

^{a,b,c} means in rows with different letters differ significantly at $p < 0.05$.

Sensory analysis comprised the evaluation of particular attributes of products (Table 2) and the indication of the most preferred product. All products were scored between 3.8 and 4.8, which indicated their good sensorial quality and there were no significant differences between the control and TP containing products. These results are in line with those of GARCÍA *et al.* (2009), who used dry tomato peel (DTP) in hamburgers and noted no significant differences between the control sample and those produced with a 3% addition of DTP in odour and texture. CALVO *et al.* (2008) also reported no differences in a hedonic test of dry fermented sausages produced with dry tomato peel up to 1.2% (w/w). Panellists more often chose products with TP (2.0% and 2.5%) as more preferred than the control (0% TP) ($P < 0.05$). The results indicate that a TP addition in the range from 2.0% to 2.5% positively affected the sensory quality of comminuted meat products. CALVO *et al.* (2008) found that the colour of meat products (sausages) influenced the preferences of consumers, although in their study the addition of dry tomato peel (0.9 and 1.2%) to sausages lowered their acceptability. Thus, it might also be concluded that the acceptability of meat products with tomato powder depends on the product type and its characteristics.

Tomato powder affected TBARS in the products during 7-day cold storage in aerobic conditions (Fig. 1). In the control sample, which did not contain TP, TBARS increased in storage time and significant differences between day 1, day 3 and day 7 were noted ($P < 0.05$). In the product, which contained 2.0%, TBARS increased between day 1 and 3 and remained unchanged between day 3 and 7, whereas in the product (which contained 2.5% TP) TBARS did not change during the storage period.

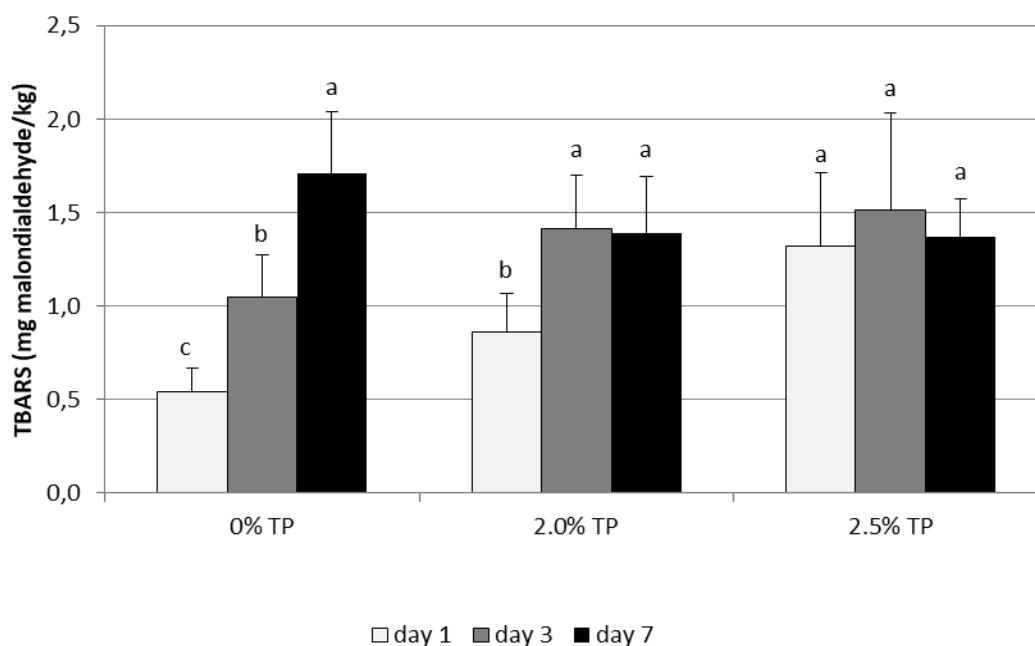


Figure 1. The influence of storage time on TBARS in meatloaves produced with the addition of tomato powder (2.0% TP, 2.5% TP) and without the tomato powder (0% TP).

^{a,b,c} means within each treatment with different letters differ significantly at $p < 0.05$.

These findings suggest that tomato powder has the potential to inhibit oxidation in meat products stored under aerobic conditions and its effectiveness depends on the amount of tomato powder. The anti-oxidative effect of lycopene added to paprika salami was also reported by ROHLÍK *et al.* (2013), which supports the results of the present study. On the other hand, it was also noted that TBARS values at day 1 were the highest for 2.5% TP, whereas they were the lowest for 0% TP products. The results indicate that the method used for TBARS determination, which is suitable for evaluation of the oxidation process in meat and meat products, might give misleading results, probably due to the elution of carotenoids from TP-containing samples, which contributes to higher absorbance values. That is why the results of TBARS were not compared between samples containing different amounts of TP, but analysed taking into consideration only the storage time. On the other hand, the increase in TBARS along with the increased amount of tomato products (powder, paste) in meat products was also noted by EYILER and OZTAN (2011), DEDA *et al.* (2007) and HAYES *et al.* (2013), who attributed it to the pro-oxidative effect of lycopene used in higher concentrations.

TBARS is used as a lipid oxidation indicator in meat and processed meat products. A threshold of 2 mg malondialdehyde/kg sample is regarded as the minimum TBARS value that causes the off-flavour in meat and meat products (EYILER and OZTAN, 2011). No samples investigated in this study exceeded 2 mg MA/kg during seven days of storage under aerobic conditions, which indicated that their quality was not reduced by excessive lipid oxidation. Similar values were noted in the previous studies, in which meat products with lycopene addition were stored vacuum-packed (MODZELEWSKA-KAPITUŁA, 2012; EYILER and OZTAN, 2011). However, in the previous studies, the antioxidant properties of lycopene in cooked meat products, stored in vacuum-packages were not proven, probably due to evacuation of the oxygen from vacuum packages.

4. CONCLUSIONS

In conclusion, tomato powder added in amounts of 2.0% and 2.5% to a comminuted meat product increased its sensory acceptability and oxidative stability, changed the colour towards more orange and decreased pH, although it had no influence on cooking losses. Thus, the meat products obtained using 2.5% of tomato powder might be a healthier alternative to the ready-to-eat meat products currently available on the market. However, before launching them on the market, the results of the present study should be complemented with an evaluation of consumer acceptability of the products and determination of microbial quality, due to the lack of preservatives such as sodium nitrite. Lycopene content in the products should also be examined, since the storage of tomato products (e.g. tomato puree) might reduce lycopene content (MARKOVIĆ *et al.*, 2007). The application of tomato powder as a source of lycopene and the lack of food additives provides the opportunity to introduce "clean label" products with nutritional benefits for the consumer.

ACKNOWLEDGEMENTS

The authors would like to thank the technical support of Natalia Wdowiak. The study was financed from the statutory funds of the Faculty of Food Sciences, University of Warmia and Mazury in Olsztyn. 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.

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Paper Received February 19, 2019 Accepted May 20, 2019

EFFECTS OF HIGH PRESSURE AND MARINATION TREATMENT ON TEXTURE, MYOFIBRILLAR PROTEIN STRUCTURE, COLOR AND SENSORY PROPERTIES OF BEEF LOIN STEAKS

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ABSTRACT

The influence of high pressure/marination treatment on the texture, myofibrillar protein structure, color and sensory properties of beef loin steaks was studied. Combined high pressure and marination treatment at 550 MPa significantly increased beef tenderness, but had a “whitening/brightening” effect on the color of the samples ($P < 0.05$). High-pressure processing caused protein degradation, leading to texture development. Furthermore, the panelists gave the highest overall impression score to the 150 MPa pressurized samples. These results show that combined high pressure and marination treatment at 550 MPa can potentially improve the textural properties of beef loin steaks, although it is less favored than pressurization treatment.

Keywords: beef, high pressure, marination, protein degradation, texture

1. INTRODUCTION

High pressure processing has been a hot topic of study among scientists because of its positive effects on the safety, quality and sensory properties of food products (EVRENDİLEK *et al.*, 2008). Addressing the increasing demand for minimally processed foods with high nutritional and sensory quality, the food industry has employed high pressure processing to develop high quality, fresh, additive-free food products with an extended shelf life. In the European Community, high pressurized foods are classified as “novel foods” (CAMPUS, 2010). This novel technology also offers an alternative to pasteurization treatment and could have great potential for heat-sensitive foods (DURANTON *et al.*, 2011).

There is particular interest in researching the effects of high pressure on the food matrix (SUN and HOLLEY, 2010). It has been discovered very recently that short-time high pressure application at lower temperatures develops tenderness in meat and meat products while minimally changing the natural characteristics of the product (BAJOVIC *et al.*, 2012). It has been hypothesized that applying pressure to meat in the postmortem period could cause changes to the enzymes and proteins, especially to the gelatin characteristics of myofibrillar proteins, color, microbial load, ultrastructure and textural properties of meat (SCHENKOVA *et al.*, 2007). In research studies, pressure levels applied to post-rigor meat generally ranged from 100 to 600 MPa with short processing times (5-20 min.) at 15-60°C, according to the purpose of the study (CHAN *et al.*, 2011; KRUK *et al.*, 2011; MCARDLE *et al.*, 2013; GROSSI *et al.*, 2014; SIKES and TUME, 2014; GIMENEZ *et al.*, 2015). Researchers have also reported that high pressure technology is a physical additive-free process for meat tenderizing and softening due to its effects on the gel-forming ability of proteins and on texture (BUCKOW *et al.*, 2013). The effective pressure levels have varied from 150 to ≥ 500 MPa (5 min, 20°C) for meat tenderization (SUN and HOLLEY, 2010). Furthermore, post-rigor meat tenderization without bleaching of color can be achieved with pressure levels up to 300 MPa for a few minutes at room temperature (CAMPUS, 2010).

Marination treatment using plant additives is another natural way to preserve meat and meat products. In recent years, natural plant extracts with high phenolic contents have been used in meats, due to their safety characteristics and beneficial effects on health, synthetic chemical preservatives. There are numerous studies about plant extracts (such as grape seed, green tea, pomegranate, peanut skin, garlic, rosemary, olive leaf, moringa leaf, nettle, myrtle, and mint leaf extracts) used in meat and meat products (AKARPAT *et al.*, 2008; ALP and AKSU, 2010; YU *et al.*, 2010; DEVATKAL *et al.*, 2010; HAYES *et al.*, 2010; COLINDRES and BREWER, 2011; RABABAH *et al.*, 2011; BISWAS *et al.*, 2012; DAS *et al.*, 2012; ÖZVURAL and VURAL, 2012; CAO *et al.*, 2013). Among these natural extracts, oleoresin rosemary (Herbalox®) has been commonly included in food processing as a shelf life extender and flavor developer (AHN *et al.*, 2007). In addition, there are many studies in the literature about the antimicrobial and antioxidant activities of rosemary in different food materials (BOTSOGLOU *et al.*, 2007; SASSE *et al.*, 2009; NIETO *et al.*, 2010; PUANGSOMBAT and SMITH, 2010; COLINDRES and BREWER, 2011; WOJCIAK *et al.*, 2011; GIBIS and WEISS, 2012; MATHENJWA *et al.*, 2012; KIM *et al.*, 2013). The literature studies reported that the effective usage level of rosemary extract varied between 0.02-10% in a marinade solution for retarding lipid oxidation and improving sensorial characteristics in meat and meat products (AHN *et al.*, 2007; AKARPAT *et al.*, 2008; ROJAS and BREWER, 2008; WOJCIAK *et al.*, 2011).

The use of a combination of pressure and marination treatment can be an alternative preservation method for meat producers. The combined treatment of pressure and marination can be more efficient at improving meat quality attributes and increasing shelf life. It was reported that the combined treatment of high pressure and natural antioxidants as a multi-hurdle approach can be an alternative treatment in the meat industry (HYGREEVA and PANDEY, 2016). However, there are relatively few studies regarding the combined use of high pressure and natural extracts in meat and meat products, and generally chemical preservatives were used for marination in these studies (SCHENKOVA *et al.*, 2007; OHNUMA *et al.*, 2013; KIM *et al.*, 2014; GIMENEZ *et al.*, 2015; RODRIGUES *et al.*, 2016). In addition, oregano, rosemary, papain plants and carvacrol were used as natural antioxidants for meat and meat products in the studies evaluating a combination of high pressure and marination treatment (BRAGAGNOLO *et al.*, 2005; GOMEZ-ESTACA *et al.*, 2007; de OLIVEIRA *et al.*, 2015). Although very few studies have been published about the effects of rosemary extract and high pressure treatments on sardines and chicken breast meat, to the best of our knowledge, there has been a lack of information about the effects of combined high pressure and rosemary extract marination on beef quality (BRAGAGNOLO *et al.*, 2005; GOMEZ-ESTACA *et al.*, 2007). The literature studies about combined use of high pressure and natural antioxidants are still in an early stage, and more studies are needed to be conducted (HYGREEVA and PANDEY 2016). According to these informations, the main goal of this study was to research the combined effects of high pressure and marination treatment on the textural, color and sensory properties of beef loin steaks and to improve natural new textured meat products.

2. MATERIALS AND METHODS

2.1. Materials

Beef loin steaks were supplied by a local retail butcher and cut into 2×10×4 (height×width×length) cm uniform portions weighing an average of 50-70 g before high pressure and marination treatment. Oleoresin rosemary extract (Herbalox® Type W seasoning oil) was supplied by Kalsec Inc. (Kalamazoo, Michigan, USA) and used for marination. It is dispersible in water (polar carriers) and oil (nonpolar carriers) with agitation and has a brown, viscous, liquid appearance.

Eight groups of samples were used in the experiments based on high pressure treatment and high pressure/marination treatment. The samples were divided into (i) 0:control (non-pressurized samples), (ii) 150/0 (150 MPa HPP), (iii) 350/0 (350 MPa HPP), (iv) 550/0 (550 MPa HPP), (v) 1: marinated, non-pressurized sample (vi) 150/1 (150 MPa HPP/marination), (vii) 350/1 (350 MPa HPP/marination) and (viii) 550/1 (550 MPa HPP/marination) groups. All experiments were carried out in triplicate.

2.2. Marination treatment

Marinades were prepared with oleoresin rosemary extract. Preliminary experiments were performed to determine the appropriate marinade concentration for preserving and developing meat quality characteristics. Each sample was placed in a polyamide/polyethylene bag (Apack Ambalaj, İstanbul, Turkey) containing 10 ml of marination solution (including 5% oleoresin rosemary extract) and was kept overnight at 4°C. On the following day, the marinades were removed from the packages, and all

samples were vacuum packaged in double pouches to prevent contamination of the samples by the pressurization medium from bags breaking due to pressurization.

2.3. High-pressure processing

High-pressure processing was applied to the non-marinated and marinated samples. As a result of adiabatic heating, pressure treatment increases the temperature of pressure-transmitting fluid and samples, depending on the product composition and initial temperature of the sample (KOCA *et al.*, 2011). For this reason, the initial temperatures of the samples were adjusted before the high pressure treatment, and the final temperature of the samples after pressurization was monitored with a computer program and found to be approximately $20\pm 2^{\circ}\text{C}$.

The high pressure process was carried out in a MSE-CIP-WB-5500 high pressure food processor (MSE Teknoloji Ltd., Gebze, Turkey) with a 0.7 L vessel volume. Propylene glycol (Kimetsan Co., Ltd., Ankara, Turkey) was used as the pressure-transmitting fluid. The pressure vessel was surrounded by coils connected to a cooling circulator (model RE1050S, Lauda Dr R. Wobser GmbH & Co. KG., Germany). The temperature of the pressure vessel and the pressure-transmitting fluid inside the pressure vessel were controlled with these coils. The inherent ramp rate was 5 MPa/s, and the pressure was increased to the test pressures of 150 MPa, 350 MPa and 550 MPa within approximately 30 s, 70 s and 110 s, respectively. The samples were held at test pressures for 5 min. After the pressurization, decompression was manually performed in approximately 20 s. During the pressure treatments, the temperature of the pressure-transmitting fluid was monitored with two K-type thermocouples mounted to the center of the top closure of the pressure chamber and positioned close to the sample. In addition, the treatment cycle was controlled by a computer program throughout the pressurization. After pressurization, all samples were stored at 4°C prior to analysis within 24 h.

2.4. Texture profile analysis

Texture profile analysis (TPA) of samples was carried out with a TA-XT Plus Texture Analyzer (Stable Micro Systems, England). Beef loin steaks were cooked in a water bath at 80°C until reaching an internal temperature of 72°C and then cooled to room temperature for 45 min before texture analysis. A 5 kg load cell was used in the experiments. The cylindrical samples (1 cm diameter and 2 cm length) were compressed across the fiber direction in two consecutive cycles to 50% of their original height using a cylindrical probe, 38 mm in diameter. The sample was placed under the probe that moved downwards at a constant speed of 2.0 mm s^{-1} (pre-test), 2.0 mm s^{-1} (test), and 5.0 mm s^{-1} (post-test). A time of 5 s was allowed to elapse between the two compression cycles. The TPA parameters (hardness, springiness, cohesiveness, gumminess, chewiness and adhesiveness) were expressed as described by MOCHIZUKI (2001). The measurements of each sample were replicated at least six times. All textural analyses were conducted using Texture Exponent software version 4.0.9.0. (Stable Microsystems Ltd., Surrey, England).

2.5. Protein solubility

Myofibrillar proteins were extracted according to the method described by CLAEYS *et al.* (1995). Samples of 2.5 g of minced meat were homogenized with 25 mL of 0.05 M Tris, 0.25 M sucrose and 1 mM EDTA buffer, pH 7.6. Homogenate was centrifuged at $1000 \times g$ for 10

min. After centrifugation, the supernatant was removed, and the pellet was suspended in 25 mL of 0.05 M Tris, 0.05 M EDTA buffer, pH 7.6 and sedimented at $1000 \times g$ for 10 min. Then, the supernatant was removed again, and the pellet was resuspended in 25 mL of 0.15 M KCl and centrifuged at $1000 \times g$ for 10 min. The same procedure was carried out three times. The myofibril solution was lyophilized and used for further analysis.

The lyophilized myofibril extracts were analyzed for protein concentration. The extracts were dissolved in sample buffer (2×Laemmli buffer, 2-mercaptoethanol, bromophenol blue, pH 6.8). Then, the dissolved extracts were placed in a water bath at 50°C overnight and filtered using Whatman no. 1 filter paper. After filtration, the protein concentration of the extracts was determined using the Bio-Rad Quick Start Bradford Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) based on the Bradford method (BRADFORD, 1976). Bovine serum albumin was used as the standard. The myofibrillar protein solubility of the samples was expressed as mg protein/mL extract solution.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of LAEMMLI (1970) using a 12% separation gel and 4.5% stacking gel (bisacrylamide: acrylamide 1:37 [w/w]). The protein concentration of the loaded sample was adjusted to 10 µg for each sample. A protein broad range marker (Bio-Rad Unstained SDS-PAGE standards, 161-0317) was used as the molecular weight standard (6.5-200 kDa). The electrophoresis run was carried out at 100 V in a Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). After the runs, the gels were stained with 0.01 Coomassie blue, 50% methanol and 10% acetic acid and then destained in 10% methanol and 7% acetic acid. The gels were visualized, and protein molecular weights were estimated using Bio-Rad Versadoc 4000 MP and Quantity One Software (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was carried out in duplicate.

2.7. Cooking loss

Beef loin steaks were placed in plastic bags and cooked in a preheated water bath until the internal temperature of the samples reached 72°C. Then, the samples were taken from the water bath, and excess moisture on the surface of the samples was removed with filter paper. Subsequently, the samples were cooled to room temperature and reweighed. The cooking loss (CL) was expressed as a percentage of the weight difference before and after cooking using the following formula described by RODRIGUES *et al.* (2016):

$$CL = (\text{initial weight} - \text{final weight}) / \text{initial weight} \times 100$$

2.8. Color measurements

The color of the samples was measured using a colorimeter (Minolta Chromameter CR-300; Minolta Camera Co., Ltd., Osaka, Japan) with illuminant D65 (light source) and a 10° observation angle. The beef loin steak packages were opened and exposed to air for 10 min prior to analysis. A CIELAB system was used to determine the color attributes, and the results were expressed as L^* (lightness), a^* (redness and greenness), and b^* (yellowness and blueness). For each sample, five color readings were taken (one at the center and the others from different sides of the sample) at room temperature. The total differences in the color reading values were calculated as described by JUNG *et al.* (2003):

$$\Delta E = \sqrt{(L^* - L_{ref}^*)^2 + (a^* - a_{ref}^*)^2 + (b^* - b_{ref}^*)^2}$$

The color values of the non-pressurized samples were used as a reference for the sample groups pressurized without marination in calculating ΔE , and the color values of marinated/non-pressurized samples were used as a reference for the marinated pressurized sample groups in calculating ΔE .

2.9. Sensory evaluation

Eight graduate students and lecturers in the Department of Food Engineering at Manisa Celal Bayar University participated in the sensory tests as panelists. The panelists were asked to evaluate the sensory parameters of appearance, color, texture, chewiness, juiciness, flavor and overall acceptability. A hedonic scale of 1-5 was used for each attribute. The 5-point hedonic scale was as follows: like very much (5), like much (4), like (3), like slightly (2) and dislike (1). Unsliced raw and cooked samples were presented to the panelists to rate their preferences in terms of appearance, color and texture attributes. In addition, cooked samples were sliced, and a sliced sample from each group was presented to the panelists to rate their preferences in terms of chewiness, juiciness and flavor attributes. The samples were served on plates that were randomly identified with three-digit codes, and a cup of water and bread were given to the panelists to eliminate the residual taste of the samples (DJENANE *et al.*, 2011).

2.10. Statistical analysis

All of the experiments were repeated on three separate occasions. The statistical analyses were performed using SPSS Version 25.0 (SPSS INC., 2017). The experimental data were expressed as the means $\pm$ standard deviations. A two-way analysis of variance was conducted to evaluate the effects of high pressure and marination treatment, and the significant differences between pairs of means were tested by Duncan's multiple range test at a confidence level of $P < 0.05$. The results of the sensory analysis using a hedonic scale were evaluated by Friedman's (non-parametric) rank test and a Wilcoxon test was used to test for pair differences ($P < 0.05$) (MEILGAARD *et al.*, 2015).

3. RESULTS AND DISCUSSION

3.1. Texture profile analysis

The textural properties of marinated and marinated pressurized samples are presented in Table 1. Both pressure and marination treatment had a significant effect on the hardness, gumminess and chewiness of the samples ($P < 0.05$). High pressure treatment alone significantly affected all texture profile parameters, whereas marination treatment was only effective on cohesiveness and adhesiveness ($P < 0.05$). These results suggest that pressure affects the normal texture, marination with rosemary extract partly affects the texture, while the pressure and marination interaction increase the effects on the textural properties of samples.

Table 1. Texture profile parameters of beef loin steaks marinated with rosemary extract and treated with high pressure.

	Hardness (N)	Springiness	Cohesiveness	Gumminess (N)	Chewiness (N)	Adhesiveness
<i>A: Pressure level</i>						
0	33.6±0.8 ^c	0.55±0.02 ^a	0.67±0.006 ^a	22.4±0.4 ^c	12.5±0.6 ^c	0.57±0.06 ^c
150	41.0±0.9 ^b	0.60±0.02 ^a	0.62±0.006 ^b	25.4±0.4 ^b	15.1±0.7 ^b	0.35±0.06 ^{ab}
350	57.9±0.8 ^a	0.55±0.02 ^a	0.59±0.006 ^c	34.2±0.4 ^a	18.7±0.8 ^a	0.47±0.06 ^{bc}
550	26.2±0.8 ^d	0.48±0.02 ^b	0.62±0.006 ^b	16.4±0.4 ^d	7.7±0.7 ^d	0.18±0.06 ^a
SL	0.0	0.04	0.0	0.0	0.0	0.01
<i>B: Marination</i>						
0	39.2±0.6	0.56±0.02	0.64±0.004 ^a	25.0±0.3	14.0±0.5	0.33±0.04 ^a
1	38.7±0.6	0.52±0.02	0.61±0.004 ^b	24.2±0.3	13.0±0.4	0.46±0.04 ^b
SL	NS	NS	0.0	NS	NS	0.04
<i>A×B</i>						
SL	0.0	NS	NS	0.0	0.0	NS
<i>Samples</i>						
150/0 ^{**}	34.9±1.2 ^d	0.63±0.05	0.64±0.013	22.4±1.2 ^d	14.0±1.2 ^b	0.27±0.03
350/0	61.9±1.3 ^a	0.54±0.04	0.61±0.012	37.7±1.2 ^a	20.3±2.7 ^{ab}	0.47±0.22
550/0	28.3±3.7 ^e	0.51±0.07	0.65±0.002	18.4±0.9 ^e	9.0±2.1 ^c	0.19±0.12
150/1	47.0±2.0 ^c	0.57±0.03	0.60±0.008	28.3±0.8 ^c	16.2±0.4 ^b	0.43±0.05
350/1	53.9±2.9 ^b	0.55±0.04	0.57±0.004	30.7±0.8 ^b	17.0±1.5 ^{ab}	0.47±0.07
550/1	24.1±1.8 ^f	0.44±0.03	0.60±0.013	14.4±1.0 ^f	6.4±0.2 ^c	0.17±0.04

*The results are the mean values of three replicates (n=8) ± standard error. Means with alphabetical superscripts (a-f) in the same column (within each main effect) are significantly different (P<0.05).

**The first number refers to the pressure level, and the second refers to the rosemary extract added (5%). 0: no added extract, 1: added extract.

***L*: lightness; a: redness and greenness; b: yellowness and blueness; ΔE: total color difference; SL: significance level; NS: not significant.

The combination of marination and high pressure treatment led to an increase in hardness at up to 350 MPa and a slight decrease in hardness at higher pressure values (550 MPa) (P<0.05). High pressure treatment alone also showed a similar trend in the hardness values of the samples, whereas marination treatment alone had no significant effect on hardness (P>0.05). Our results were in agreement with those of MA and LEDWARD (2004), who reported that high pressure treatment at or above 200 MPa increased meat hardness. These results could be attributed to the aggregation of pressure-treated myofibrillar proteins at 100-300 MPa, causing increased hardness (MA and LEDWARD, 2004; SIMONIN *et al.*, 2012; RODRIGUES *et al.*, 2016). The hardness values of all of the samples were significantly decreased at 550 MPa high pressure treatment (P<0.05). In the literature, the decrease in hardness at high-pressure values was explained by the enzymatic hydrolysis of muscle proteins (MALINOWSKA-PANCZYK *et al.*, 2013). Furthermore, the lowest hardness values were observed in marinated pressurized (550

MPa) samples. These results showed that pressure treatment of previously marinated meat can be more effective for providing softer texture than pressure treatment alone.

The secondary parameters of gumminess and chewiness showed similar changes with hardness. The gumminess and chewiness values of the samples increased with a high-pressure treatment up to 350 MPa and decreased significantly at the higher pressure value of 550 MPa. There was also a significant interaction between pressure level and marination on gumminess and chewiness values ($P < 0.05$). The results indicated that the marinated pressurized beef samples were more tender, less gummy and less chewy than the samples that were pressurized alone. It was reported that the loss of myosin structure induced a decrease in gumminess when the texture profile of pressure treated samples was examined with thermograms (ANGSUPANICH and LEDWARD, 1998).

No significant interaction was found between pressure level and marination for springiness, cohesiveness and adhesiveness of the samples ($P > 0.05$). The springiness, cohesiveness and adhesiveness values of the samples changed variably at different pressure levels. Pressure treatment alone had a significant effect on these texture attributes of the samples, whereas marination treatment alone was only effective on cohesiveness and adhesiveness ($P < 0.05$). At the 150 MPa high pressure treatment, the springiness values increased while the cohesiveness and adhesiveness values decreased. A similar relationship was found by ANGSUPANICH and LEDWARD (1998) and ASHIE *et al.* (1997). On the other hand, some opposing results were found by MALINOWSKA-PANCZYK *et al.*, (2013). At 350 MPa and 550 MPa, the springiness, cohesiveness and adhesiveness decreased. This could be explained by the protective effects of high pressure against heat denaturation of meat proteins (FERNANDEZ-MARTIN *et al.*, 1997).

In general, fresh meat tenderization depends on resolving two components: actomyosin toughness and background toughness. Actomyosin toughness is related to myofibrillar proteins, while background toughness is related to connective tissue and stromal proteins (SUN and HOLLEY, 2010). The effects of high pressure treatment on meat tenderization can be explained by the changes to myofibrillar protein structure. Two possible mechanisms cause myofibrillar protein dissociation and the subsequent decrement in toughness of the meat: thermal degradation of muscle proteins and the enzymatic hydrolysis of proteins (SIMONIN *et al.*, 2012). Pressure breaks up the myofibrillar structure and accelerates enzyme activation in meat as mentioned above. In the present study, rosemary extract also showed a positive effect on some textural properties of samples; however, the higher tenderization effect on beef loin steaks was achieved by the combination of high pressure and marination treatment.

3.2. Protein solubility

Protein solubility is one of the most important functional properties of meat proteins (VAN LAACK *et al.*, 2000). As a consequence of increased interactions between protein constituents and water, protein solubility can change and cause significant alterations to meat texture (CHEFTEL and CULIOLI, 1997).

The effects of high pressure and marination treatment on the solubilization of myofibrillar proteins are shown in Fig. 1. According to the results, the high pressure and marination treatment had no effect on myofibrillar protein solubility in the samples. However, the protein solubility of the samples generally decreased with increasing pressure when compared to the untreated group. Similar results have been found in previous studies. CHAPLEAU *et al.* (2003) found decreased myofibrillar protein solubility in beef samples subjected to pressure treatment (≤ 600 MPa) compared to control samples.

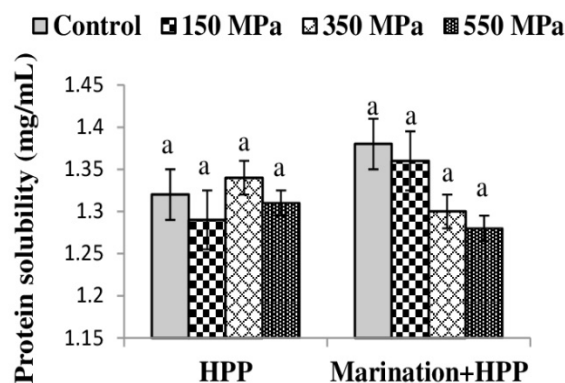


Figure 1. Effects of high pressure/marination treatment on the myofibrillar protein solubility of beef loin steaks.

Furthermore, MALINOWSKA-PANCZYK *et al.* (2013), SOUZA *et al.* (2011), GROSSI *et al.* (2012) and CHAN *et al.* (2011) also reported similar decreases in protein solubility for cod, salmon, pork and beef samples (>60 MPa pressure treatment), pork (215 and 600 MPa pressure treatment) and turkey meat (≤ 600 MPa pressure treatment), respectively. The literature reports that protein solubility is a good indicator of protein denaturation (VAN LAACK *et al.*, 2000). Additionally, protein solubility decreases with increasing pressure due to the formation of insoluble protein aggregates that can no longer be extracted (MARCOS and MULLEN, 2014).

3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Postmortem degradation of myofibrillar proteins has been reported to be an essential part of postmortem tenderization. The increase in protein degradation reflects lower mechanical tenderness and promotes the development of meat texture (SOUZA *et al.*, 2011). For this reason, it is of great importance to understand the effects of high pressure processing on myofibrillar proteins in considering textural changes in pressurized meat, as described above.

Fig. 2 shows the SDS-PAGE profile of myofibrillar proteins from each of the high-pressure and marination-treated samples. The volume intensity of each protein band is also presented in Fig. 3. The protein bands extracted from the samples for myosin heavy chain (MHC) (200 kDa), C-protein (135 kDa), α -actinin (95 kDa), desmin (53 kDa), actin (43 kDa), tropomyosin (36 kDa) and myosin light chain (MLC1, MLC2) (24 kDa, 14 kDa) were identified on a SDS-PAGE gel. Similar myofibrillar proteins were also identified by CHAPLEAU *et al.* (2003), CHAN *et al.* (2011), OMANA *et al.* (2011) and SPERONI *et al.* (2014) in beef, turkey, poultry and meatball samples, respectively.

In general, increasing the applied pressure reduced the band intensities of the myofibrillar proteins. On the other hand, the SDS-PAGE profile of the marinated pressurized samples was similar to that of the samples that were pressurized alone; therefore, we suggest that marination treatment had no effect on myofibrillar protein degradation. The pressure-treated samples had the lowest protein band intensities, and the molecular weights of

mainly degraded myofibrillar proteins ranged from 53 kDa to 200 kDa (MHC, C-protein, α -actinin and desmin).

The band intensities of MHC, C-protein, α -actinin and desmin extracted from the pressurized samples were noticeably decreased compared to those of the control samples. The decreased band intensities may have been caused by protein aggregation due to intermolecular disulfide bond formation at the higher pressure levels (ANGSUPANICH *et al.*, 1999). Myofibrillar proteins were partly degraded with high pressure treatment, and MHC protein was the most degraded protein in the SDS-PAGE profile. However, MLC2 protein was found to be unaffected or even decreased in intensity with applied pressure. This may be because myosin aggregation mechanisms involve the dissociation of heavy chains from light chains, so that only myosin heavy chains form aggregates under pressure (SPERONI *et al.*, 2014).

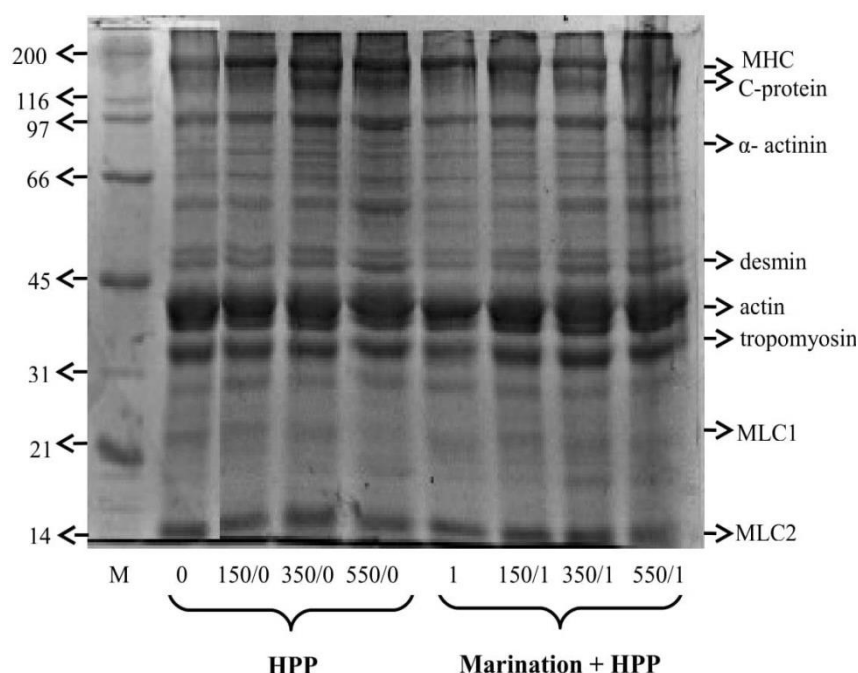
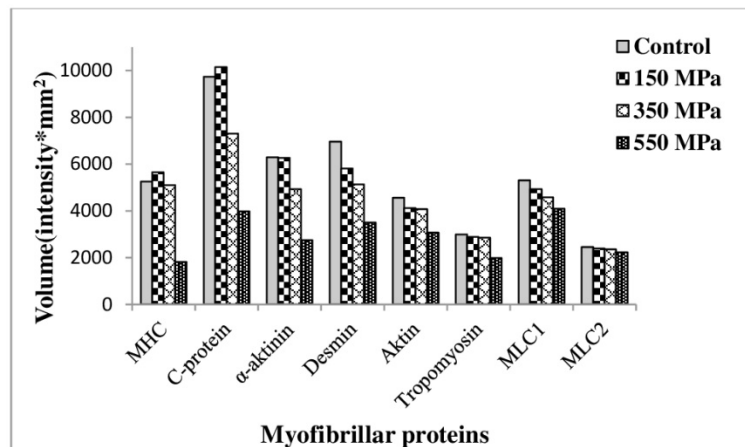


Figure 2. SDS-PAGE patterns of myofibrillar proteins isolated from beef loin steaks. M: Marker, 0: no added extract (control), 150/0, 350/0, 550/0: pressurized, 1: added extract, 150/1, 350/1, 550/1: marinated pressurized

A



B

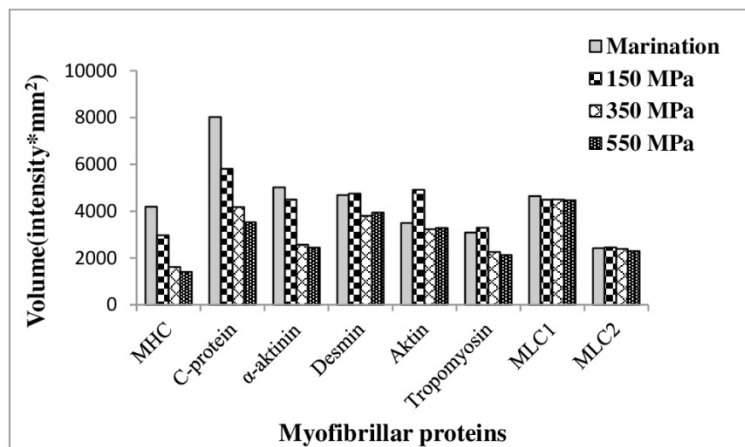


Figure 3. A: The volume intensity of the different protein bands from SDS-PAGE for the high-pressurized sample groups. B: The volume intensity of the different protein bands from SDS-PAGE for the marinated high-pressurized sample groups.

The changes in band intensities of myofibrillar proteins under pressure are attributed to conformational changes in proteins and thereby decreased solubility due to denaturation following covalent linking or increased solubility due to degradation into lower molecular weight compounds. Our results are in accordance with this explanation. The protein band intensities and solubilities decreased in parallel with increasing pressure.

3.4. Cooking loss

Table 2 shows the cooking loss values of the samples. The results indicated that there was no significant interaction between pressure level and marination for cooking loss values of the samples ($P > 0.05$). However, it was found that pressure level and marination separately had a significant effect on cooking loss ($P < 0.05$). A significant difference was observed in all pressure levels compared to the control group ($P < 0.05$).

Table 2. Color and cooking loss values of beef loin steaks marinated with rosemary extract and treated with high pressure.

	L^*	a^*	b^*	ΔE	Cooking Loss (%)
<i>A: Pressure level</i>					
0	40.86±0.5 ^c	9.4±0.5 ^b	11.8±0.3 ^d		40.60±0.6 ^a
150	40.69±0.5 ^c	10.0±0.5 ^{ab}	12.8±0.3 ^c	2.8±0.4 ^c	38.03±0.6 ^b
350	54.41±0.5 ^b	11.3±0.5 ^a	18.6±0.3 ^a	15.4±0.4 ^b	37.55±0.6 ^b
550	57.20±0.5 ^a	8.7±0.5 ^b	17.6±0.3 ^b	17.5±0.4 ^a	39.22±0.6 ^{ab}
SL	0.0	0.01	0.0	0.0	0.0
<i>B: Marination</i>					
0	46.25±0.3 ^a	10.4±0.3 ^a	14.3±0.2 ^a	12.0±0.4	37.64±0.4 ^a
1	50.33±0.3 ^b	9.3±0.3 ^b	16.1±0.2 ^b	11.8±0.4	40.05±0.4 ^b
SL	0.0	0.02	0.0	NS	0.0
<i>A×B</i>					
SL	0.04	NS	NS	NS	NS
<i>Samples</i>					
150/0 ^{**}	37.50±0.7 ^e	10.4±1.9	12.1±1.2	3.6±0.9	36.18±0.9
350/0	52.20±0.9 ^c	12.3±0.9	17.3±1.0	14.6±0.9	36.24±2.1
550/0	56.06±1.4 ^b	9.1±1.5	16.7±0.9	17.8±0.9	38.19±0.9
150/1	43.89±1.2 ^d	9.6±0.7	13.6±0.4	2.1±0.8	39.87±0.9
350/1	56.63±0.9 ^{ab}	10.2±0.3	20.0±0.6	16.2±0.6	38.85±0.4
550/1	58.34±1.8 ^a	8.3±1.2	18.4±0.5	17.2±1.9	40.24±0.7

*The results are the mean values of three replicates (n=8) ± standard error. Means with alphabetical superscripts (a-d) in the same column (within each main effect) are significantly different (P<0.05).

**The first number refers to the pressure level, and the second refers to the rosemary extract added (5%).
0: no added extract (control), 1: added extract.

*** L^* : lightness; a^* : redness and greenness; b^* : yellowness and blueness; ΔE : total color difference;
SL: significance level; NS: not significant.

Both pressure and marination treatment generally resulted in an increase in cooking loss values except for the marinated pressurized (350 MPa) group, but the differences were not significant (P>0.05). The cooking loss values of the samples that were pressurized alone increased with increasing pressure. A similar trend was determined by KIM *et al.* (2014) who reported increased cooking loss values in beef samples pressurized at 300, 450 and 600 MPa compared to the control group. In addition, NETO *et al.* (2015) reported that 100, 200, 300 and 400 MPa high-pressure treatment led to increased cooking loss values in beef samples. These authors also reported that high pressure levels and changes in myofibrillar protein structure at these pressures had a negative effect on the water holding capacity of meat and consequently increased cooking loss. In addition, MARCOS *et al.* (2010) explained that sarcoplasmic proteins decreased high-pressure effects on cooking loss but that the increased denaturation of sarcoplasmic proteins induced by pressure had a negative effect on the cooking loss values of meat. The cooking loss values of the marinated pressurized samples decreased at 350 MPa and then increased with increasing pressure. Similar results were obtained by BARBANTI and PASQUINI (2005) in marinated meat. Increasing cooking loss values might be attributed to lower water binding capacity and moisture loss during cooking.

3.5. Color

The color measurements of beef loin steaks marinated with rosemary extract and subjected to high pressure are shown in Table 2. Statistical analysis showed a two-way interactive effect between pressure level and marination for L^* values of the samples ($P < 0.05$). Pressure level and marination separately had a significant effect on the a^* and b^* values of the samples ($P < 0.05$).

The L^* values showed an increasing trend, while the a^* and b^* values increased up to 350 MPa and then decreased. Marination with rosemary extract also caused a significant increase in L^* and b^* values and decrease in a^* values ($P < 0.05$). Pressure level had a significant effect on L^* values at pressures above 150 MPa compared to the control group ($P < 0.05$). It was also found that the a^* values significantly changed at 150 and 350 MPa pressure levels, whereas the b^* values significantly changed at all pressure levels ($P < 0.05$). Similar results were also reported by KIM *et al.* (2014), MARCOS *et al.* (2010), MCARDLE *et al.* (2010), OHNUMA *et al.* (2013) and RODRIGUES *et al.* (2016) for beef *M. Longissimus dorsi*, beef supplemented with conjugated linoleic acid, beef *Longissimus lumborum*, beef *M. Pectoralis profundus* and beef treated with sodium hydrogen carbonate, respectively. The highest L^* values were determined in marinated pressurized (550 MPa) samples. These increases in L^* values caused discoloration of the beef samples, which was attributed to the whitening/brightening effect between the range of 200 to 350 MPa and ferrous (Fe^{2+}) myoglobin oxidation to ferric (Fe^{3+}) metmyoglobin at pressures above 400 MPa in the literature (SIMONIN *et al.*, 2012; BUCKOW *et al.*, 2013). The whitening/brightening effect occurred due to the following changes: (i) protein coagulation causing loss of sarcoplasmic and myofibrillar protein solubility, affecting their structure and surface properties and (ii) myoglobin denaturation and the displacement or release of the heme group (BUCKOW *et al.*, 2013). MUSSA (1999) also reported that the lighter color in pressurized meat could be related to alterations in the water content of samples due to drip loss. In the present study, visual color observations of the samples are also shown in Fig. 4.

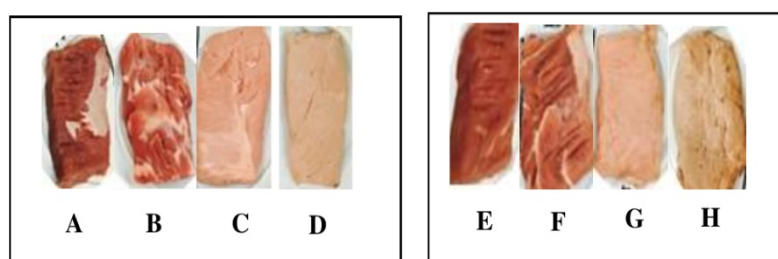


Figure 4. Visual color observation of beef loin steaks. A: control (unpressurized), B: 150 MPa, C: 350 MPa, D: 550 MPa, E: marinated/unpressurized, F: marinated/150 MPa, G: marinated/350 MPa, H: marinated/550 MPa.

Increasing pressure caused the increase in a^* values of samples up to 350 MPa; then, the a^* values of the samples decreased at pressures above 350 MPa. JUNG *et al.* (2003) found that pressure treatment up to 300 MPa decreased metmyoglobin content and higher pressures led to increased metmyoglobin content in the beef samples. These authors also explained

the increases in a values at pressures up to 300 MPa by the activation of enzymes causing metmyoglobin reduction. Our results are in agreement with previous reports for beef samples (JUNG *et al.*, 2003; MARCOS *et al.*, 2010; LOWDER *et al.*, 2014).

The b values represent the intensity of yellowness and blueness in the samples. In the present study, b values showed a similar trend with a values. Increasing pressure caused by the increase in b values of samples up to 350 MPa and then the b values of the samples decreased at pressures above 350 MPa. Similar results were found by MCARDLE *et al.* (2010), who reported higher b values in beef samples pressurized at 300 MPa compared to the samples pressurized at 200 MPa and lower b values in beef samples pressurized at 400 MPa compared to the samples pressurized at 300 MPa. On the other hand, GOUTEFONGEA *et al.* (1995) reported an increase in b values for minced meat samples pressurized at 600 MPa (20°C for 30 min). These authors related the increase in b values to the change of the myoglobin chemical state. CARLEZ *et al.* (1995) also stated that the increase in b values was due to metmyoglobin formation.

The total color difference (ΔE) indicates the evaluation of color changes. The results revealed that there was no significant interaction between pressure level and marination for ΔE values of the samples ($P>0.05$). However, the pressure level had a significant effect on ΔE values ($P<0.05$). An increase of 10 units in ΔE is thought to significantly change the appearance of meat color (JUNG *et al.*, 2003). In the present study, an increase of 10 units in ΔE values was found in the samples pressurized at 350 and 550 MPa.

3.6. Sensory evaluation

The sensory evaluation results of the raw and cooked samples are shown in Table 3 and Table 4. In general, the addition of rosemary extract (5%) did not positively affect the sensory scores of the samples. It was observed that pressurized samples were evaluated as better than marinated pressurized samples. Increasing pressure caused a decrease in the sensory scores of the samples. The panelists showed slightly Friedman rank test significant preferences in appearance, color and texture attributes of raw samples and chewiness, juiciness and overall impression attributes of cooked samples ($P<0.05$). According to the results of the raw samples, the control samples received the highest score for appearance and texture, whereas 150 MPa pressurized samples were rated highest for color. The results of cooked samples also showed that 150 MPa pressurized samples received the highest score for appearance, color, texture, chewiness and overall impression, while 350 MPa pressurized samples were rated highest for juiciness.

The panelists did not notice the color difference between the pressurized and non-pressurized cooked samples. It has been reported that high pressure treatments caused visible color changes in raw meat, but the color difference decreased extremely after cooking. Our results are in agreement with those of MOR-MUR and YUSTE (2003) and SIMONIN *et al.* (2012). In addition, the panelists recognized the color differences in the raw samples. According to the pair comparisons, there was a significant difference between the control samples and pressurized as well as marinated pressurized samples ($P<0.05$) in color scores. Similarly, the appearance of the raw samples was also significantly influenced by the treatments ($P<0.05$). These results were also supported by the color measurements shown in Table 2.

Table 3. Sensory evaluation of pressurized and marinated pressurized raw samples

	Appearance	Color	Texture
0	4.80±0.4	4.70±0.6	4.30±0.7
1	4.10±0.6	4.15±0.7	4.05±0.7
150/0**	4.75±0.4	4.75±0.4	4.20±0.8
350/0	2.60±1.2	2.65±1.2	3.70±1.0
550/0	2.00±1.0	2.00±0.9	3.35±0.8
150/1	3.60±0.8	3.75±0.9	3.80±0.8
350/1	2.15±0.8	2.25±0.9	3.70±1.0
550/1	1.70±0.9	1.90±1.1	3.40±0.9
SL	0.0	0.0	0.0

*The results are the mean values of three replicates (n=8) ± standard error.

**The first number refers to the pressure level, and the second refers to the rosemary extract added (5%).

0: no added extract (control), 1: added extract, SL: significance level.

The panelists tended to give lower scores for the texture attributes of cooked and raw samples than for the control group. The pair comparisons of texture scores of the control group and the other sample groups were significant except for 150 MPa pressurized groups (150/0, 150/1) and marinated 350 MPa pressurized group ($P < 0.05$). Surprisingly, no significant difference was found in the texture attributes of the cooked samples ($P > 0.05$). These results were not in agreement with TPA measurements reported in the previous sections, which were significantly affected by pressure ($P < 0.05$). The contrast between the results could be due to the different preferences reflected by the panelists regarding texture. On the other hand, the panelists gave higher chewiness scores to the pressurized samples (150/0, 350/0) and marinated pressurized samples (350/1) compared to the control group, and the pair significant differences were found between the control group and the 350 MPa pressurized group (350/0, 350/1) ($P < 0.05$).

Table 4. Sensory evaluation of pressurized and marinated pressurized cooked samples

	Appearance	Color	Texture	Chewiness	Juiciness	Flavor	Overall Impression
0	3.80±1.0	4.20±0.8	3.95±0.8	3.45±1.2	3.50±1.1	3.80±1.0	3.55±1.1
1	4.00±0.9	4.20±0.7	4.00±0.8	3.55±1.1	3.35±1.1	3.65±1.0	3.55±0.9
150/0**	4.20±0.8	4.25±0.8	4.10±1.1	4.20±0.8	4.00±0.8	4.10±0.9	4.10±1.0
350/0	4.05±0.9	3.05±1.1	3.95±0.8	4.15±0.7	4.25±0.9	4.30±0.8	4.00±0.7
550/0	4.00±0.9	3.05±1.1	3.55±0.9	3.50±1.4	3.50±1.2	3.90±1.0	3.40±1.1
150/1	3.60±1.1	3.60±0.8	3.40±0.9	3.55±0.8	3.40±0.8	3.60±0.7	3.50±0.8
350/1	3.70±1.0	2.90±1.0	3.80±0.8	3.70±1.1	3.65±1.0	3.60±1.0	3.20±1.0
550/1	3.80±1.0	3.15±1.3	3.55±1.1	3.20±1.0	3.15±0.9	3.65±0.8	2.75±0.8
SL	NS	NS	NS	0.0	0.0	NS	0.0

*The results are the mean values of three replicates (n=8) ± standard error.

**The first number refers to the pressure level, and the second refers to the rosemary extract added (5%).

0: no added extract (control), 1: added extract, SL: significance level.

It was determined that the samples pressurized up to 350 MPa had higher juiciness scores than the control group, while the juiciness scores decreased at 550 MPa pressure levels. MACFARLANE (1973) stated that decrements in juiciness scores were attributed to increased moisture retention based on the defragmentation of structural proteins into hydrophilic peptides/free amino acids.

The panelists gave the highest overall impression scores to the 150 MPa pressurized samples; however, no significant differences were found between the overall impression scores of 150 MPa pressurized samples and the control group ($P>0.05$). The 350 MPa pressurized samples also received the highest scores for flavor in all sensory characteristics of the cooked samples. According to the sensory evaluation results, the panelists preferred the 150 MPa and 350 MPa pressurized samples most, which were more tender, chewier, juicier and tastier than the control group. In addition, the marinated pressurized samples had lower sensory scores than the samples that were pressurized alone. It was reported that the sensory acceptance among panelists of high pressurized meat products varied and that they generally reported good sensory scores, although with some alterations in aroma and taste components (SIMONIN *et al.*, 2012).

4. CONCLUSION

A pressure of 550 MPa improved the tenderness of beef loin steaks but caused a light color in the meat appearance. High pressure processing caused protein degradation, leading to a great change in the protein profile of samples and thereby the development of meat texture. The panelists preferred the samples pressurized at 150 MPa and 350 MPa in the sensory panel. The best overall beef quality was achieved with the combined application of high pressure and marination. High pressure treatment has important positive effects on beef quality due to limitations regarding meat discoloration. In this regard, high pressure and marination treatment present a good alternative strategy for developing reliable and healthier meat products with desirable texture. However, further studies are needed for process optimization.

ACKNOWLEDGEMENTS

We would like to thank the Scientific Research Project Office of Manisa Celal Bayar University (Project no: BAP2015-091) for financial support.

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Paper Received November 4, 2018 Accepted April 10, 2019

INFLUENCE OF FERTILISATION TYPE ON THE QUALITY OF VIRGIN RAPESEED OIL

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ABSTRACT

The aim of the study was to determine whether, and to what the type of fertilisation employed can affect the quality of the harvested spring rape and the oil extracted therefrom through virgin oil pressing. The article presents the results of research conducted with the use of ATR-FTIR absorption spectroscopy of selected cold pressed vegetable oils. The study included three types of rapeseed oil from spring plants grown with traditional NPK mixture fertilisation and digestate. The oxidative stability of the pressed oil was also determined and soil parameters such as: content of absorbable nutrients and acidity in the experimental plots.

Keywords: ART-FTIR spectroscopy, cold pressed oils, fat, fatty acids, fertiliser, oxidative stability

1. INTRODUCTION

With the view of increasing rapeseed yields, numerous artificial fertilisers have been used in recent years to modify the soil content of micro-organisms and drastically alter the general environmental conditions of the soil. This situation has a negative impact on both the sustainable development of soil fertility and, above all, quality of the natural environment (RAMIREZ *et al.*, 2012). Health and environmental concerns force societies to support a revival of traditional methods of food production that are consistent with the guidelines for sustainable agriculture (CETIN and SEVIK, 2016). The trend led to the development of the concept of the so-called eco-efficiency, which entails the use of natural fertilisers produced through natural processing of agricultural waste in agricultural biogas plants (CZEKALA *et al.*, 2012; DENNEHY *et al.*, 2016). As observed by MÖLLER and MÜLLER (2012), the use of digestate containing large amounts of macro- and micro-nutrients normally found in the natural environment provides a viable alternative to mineral fertilisers, capable of improving soil fertility and quality of farm produce by providing plants with easily absorbable elements (such as nitrogen, phosphorus and potassium). Depending on the quality and nutritional status, digestate application has been shown to be advantageous in terms of crop yield, plant nutrient uptake (TERHOEVEN-URSELMANS *et al.*, 2009; ANDRUSCHKEWITSCH *et al.*, 2013) soil health (VANEECKHAUTE *et al.*, 2013), and enhancement observed in dehydrogenase activity (GARCÍA-SANCHEZ *et al.*, 2015).

The technological and nutritional value of rapeseeds is determined by their chemical composition, in particular the content of nutrients and anti-nutrients, which in turn is closely related to the qualities of the plant species itself and primarily defined by genetic factors. Under specific conditions, the nutrient content can, however, vary significantly depending on the cultivar, soil type, fertilisation, weather conditions, and technological processing procedures employed (heating, steaming, autoclaving, etc.) (KRASUCKI *et al.*, 2001). Low quality raw material – containing immature seeds, contaminated, damaged, excessively moist, affected by the processes of fat hydrolysis and oxidation – will produce low quality oil characterised by short shelf life. Consequently, the most valuable oils are those extracted from the freshest, highest quality material in which the oxidative processes are still relatively unadvanced. The process of fat oxidation is the primary cause of diminished quality manifested in the loss of oil's organoleptic and nutritional value (ZIEMLAŃSKI and BUDZYŃSKA-TOPOLOWSKA, 1991; POYATO *et al.*, 2014). The oxidation rate is conditioned by a number of factors, including the content of fatty acids, presence of pro- and antioxidants and, particularly in the case of cold pressed oils, the storage conditions (WRONIAK and ŁUKASIK, 2007).

The growing interest in natural and safe food, including oils and fats, is a testament to its value as a rich source of healthy nutrients, whose consumption facilitates prevention of numerous, particularly digestive diseases and is beneficial to the overall health of organisms (HAREL *et al.*, 2002). The seeds of both spring and winter rape are used in the production of cooking oil and provide a key raw material in the production of (increasingly popular and sought-after) biofuels for compression-ignition engines, particularly in Europe (VAN DUREN *et al.*, 2015, ATABANI *et al.*, 2013) and certain countries in Africa and Asia, including China (WU and LEUNG, 2011; SHIN *et al.*, 2012). It can be safely assumed that Europe is currently the leading global producer of rapeseed (USDA, 2016). Compared to other oils such as sunflower oil, corn oil, sesame oil or olive oil, rapeseed oil is characterised by a particularly rich chemical composition. The high content of fat and protein as well a wide range of fatty acids, including large quantities of

n-3 fatty acids such as α -linolenic acid (C18:3), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid, as well as its n-6/n-3 ratio, make it an attractive offering for various consumer groups. Said acids are very beneficial from the perspective of human health. However, high content of α -linolenic acid, often exceeding 10%, increases the rapeseed oil's susceptibility to autooxidation, thus negatively affecting its oxidative stability (SHAHIDI, 2005; PARRY *et al.*, 2005). The presence of lipids, carbohydrates, sterols, aliphatic alcohols, tocopherols and a combination of pigments ensures antioxidative activity and high quality, both in terms of nutritional and sensory value (DAMERAU, 2015; TSAKNIS *et al.*, 1997; SZYDŁOWSKA-CZERNIAK *et al.*, 2013). Notably, as reported in a number of studies comparing cold pressing to other methods of oil extraction, due to the presence of anti-oxidants (carotenoids, tocopherols), as well as other phenolic compounds whose small quantities are introduced into the oil in the process of pressing the seeds, cold pressed oils are characterised by one of the highest values of oxidative stability (CICHOSZ and CZECHOT, 2011).

It is noteworthy that the final quality of both the seeds themselves and the oil extracted therefrom is dependent on a variety of diverse factors, including climate, type of plantation, methods of harvest, storage, preliminary processing and, above all, the process of cold pressing itself.

The increased awareness of the value of oil consumption stimulated the development of better quality products and renewed interest in methods of extracting vegetable oil without the use of chemical reagents, or at least with minimum use thereof. Due to the above, in recent years, we have observed a turn back to the methods of oil extraction involving cold pressing and virgin oil pressing (SZYDŁOWSKA-CZERNIAK *et al.*, 2013) under which, due to the low temperatures under which the process takes place, the extent of degradation of bioactive compounds can be substantially reduced (RAMADAN, 2013). The simplicity of the process of extracting the oils selected for the present study simultaneously allows them to preserve much of their original colour, high nutritional value, high bioactive compounds content, as well as the characteristic taste and smell.

The aim of the study was to: 1. determine and compare selected growing conditions based on soil analysis after fertilisation with a mineral NPK mixture and digestate. 2. analyse the quality of seeds in terms of fat and protein content in spring rape seeds after using fertilisers containing different amounts of nitrogen. 3. analyse selected quality parameters of seeds intended for oil extraction and the quality of the thus obtained virgin rapeseed oil. The scope of the study included: determination of the content of moisture, fats, fatty acids, and protein in the respective seeds, an analysis of the oil extracted therefrom and determination of its oxidative stability. A comparative analysis of ATR-FTIR spectra was employed for the purposes of identifying/highlighting differences in the chemical makeup of the respective oils.

2. MATERIALS AND METHODS

The research material comprised three spring rape cultivars: 'Markus' – 'M', 'Bios' – 'B' and 'Feliks' – 'F' obtained from Hodowla Roślin Strzelce Sp. z o.o. The aforementioned rapeseed cultivars were grown in two combinations on experimental plots of 27 m² located in the Lublin Voivodship (51°16'19.8"N 22°24'34.0"E), Poland (Fig. 1). In the first version of the experiment, natural fertiliser (digestate) obtained from the agricultural biogas farm Wikana Bioenergia Sp. z o.o. in Piaski, Lublin Voivodship, was used, while in the second

version, the traditional multi-compound fertilizer Yara NPK 5-14-28 was applied (ammonium nitrogen - 5 %, P- 14% K - 28 %, SO₃ – 12.5%, CaO - 3%). The digestate in the amount of 97 L was distributed in a plot of 27 m² (36,000 l/ha). The pH of the digestate used in the cultivation of spring rape was 8.73. The cultivars grown in the control plot – C did not receive any of the aforementioned fertilisers. The products included in the study were obtained from a two-year experimental plot cultivation (2015 and 2016). After harvest, the seeds were cleaned and stored in laboratory conditions for a period of one month in 20°C ambient temperature and 70% ambient humidity to equalise the moisture content. Subsequently, the seeds were cold pressed to extract oil. The oil was then refrigerated for a period of one week in 4°C, in dark bottles, to separate the oil from impurities. Calculations were conducted for triplicate mean values. All the results obtained in the course of the two-year experiment were averaged and tabularised (see tables below).

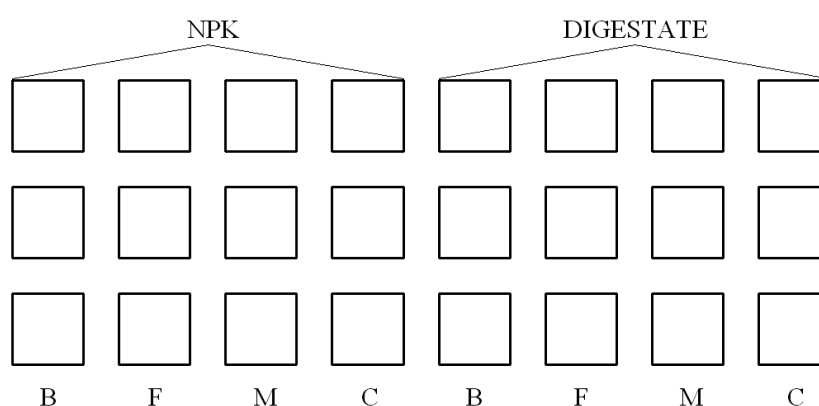


Figure 1. Distribution of experimental spring rape plots.

Weather information relating to the period from March to September 2015 and 2016 was obtained from the Institute of Meteorology and Water Management National Research Institute IMGW-PIB as recorded at the weather station Lublin-RADAWIEC.

The measurements of soil microelement content prior to the application of the selected fertilisers and after the harvest were conducted at the Regional Chemical-Agricultural Station in Lublin. Soil samples were tested for the available nutrient content, in particular phosphorus and potassium, in accordance with the applicable norms with respect to those nutrients, i.e. PN-R-04024:1997, as well as magnesium in mg per 100g of soil, in accordance with PN-ISO 10390:1997. Soil acidity, pH (KCl) and potential liming requirements were also considered.

The digestate content of major nutrients and heavy metals was determined. The laboratory tests were conducted at the Regional Chemical-Agricultural Station in Lublin in accordance with KQ/PB-17-76-77 rev. 04 of 02.07.12. The biogas plant feedstock used in the production of biogas and digestate consisted of: corn silage, whey and plant waste.

Rape seeds were analysed in terms of fat content. The fat content was determined using Soxhlet method in accordance with PN- EN 1163:1999, i.e. by multiple, continuous extraction from pulverised and pre-dried product using an organic extraction solvent, followed by removal of the solvent and determination of the fat content by weight. The

tests were conducted at the Central Agro-Energy Laboratory of the University of Life Sciences in Lublin.

Rapeseeds were tested for fatty acids content in accordance with PM-EN ISO 5509:2001. "*Vegetable and animal fats and oils*" – The analysis of fatty acid methyl esters was performed by way of gas chromatography at the Central Agro-Energy Laboratory of the University of Life Sciences in Lublin.

Rape seeds were analysed in terms of protein content. The protein content was determined using the Kjeldahl method, in accordance with PN – A – 04018/A z 3:2002, i.e. by way of sulphation of organic nitrogen compounds using concentrated sulphuric acid in the presence of a catalyst, and subsequent alkalization of the solution, distillation and titration with sulphuric acid of ammonia bound by boronic acid.

The oil was extracted using a continuous action screw press with exchangeable nozzles, 8 mm in diameter with a set of microscopic sieves, manufactured by Farnet DUO. Before activation, the press was preheated to 60°C ±10°C. Once the press was active and the pressing temperature equalised, the pressing process itself was initiated. Stabilisation was achieved after pressing oil from approximately 1kg of seeds, with the temperature stabilised at approximately 70°C. The pressing temperature was measured using an ama-digit thermometer. After the extraction, the oil was stored in dark-glass bottles at 5°C to achieve natural decantation over a period of 6 days. Afterwards, the oil was ready for laboratory analyses.

The oxidative stability was measured using a Rancimat 670 apparatus (Metrohm AG, Herisau, Switzerland). Oil samples (2.5 g) were weighed into reaction vessels and heated to 120°C under a dry air flow of 20 l h⁻¹. The volatile compounds released during oxidation were collected into a cell containing distilled water, and the increasing water conductivity was continually measured. The time taken to reach the conductivity inflection point was recorded as the induction period (IT) expressed in hours. All determinations were carried out in triplicate. The normal time was calculated using StabNet software, which controlled the Rancimat apparatus.

Infrared absorption spectra were recorded with a Vertex 70 FTIR (Bruker) spectrometer. The attenuated total reflection (ATR) configuration was used with 20 internal reflections of the ZnSe crystal plate (45° cut). Typically, 16 scans were collected, Fourier-transformed, and averaged for each measurement. Absorption spectra at a resolution of one data point per 1 cm⁻¹ were obtained in the region between 4000 and 600 cm⁻¹. The instrument was continuously purged with N₂ for 40 min. before and during measurements. The ZnSe crystal plate was cleaned with ultra-pure organic solvents from Sigma-Aldrich. The spectral analysis was performed with Grams/AI software from ThermoGalactic Industries (USA). All experiments were carried out at 25°C.

In the recent years, chemometric methods have played an important role in studies on food quality as well as the identification and quantification of major constituents of foods. The use of chemometric methods combined with spectroscopic techniques has been analysed in previous research. The literature is extensive and details the theoretical aspects of applying such methods as e.g. Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) or Partial Least Squares Regression (PLS). Examples are the analysis pork fat or lard as a whole of matter extracted from pork (ROHMAN *et al.*, 2011), the determination of fat in milk samples (INON *et al.*, 2004), analysis of extra virgin olive oil adulterated with palm oil (ROHMAN and CHE MANA, 2010). Principal Component Analysis (PCA) is the most popular mathematical method of reducing data dimensionality with a minimum loss of information. PCA allows us to visualize and interpret data. Another multivariate method with which we can hierarchize the data is

Hierarchical Cluster Analysis (HCA), which searches for objects, which are close together in the variable space. HCA and PCA methods combined with spectroscopic techniques have been successfully used for the purposes of determining food quality as well as identifying and quantifying major constituents of foods.

The objective of the present study was to overview the similarities and differences observed between oil samples extracted through cold pressing and also involved the content of fatty acid composition in rapeseeds.

The results obtained for the selected oil products were analysed statistically by way of mean value and $\pm$ standard deviation determination, as wells as post-hoc Tukey's test, with the level of significance of $p < 0.05$, using Statistica 10 software. Furthermore, correlations between the respective variables were evaluated using PCA and HCA. PCA transforms the original, measured variables into new uncorrelated variables. HCA was applied to the data in order to identify similarities between different oils samples. HCA calculates the distances (or correlations) between all oils samples based on Euclidean distance.

3. RESULTS AND DISCUSSION

Table 1 presents the relevant weather conditions including atmospheric pressure, air temperature and rainfall in the years of cultivation, 2015 and 2016, for the period from March (sowing) to August when the crop was collected by way of two-stage harvest. As shown in the relevant data, 2015 was characterised by lower rainfall (10.08 mm H₂O) relative to 2016, when the total rainfall in the analysed period was (12.59 mm H₂O). The mean daily air temperature in 2015 was higher than in 2016, with the exception of April and May, respectively 7.75 and 12.37°C.

Table 1. Weather conditions in 2015 and 2016.

Month	Year 2015			Year 2016		
	Pressure (hPa)	Temperature (°C)	Rainfall (mm H ₂ O)	Pressure (hPa)	Temperature (°C)	Rainfall (mm H ₂ O)
3	991.41	4.70	1.31	985.26	3.46	2.04
4	986.86	7.75	1.20	983.69	8.91	1.20
5	986.75	12.37	2.20	985.82	14.44	1.09
6	989.60	26.63	0.63	986.30	18.34	1.78
7	986.81	21.60	1.42	987.12	18.91	4.49
8	990.38	25.15	0.30	990.73	18.07	1.52
9	989.19	14.63	3.02	990.73	15.15	0.47

An important gap given that a significant popularisation of inorganic N fertilizers in the coming decades is expected to occur in developing countries as a consequence of population growth and increasing food demands (FARNWORTH *et al.*, 2017). Oil and nitrogen (N) content in soil and rapeseed is an important quality indicator affecting oil yield and protein content of the meal (GRANT *et al.*, 2011). Nitrogen content has a significant impact on protein synthesis and must be supplied in adequate amounts to

ensure optimum crop yield. Too much N in the soil can lead to a nutrient imbalance that can restrict protein synthesis and reduce rapeseed growth and seed yield (GRANT *et al.*, 2012; LEMKE *et al.*, 2009). According to RATHKE *et al.* (2005), the yield of oilseed rape involves balancing the synthesis of oil and crude protein in the seeds, as well as the energy and carbon dioxide (CO₂) budget of the photosynthetic pool.

Table 2 presents the results of the analysis of major nutrient and heavy metal content in the digestate used for the spring rape cultivation. The same confirm the presence of numerous major nutrients. However, one should not that with respect to primary major nutrients such as: nitrogen, phosphorus and potassium, the levels observed in the applied digestate (respectively: 0.119; 0.12; 5.37 g/L) were significantly lower than those present in industrial fertilisers. ODLARE *et al.* (2008) reported in his research high content of mineral nutrients (nitrogen, phosphorus, potassium) in fermentation wastewater, while Rehl (2011) observed that in terms of response time digestate was in fact comparable to mineral fertilizers due to that fact that the N, P, and K nutrients were more readily accessible and available for the plants. Based on the subsequent analyses of the obtained results it can be reported that the tested digestate samples contained virtually no heavy metals, while the overall high major nutrient content supported the usability of digestate as a potential alternative fertiliser. Furthermore, COMPARETTI (2013) and KOURIMSKÁ *et al.* (2012) observed that digestate contains organic matter that has a significant positive influence on the physicochemical quality of the fertilised soil.

Table 2. Content of major nutrients and selected heavy metals in digestate used for cultivation of spring rape.

Element	Unit	Content
Nitrogen	(g/l)	0.119
Phosphorus	(g/l)	0.12
Potassium	(g/l)	5.37
Calcium	(g/l)	0.28
Magnesium	(g/l)	0.07
Cadmium	(mg/l)	<0.43
Lead	(mg/l)	<0.43
Nickel	(mg/l)	<0.43
Chromium	(mg/l)	<0.43
Copper	(mg/l)	0.43
Zinc	(mg/l)	2.00
Manganese	(mg/l)	2.26
Iron	(mg/l)	70.82

Table 3 presents the averaged results obtained in the course of the two-year study in terms of major nutrient content in the soil prior to an after the application of selected fertilisers for the purposes of spring rape cultivation. Based on the obtained, data it was observed that the pH of the soil in both cases remained relatively stable and did not change significantly after the sowing. With respect to nutrient content in plants relative to the levels or phosphorus, potassium and magnesium, the values varied significantly in the samples selected for the study. In all cases, the content of said nutrients was significantly

higher in the fertilised plots when compared to the control plots, both prior to sowing and after the harvest.

The analysis of soil content after the harvest revealed similar values for both experimental variants, which in both cases remained higher than those observed for the control, and were e.g.: 64.5 mg /100g of phosphorus in the plot fertilised with digestate compared to 25.1 mg /100g in the control plot. In the case of potassium, its concentration in the control plot was 20.04 mg/100g, while in the digestate plot it was 60.05 mg/100g. Data related to magnesium content analyses returned very similar results, i.e. 9.6 mg/100g in control plots and 21.8 mg/100g in lots fertilised with digestate. Given the above, the primary agricultural use of digestate, given its physical and chemical properties, is biofertilisation. Such usability of fermentation wastewater was also suggested, based on obtained research results, by KOUŘIMSKÁ *et al.*, (2012), EICKENSCHIEDT *et al.*, (2014), VÁZQUEZ-ROWE *et al.*, (2015). KOUŘIMSKÁ *et al.*, (2012) reported that digestate used in fertilisation improved soil fertility, as well as quality of the crop and the plants' resistance to biotic and abiotic factors.

The statistical analysis using the post-hoc Tukey test at the significance level of $p < 0.05$ also revealed statistically significant differences in the content of the respective nutrients between the control plots and the experimental fertilised plots. Statistically significant differences were also observed between the content of the analysed nutrients in the soil prior to sowing and after the harvest, with the exception of control plots relate to the use of traditional NPK fertilisation.

Table 3. Content of available nutrients and soil acidity.

Experimental variant	Plot type	Acidity		Liming needed	Content of available nutrients (mg 100/g)		
		pH in KCL	reaction		Phosphorus P ₂ O ₃	Potassium K ₂ O	Magnesium Mg
Prior to sowing							
Digestate	Control	6.97	neutral	no	23.8a	16.3a	10.3a
	Exp. plot	7.22	alkaline	no	42.17b	53.17b	13.75b
NPK fertilizer	Control	7.06	alkaline	no	21.1c	15.7a	9.5a
	Exp. plot	5.51	acidic	yes	23.57a	20.55c	14.05b
After harvest							
Digestate	Control	6.99	neutral	no	25.1d	20.04c	9.6a
	Exp. plot	7.52	alkaline	no	64.5e	60.5e	21.8c
NPK fertilizer	Control	7.06	alkaline	no	21.6c	16.7a	9.55a
	Exp. plot	5.37	acidic	yes	26.5d	23.3d	17.2b

a, b, c, d, e – statistically significant cultivar differences relative to the control; mean values marked with the same letter are not statistically significantly different ($p > 0.05$).

The yield obtained from spring rape plants in 2015/2016 was below expectations and the unfavourable weather conditions observed in the summer in both years resulted in yield discrepancies. It is assumed that the correct moisture content of rapeseed suitable for further storage ought to be between 5 and 9%, with 10% being within the admissible range in some countries, e.g. Canada (GAWRYSIK-WITULSKA *et al.*, 2012). Table 4 presents

the values of initial moisture during harvest, moisture reported during analyses, as well as fat and protein content of the rapeseeds. The initial moisture content during harvest was between 8.3 and 10% dry mass, respectively for all the samples. During storage, the value dropped on average by 1% relative to the initial moisture content, which rendered it acceptable by oil and fat industry standards, i.e. the harvested seeds would be accepted for further potential storage.

The tests conducted with the aim of determining the fat content in seeds revealed that this particular characteristic was primarily dependent on genetic conditions. Seeds of spring rape are characterised by slightly lower fat and protein content (respectively 48.6% and 24%) compared to winter rapeseed (WARMIŃSKI *et al.*, 2001). In our study, the protein concentration wasn't strongly affected by the application of N, regardless of the fertilizer used. Since N is a major structural component of protein, increasing N supply frequently leads to an increase in protein concentration (MALHI and GILL, 2007). In both experimental cases, the fat content turned out to be fairly similar, varying between 40.23 and 42.57% for digestate cultivations, with the lowest value recorded for 'Feliks' control, and between 41.33 and 42.53% for 'Feliks' control cultivar on traditional NPK fertiliser.

The protein content is undoubtedly an important parameter influencing the usefulness of various oils, which is why its measurement is necessary when determining the potential usability of the fat-free leftover material found in rapeseeds as e.g. raw material in the production of dietary protein supplements (GAWRYSIAK-WITULSKA *et al.*, 2012). The analysis of protein content revealed that the same was slightly higher in seeds cultivated using natural fertiliser (digestate) and was 23.30% (for 'Bios' cultivar) and between 23.23% and 22.47% (for 'Feliks' cultivar). We concluded that the application of digestate had a subtle but noticeable influence on yield and quality compared to mineral fertilization. The obtained results in terms of fat and protein content were consistent with values reported by other authors (MURAWA and WARMIŃSKI, 2005). In the research of RATHKE *et al.* (2005) analyzing the effect of applying cattle manure, it was observed that seed yield of winter oilseed rape tended to increase with increasing N fertilization rate, while the oil content in the seeds declined. CHEEMA *et al.*, (2001) and MASON and BRENNAN, (1998) reported the negative influence of N fertilization on the oil (fat) content in seeds.

The statistical post-hoc Tukey test conducted at the significance level of $p < 0.05$ revealed no statistically significant differences in terms of the protein content between the respective rapeseed cultivation variants. At the same time, such significant differences were observed for the fat content. MENSINK and KATAN (1990) say that high fatty acids content in oils is desirable because of their health benefits. The fatty acid content varies significantly between vegetable oils obtained from different sources, particularly depending on the actual plant cultivar used and plant maturity. Other factors with significant influence include: the region in which the plants are grown and specific climatic conditions (MURKOVIC *et al.*, 1996).

Given the above, Table 4 presents the primary fatty acids content in spring rapeseeds during the first stage of storage treated as the point of reference for the subsequent extracted oil measurements. The fatty acid content in oils was determined for specific rape cultivars (as reflected by the obtained measurement results) and returned the approximate percentages of 60% oleic acid, 21% linoleic acid, and 10% linolenic acid (KRYGIER, 1997). Comparable results were obtained in the cultivation and harvest of winter rape, where the highest reported content of MUFAs and PUFAs was respectively 68.33% and 34.55% (TYS *et al.*, 2006). In terms of the analysed seeds, the fatty acid content was within the range approved by the CODEX ALIMENTARIUS (2011).

Table 4. Moisture, fat and protein content in seeds.

Experiment	Cultivar	Initial moisture (%)	Moisture content (%)	Fat content (% dry mass)	Protein content (% dry mass)
Control	'Bios'	10.00±0.06aA	9.93±0.06aA	42.57±0.15aA	23.35±0.35aA
	'Feliks'	9.50±0.06aA	8.80±0.10bB	40.23±0.85cB	22.47±0.21aA
	'Markus'	9.90±0.08aA	8.93±0.21bA	40.73±0.64cB	23.37±0.15aB
Digestate	'Bios'	9.20±0.05bA ^a	8.17±0.06aA ^a	41.37±0.12bA ^a	23.30±0.10aA ^a
	'Feliks'	8.30±0.06bA ^a	7.27±0.06aA ^a	41.67±0.38bA ^a	23.23±0.06aA ^a
	'Markus'	9.03±0.07bA ^a	7.97±0.12aA ^a	41.97±0.06bA ^a	22.83±0.21aA ^a
NPK fertilizer	'Bios'	8.75±0.05bA ^a	7.97±0.06aA ^a	41.80±0.10aA ^a	22.73±0.06aA ^a
	'Feliks'	8.60±0.05bA ^a	7.57±0.06aA ^a	42.00±0.44bA ^a	23.57±0.31aA ^a
	'Markus'	8.95±0.09bA ^a	7.67±0.06aA ^a	41.33±0.06bA ^a	22.37±0.40aA ^a

a, b, c - statistically significant cultivar differences relative to the control;

A, B - statistically significant differences between cultivars under the same field experiment;

^{a, b} - statistically significant differences between the respective fertilizer type experiments relative to the same parameters; mean values marked with the same letter are not statistically significantly different ($p > 0.05$).

Based on the obtained results (Table 5) it can be concluded that in the case of seeds grown with the use of digestate, the content of oleic acid (C18:1) and linoleic acid (α -C18:3) was lower in the control than in the "ecologically" grown samples of 'Bios', 'Markus' and 'Feliks' cultivars. An analogous situation was also observed in seeds cultivated using traditional NPK fertilisation. The statistical Tuckey's post-hoc analysis revealed statistically significant differences in terms of acid content between the cultivars and their respective control, in terms of C18:1n9c for 'Bios' and 'Feliks', in terms of C18:2n6c (only for 'Feliks'); and in terms of C18:3n3 again for 'Bios' and 'Feliks', where the experimental plots were cultivated on digestate. Statistical differences were observed in terms of the C18:1n9c content between particular seed cultivars from the cultivation fertilized traditionally with NPK, specifically between 'Bios' seeds on the one hand, and 'Feliks' and 'Markus' seeds on the other. Differences were also observed in terms of C18:3n3 acid between seed cultivars grown on digestate, specifically between 'Bios' seeds on the one hand, and 'Feliks' and 'Markus' seeds on the other.

In terms of SFAs in the 'Bios' cultivar, the highest values were observed in the control, where it reached 7.85%, compared to 7.53% for traditionally fertilised crops. In terms of the other two cultivars, the highest Saturated Fatty Acids content was observed in the digestate cultivations 7.99% followed by the control 7.76% ('Feliks') and 7.76% for traditional fertilisation ('Markus'). The lowest content of those fatty acids was observed in 'Markus' seeds grown on digestate: 7.55%.

A very similar situation could be observed with respect to Monounsaturated Fatty Acids. After analysing the results obtained for Omega 3 acids, it can be concluded that the same fluctuated between 6.22 and 7.64%, with the lowest values observed for Feliks seeds both in the control (6.90%) and the experimental cultivations, respectively 6.22% for digestate and 6.31% for NPK. The highest content of the same was observed for 'bios' seeds cultivated using the biofertilizer (7.64%).

The content of Omega 6 acids in the seeds of analysed cultivars varied between 17.90 and 20.98%. The highest values were observed for the control 'Feliks' cultivar (20.48%) and the lowest for 'Markus' at 17.90% (on traditional fertiliser).

The statistical analysis using Tukey's post-hoc test at the level of significance of $p < 0.05$ revealed clear statistical difference in the content of MUFAs between the control plots and plots fertilised with digestate for the 'Bios' cultivar, as well as between the control plots and experimental plots fertilised with either digestate or NPK mixture for the 'Feliks' cultivar. The statistical analysis also revealed significant differences in terms of the content of MUFA acids in the analysed seeds, specifically for 'Bios' vs. 'Feliks' and 'Markus' grown on digestate, and between 'Bios' and 'Markus' in the NPK cultivation.

In terms of PUFAs, statistical differences were also observed for the 'Bios' and 'Feliks' cultivars relative to seeds obtained from their respective control plots. The statistical analysis revealed the existence of certain differences between the respective analysed cultivar types. Significant differences in the content of PUFAs were observed in the control cultivation between 'Bios' and 'Markus' seeds on the one hand and 'Feliks' seeds on the other. Moreover, Discrepancies were observed between the three cultivars grown on digestate as well as between 'Bios' on the one hand and 'Markus' and 'Feliks' on the other in the NPK cultivation.

Statistical differences were also observed in the content of Omega 6 acids for the 'Feliks' cultivar when comparing the control plots to the experimental plots, regardless of the fertilisation method. Furthermore, significant differences in terms of their levels were noted in the control between 'Bios' and 'Markus' seeds on the one hand and 'Feliks' seeds on the other.

Simultaneously, the statistical analysis revealed no statistical differences in terms of the content of Omega 3 acids between the experimental cultivations and the control. Differences of borderline statistical significance were observed between particular cultivars fertilized with digestate, specifically between 'Bios' seeds on the one hand and 'Feliks' and 'Markus' on the other.

According to JELEŃ *et al.* (2000) major components of vegetable oils are fatty acids, both saturated and unsaturated, mainly bound to glycerol as triacylglycerols. The allyl groups in unsaturated fatty acids are highly susceptible to free-radical reactions. DE LEONARDIS and MACCIOLA (2012) reported that the influence of fatty acid composition on oil stability has been varied between respective cases. In general, oils that are more unsaturated oxidise more readily than their less unsaturated counterparts. In the presence of oxygen, unsaturated fatty acids undergo decomposition even at low temperatures. The process of lipid oxidation results in deterioration of food quality, which can manifest itself through unpleasant odour leading consumers to reject the product (SHAHIDI, 2005). It is the decomposition of hydroperoxides that constitute the main non-volatile compounds directly involved in the decomposition of volatile compounds, such as alkenes, aldehydes, alcohols, esters, acids and carbohydrates, that deteriorates the taste of food and is responsible for the rancid smell of soap. Moreover, the oxidative degradation of lipids can damage biological membranes and protein, and as such can pose a direct threat to human life (MALHEIRO *et al.*, 2013).

Table 5. Fatty acid content of the rapeseeds.

Specification (%)	Rapeseeds								
	Control ,B'	'B' - digestate	'B' – NPK fertilizer	Control 'F'	'F' - digestate	'F' – NPK fertilizer	Control 'M'	'M' - digestate	'M' – NPK fertilizer
C14:0	0.07±0.02 aA ^a	0.08±0.02aA ^a	0.07±0.02aA ^a	0.09±0.02aA ^a	0.08±0.02aA ^a	0.09±0.02aA ^a	0.09±0.02aA ^a	0.08±0.02aA ^a	0.07±0.02aA ^a
C16:0	4.47±0.71aA ^a	4.24±0.68aA ^a	4.26±0.68aA ^a	4.74±0.76aA ^a	4.66±0.74aA ^a	4.52±0.72aA ^a	4.39±0.70aA ^a	4.46±0.71aA ^a	4.39±0.70aA ^a
C16:1	0.27±0.01aA ^a	0.25aA ^a	0.28aA ^a	0.31±0.01aA ^a	0.30±0.01aA ^a	0.28aA ^a	0.27±0.01aA ^a	0.27aA ^a	0.26aA ^a
C17:0	0.05±0.01aA ^a	-	-	0.05±0.01aA ^a	-	-	0.05±0.01aA ^a	-	-
C17:1	0.07±0.01aA ^a	0.10±0.01aA ^a	0.08aA ^a	0.08±0.01aA ^a	0.08±0.01aA ^a	0.08aA ^a	0.09±0.01aA ^a	0.07aA ^a	0.07±0.01aA ^a
C18:0	2.05±0.21aA ^a	1.99±0.21aA ^a	2.01±0.21aA ^a	1.71±0.18aA ^a	2.04±0.21aA ^a	2.08±0.22aA ^a	1.91±0.20aA ^a	1.87±0.19aA ^a	2.11±0.22aA ^a
C18:1n9c +C18:1n9t	65.01±0.60aA ^a	63.81±0.48bB ^a	64.24±0.52aA ^a	62.41±0.33cB ^b	65.76±0.67aC ^a	65.56±0.65aC ^a	64.60±0.56aA ^a	64.69±0.57aB ^a	65.24±0.62aC ^a
C18:2n6c+C18:2n6t	18.19±0.57aA ^a	18.74±0.74aA ^a	18.52±0.67aA ^a	20.48±0.27aB ^b	17.99±0.51aA ^a	18.04±0.53aA ^a	18.49±0.66aA ^a	18.78±0.75aA ^a	17.90±0.48aA ^a
C18:3n6 (gamma)	-	-	-	-	-	-	-	-	-
C18:3n3 (alpha)	6.72±0.19aA ^a	7.64±0.22aA ^a	7.14±0.20aA ^a	6.90±0.20aA ^a	6.22±0.18aB ^a	6.31±0.18aA ^a	7.17±0.20aA ^a	6.38±0.18aB ^a	6.50±0.18aA ^a
C20:0	0.69±0.06aA ^a	0.68±0.06aA ^a	0.67±0.06aA ^a	0.63±0.06aA ^a	0.69±0.06aA ^a	0.72±0.06aA ^a	0.64±0.06aA ^a	0.65±0.06aA ^a	0.72±0.06aA ^a
C20:5	-	-	-	-	-	-	-	-	-
C20:1	1.50±0.23aA ^a	1.54±0.23aA ^a	1.65±0.25aA ^a	1.66±0.25aA ^a	1.38±0.21aA ^a	1.44±0.22aA ^a	1.45±0.22aA ^a	1.65±0.25aA ^a	1.77±0.27aA ^a
C22:0	0.35±0.03aA ^a	0.34±0.03aA ^a	0.35±0.03aA ^a	0.37±0.03aA ^a	0.36±0.03aA ^a	0.37±0.03aA ^a	0.33±0.03aA ^a	0.35±0.03aA ^a	0.33±0.03aA ^a
C22:2	-	-	-	-	-	-	-	-	-
C24:1	0.17±0.03aA ^a	0.14aA ^a	0.15aA ^a	0.19±0.01aA ^a	0.14aA ^a	0.14aA ^a	0.14±0.03aA ^a	0.16±0.03aA ^a	0.10aA ^a
SFA	7.85aA ^a	7.49aA ^a	7.52aA ^a	7.76aA ^a	7.99aA ^a	7.95aA ^a	7.56aA ^a	7.55aA ^a	7.76aA ^a
MUFA	67.13aA ^a	65.98bA ^a	66.68aA ^a	64.71bB ^b	67.66aB ^a	67.49aA ^a	66.67aA ^a	67.15aB ^a	67.74aB ^a
PUFA	25.10aA ^a	26.60bA ^a	26.03bA ^a	27.53aB ^b	24.27bB ^a	24.41bB ^a	25.87aA ^a	25.53aC ^a	24.70aB ^a
OMEGA 3	6.72aA ^a	7.64aA ^a	7.14aA ^a	6.90aA ^a	6.22aB ^a	6.31aB ^a	7.17aA ^a	6.38aB ^a	6.50aA ^a
OMEGA 6	18.19aA ^a	18.74aA ^a	18.52aA ^a	20.48bB ^b	17.99aA ^a	18.04aA ^a	18.49aA ^a	18.78aA ^a	17.90aA ^a

a, b - statistically significant cultivar differences relative to the control; A, B - statistically significant differences between cultivars under the same field experiment; ^{a, b, -} - statistically significant differences between the respective fertilizer type experiments relative to the same parameters; mean values marked with the same letter are not statistically significantly different (p > 0.05).

The oxidative stability of the analysed oil samples was determined using the Rancimat method. Said stability is affected by a number of factors, including above all: the content of fatty acids, antioxidants and a whole range of other minor compounds. Table 6 presents the results of analyses performed in relation to the oxidative stability of the studied oils. In the case of oils extracted from plants cultivated on digestate, the shortest induction time was observed for the 'Bios' cultivar and was 5.21 h, while in the case of the respective control the same was three hours longer (8.31 h). The longest period of oxidative stability was recorded for the 'Feliks' oil, specifically 8.17 h, the same result as that observed in the respective control – 8.17 h. The results of measurements conducted for oil extracted from seeds cultivated using NPK and digestate fertilisation revealed relatively high oxidative stability compared to that reported by other authors. In a study by CICHOSZ and CZECHOT (2011), the induction time of oxidative processes (Rancimat test) in pressed and refined rapeseed oil was respectively: 4.5 and 4.7 h. Results obtained by WRONIAK and ŁUKASIK (2007) with respect to the oxidative stability of cold-pressed rapeseed oils with various antioxidative additives varied between 3.76 and 4.38 h.

In the case of oils obtained from traditionally fertilised cultivations, the longest induction time was observed for control 'Markus' oil – 9.30 h, and the shortest for 'Bios' oil – 8.41. In terms of oxidative stability of oils analysed relative to normal time, a discrepancy was observed for 'Bios' oil obtained from seeds grown on digestate, NPK mixture and the respective control, where it was 24.4 h (control), 20.96 h (digestate) and 22.02 h (NPK). A similar situation was recorded for 'Markus' oil (Table 6).

The conducted statistical analysis using Tukey's post-hoc test for the significance level of $p < 0.05$ also revealed slight statistical induction time differences between 'Bios' grown on digestate and the respective control plants. In the case of the 'Markus' cultivar, slight statistical differences were observed between the control and oil from the NPK plots. The results of the statistical analysis also indicated the presence of significant differences between the respective cultivars from particular field experiments. Seeds grown on digestate showed differences in terms of the time of oil induction, specifically between 'Bios' seeds on the one hand and 'Markus' and 'Feliks' seeds on the other. Significant differences were also observed between the fertilizer types for the respective rape cultivars. Specifically, for 'Bios' seeds in the case of the control and 'Bios' and 'Markus' seeds grown on digestate and NPK.

In the discussed case it should be noted that the seeds were subjected to no heating processes prior to extraction. The obtained oxidative stability values were considerably higher than those reported in other studies (4.35 or 4.39h) (KONDRATOWICZ-PIETRUSZKA, 2014; CIEMNIEWSKA-ŻYTKIEWICZ *et al.*, 2014).

Fig. 2 presents the ATR-FTIR spectra for selected oil samples. For easier interpretation of the results, Table 7 provides a breakdown of all characteristic bands (maximums) for selected samples together with the corresponding vibration of the respective functional groups. Samples were placed on a ZnSe crystal under N₂ atmosphere (as described above in Materials and Methods).

Spectra in the infrared region (ATR-FTIR) have well resolved bands that can be assigned to functional groups of particular components of food and biodiesel. Edible fats and oils as well as some biodiesel substances consist basically of triglycerides groups with different substitution patterns, which are mainly differentiated by the degree and form of unsaturation of the acyl groups and their length (GUILLEN and COBO, 1997; HONG *et al.*, 2005; SCHOLZE and MEIER, 2005; ROHMAN and CHE MAN, 2010; Van de VOORT, 1992). Fig. 2 shows the infrared spectrum of selected oil samples chosen for the spectroscopic research.

Table 6. Analysis of the induction time for oils.

Parameter	Experiment	Cultivar	Measurements
Oxidative Stability (h)	Induction time	'Bios'	8.31±0.02a ^a
	Normal time		24.40±0.53a ^A
	Induction time	Control	8.58±0.55a ^A
	Normal time		21.39±0.46a ^B
	Induction time	'Feliks'	8.17±0.04a ^a
	Normal time		21.11±0.93a ^B
Oxidative Stability (h)	Induction time	'Bios'	5.21±0.13b ^b
	Normal time		20.96±0.02b ^A
	Induction time	Digestate	7.71±0.21a ^B
	Normal time		21.42±0.11a ^A
	Induction time	'Feliks'	8.17±0.05a ^B
	Normal time		21.66±0.38 a ^A
Oxidative Stability (h)	Induction time	'Bios'	8.41±0.07a ^a
	Normal time		22.02±0.23b ^A
	Induction time	NPK fertilizer	9.30±0.08c ^b
	Normal time		23.89±0.65a ^B
	Induction time	'Feliks'	8.51±0.18a ^a
	Normal time		21.77±0.77a ^B

a, b, c - statistically significant cultivar differences relative to the control;

A, B - statistically significant differences between cultivars under the same field experiment;

a, b, c, - statistically significant differences between the respective fertilizer type experiments relative to the same parameters; mean values marked with the same letter are not statistically significantly different ($p > 0.05$).

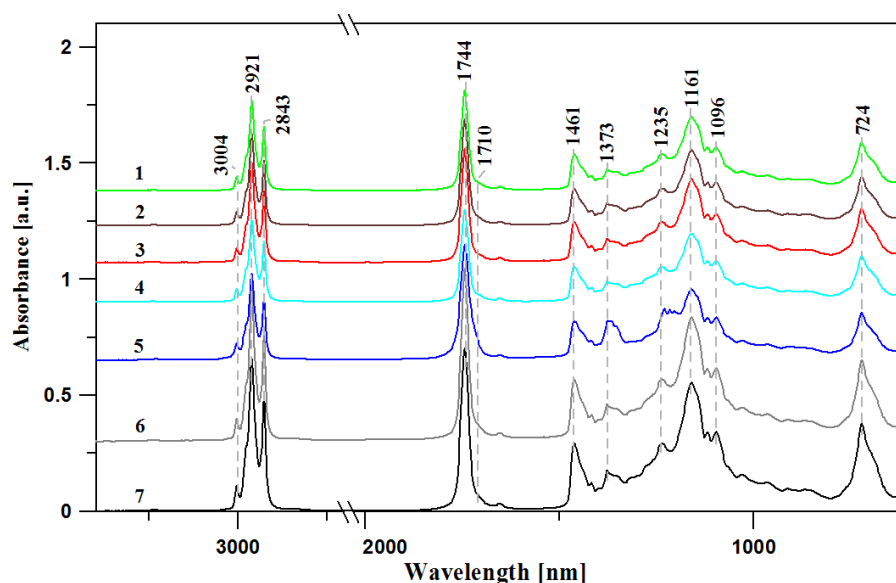


Figure 2. ATR-FTIR spectra for selected rapeseed oil samples for the following cultivars: 'Bios', 'Feliks' and 'Markus', respectively, 'Bios' NPK– (7) solid black line, 'Bios' digestate – (6) solid grey line, 'Feliks' NPK – (5) solid blue line, Feliks digestate – (4) solid light-blue line and 'Markus' NPK – (3) solid red line, 'Markus' digestate – (2) solid brown line. The spectra are presented in the spectral range of 600 – 3800 /cm.

Although some authors have suggested specific assignments of spectral bands in oils and fats (GUILLEN and COBO, 1997; LI *et al.*, 2013; QIAN *et al.*, 2007), there are some, which are notoriously difficult to decidedly assign to a specific group. Table 7 shows the frequencies of the characteristic bands or shoulders in the spectra of 7 oil samples (GUILLEN and COBO, 1997), as well as their assignment to respective functional groups (GUILLEN and COBO, 1997; YANG *et al.*, 2005; HONG *et al.*, 2005; SCHOLZE and MEIER, 2005; ROHMAN and CHE MAN, 2010), their vibration mode and intensity in spectra typical for the IR region. It should be noted that the assignment of bands corresponding to the stretching vibration in FTIR modes is usually easier than the assignment of bands corresponding to the bending vibration modes due to overlap or vibration of this group. Thus, in the absorption spectra of our oil samples, we can observe that some bending vibrations of the methylene group are produced between 1350 and 1150/ cm. This stretching vibration originates from $-C-H$ vibrations in $-CH_2$ ($\sim 1350/cm$) groups and deformation vibrations in this group ($\sim 1150\text{ cm}^{-1}$).

On the other hand, stretching vibrations of the C-O bond of esters are composed of two coupled asymmetric vibrations, $C-C(=O)-O$ and $O-C-C$, the first being more important (GUILLEN and CABO, 1997; HONG *et al.*, 2005; ROHMAN and CHE MAN, 2010); these bands occur in the region between 1300 (some $C-C(=O)-O$) and 1000/cm (of this combination's group). The $C-C(=O)-O$ band of saturated esters appears between 1240 and 1163/cm, and in unsaturated esters the vibration is produced at lower frequencies. On the other hand, the $O-C-O$ band of esters derived from primary alcohols appears in the zone between 1064 and 1031/cm, while for those derived from secondary alcohols the band appears approximately at 1100/cm (ROHMAN and CHE MAN, 2010; LI *et al.*, 2013). Both kinds of esters are present in triglyceride molecules. However, some authors assign the band at about 1238/7 /cm exclusively to out-of-plane bending vibrations of the methylene group (GUILLEN and CABO, 1997; LI *et al.*, 2013; QIAN *et al.*, 2007). Two bands in Table 7 have been assigned in a different way: the bands at approximately 1400/cm and 1319/cm. The first band, at about 1400/cm, has been assigned by some authors to terminal methyl groups on the aliphatic chains of oil components (GURDENIZ and OZEN, 2009). The second band, at about 1319/cm, was observed in all samples where absorption reached between 967 and 914/cm. In this context, it must be pointed out that the band at about 914/cm, which appears in all oil samples, has been related to the bending vibration of *cis*-disubstituted olefinic groups (GUILLEN and CABO, 1997; QIAN *et al.*, 2007) and to vinyl groups. Although the oil spectra examined in this articles seem to be similar, they show differences in the intensity of their bands as well as in the exact frequency at which the maximum absorbance is produced in each case, due to the different nature and composition of the respective oil samples under study. Further characteristic areas of vibration are found in the bands with the maximum of approximately 1740/cm, typically associated with vibrations of the $C=O$ carbonyl group in esters (HONG *et al.*, 2005; SCHOLZE and MEIER, 2005). At the same time, a very weak band, or more specifically extension of the band with the maximum at approximately 1705/cm, reveals very weak vibrations of the carbonyl group in acids. In turn, bands with the maximum at approximately 1650/cm correspond to vibrations originating from stretching vibrations of the $-C=C-$ group (due to *cis*- transformation). A characteristic area is also found in bands with the maximum of approximately 1460/cm originating from deformation vibrations in the $-C-H$ groups of CH_2 and CH_3 (bending (scissoring)). The subsequent area of bands from 890 to 660/cm represents the characteristic deformation vibrations of $-HC=CH-$ groups (*cis*- conformation, extra plain) and ring vibrations of the groups $(\delta(-(CH_2)_n-$ and $-$

HC=CH- (*cis*-)) (GUILLEN and CABO, 1997; SCHOLZE and MEIER, 2005; LI *et al.*, 2013; QIAN *et al.*, 2007).

As we progress to vibrations within higher wavenumber ranges, one should mention the significant stretching vibrations =C-H (*trans*-) with the maximum at approximately 3060/cm which originate from the vibrations of triglyceride fractions. A very characteristic feature of stretching =C-H vibrations in the *cis*- configuration are the relatively intensive vibrations at approximately 3006/7 /cm. Next, a number of vibrations with the maxima at approximately 2955, 2920 and 2855/cm originate from the stretching vibrations of -C-H in the respective -CH₂, CH₃ groups belonging to the aliphatic groups in triglycerides (HONG *et al.*, 2005; SCHOLZE and MEIER, 2005; QIAN *et al.*, 2007). Characteristic vibrations associated with specific functional groups were described in detail for all studied samples in Table 7.

Furthermore, it is noteworthy that in the spectra of the studied samples of oil extracted from rape grown on digestate and traditional fertilizer mixture (Fig. 2), there are clearly visible differences in terms of the shape of spectra in the 1770 – 1670/cm area. In most of the studied samples there is apparent, slight amplification of the band at 1742/3/cm (responsible for vibrations of the C=O group) on the side of lower wave numbers, with the clearly defined extreme at approximately 1710/cm (with the relatively lowest intensity in the control spectrum), which can be associated with the formation of a hydrogen bond between the C=O-H-O-H groups in oil samples selected for the study. Simultaneously to the appearance of the band at 1710/cm (under both fertilisation regimens), there is a clear increase in the intensity of bands at approximately 1360, 700/cm, which is associated to stretching vibrations in C-O and C-C groups (as described above).

The area between 1100 – 1300/cm is also responsible for the stretching vibrations of the C-O group, but the same varied only slightly between the studied oil samples, regardless of their origin (i.e. cultivation with the use of digestate or traditional fertiliser) (LI *et al.*, 2013; QIAN *et al.*, 2007).

With the reducing affinity of the constituent molecules for the formation of the hydrogen bond between C=O-H-O-H, the bands show a slight increase in intensity. A similar correlation can be observed both in rapeseed oil samples originating from traditionally fertilised plots and from plots fertilised with digestate.

Moreover, it would be prudent to once again mention bands from the range of 2960 to 2840/cm, which are responsible for the symmetrical and asymmetrical vibrations of CH₂ and CH₃ groups in the aliphatic sections of triglycerides. Their rates vary significantly depending on the origin of the respective samples, i.e. from rapeseed grown in plots fertilised traditionally or with the use of digestate. The variations observed in this area are very well correlated with variations in the fatty acids profile presented in Table 5. The aforementioned spectral changes also confirm the slight discrepancies in the overall fat content in the studied oil samples, as presented in Table 4 above. One should also point out the clear discrepancies in terms of band intensity between the particular rapeseed cultivations, relative to the method of fertilisation. In all spectrum-wide band intensity measurements the same was clearly lower in the case of plots fertilised with digestate as compared to traditional fertilisation. With regard to a comparison between specific cultivars, the most intensive bands were observed for 'Bios', followed by 'Markus', with the lowest values observed for 'Feliks'. The described differences correspond to the respective overall nutrient content in the studied samples of rapeseed oil. The main band differences for all samples, relative to the method of fertilisation, can be observed in bands with the respective maxima at 1745, 1709, 1368, 1220, 1154 and 1146/cm, which confirms the discrepancies in the overall fatty acids profile of the analysed samples.

Table 7. Position of the maxima of absorption spectra in relation to the respective vibrations for selected spring rapeseeds grown with the use of traditional and digestate fertilisation, within the spectral range of 3800-550/cm.

FTIR									Types and origin of vibration
Position of the maximum (/cm)									
NPK fertilizer			Digestate			Control			
'B'	'F'	'M'	'B'	'F'	'M'	'B'	'F'	'M'	
—	3463	3476	3476	3474	3476	3474	3476	3474	-C=O _w (overtone)
3005	3066	3064	3063	3061	3066	3067	3181	—	and $\nu(=C-H_{vw}, trans-)$
—	3006	3007	3007	3007	3007	3006	3059/ 3007	3062/ 3007	$\nu(=C-H_m, cis-)$
—	2969	2954	2954	2953	2955	2954	—	—	$\nu_{as}(-C-H_m, -CH_3)$
—	2955	2923	2922	2921	2922	2922	2953	2956	$\nu_{as}(-C-H_{vst}, -CH_a)$
2922	2923	—	—	—	—	—	2922	2923	and
2854	2871/ 2853	2853	2853/ 2872	2853/ 2870	2853/ 2872	2852/ 2872	2854	2852	$\nu_s(-C-H_{vst}, -CH_a)$ (aliphatic groups in triglycerides)
2731	2731	—	2731	2730	—	2729	2730	2729	
2676	2677	2676	2635/ 2680	2675	2674	2678	2678	2679	
—	2584	—	—	—	—	2335/ 2361	—	2339/23 60	-C=O _{vw} Fermi Resonance
2360/ 2336	2362/ 2337	2361	—	—	2362	—	—	—	
1743	1743	1744	1743	1743	1743	1743	1743	1744	$\nu(-C=O_{vst})$ in ester
1704	1704	1704	1705	1705	1705	1704	1702	1705	$\nu(-C=O_{vw})$ in acid
—	1654	1654/ 1559	1654	1654	1654	1653/ 1558	1653	1653	$\nu_{vw}(-C=C-, cis-)$
1462	1462	1462	1462	1462	1462	1463	1462	1462	$\delta_{vw}(-C-H)$ in CH ₂ and CH ₃ group, bending (scissoring)
1417	1441/ 1418	1440/ 1417	1416	—	1418	1440/ 1418	1439/ 1418	1440/ 1418	$\nu_{vw}(-C-H, cis-)$ bending (rocking)
—	1401	1400	1400	—	1400	1400	1400	1401	
1377	1376	1377	1377	1377	1351/ 1377	1377/ 1350	1376/ 1349	1377	
	1367	—	—	—	—	—	—	1365	$\nu_{w, m, vw}(-C-H, -CH_3)$, bending
1349	1353	1350	1349	1350	1318	1316	1317	1351	
1317	1318	1318	1317	1318/ 1302	1301	1300	1300	1300	
1300	1301	1299	1279	1277	1278	1278	1277	1279	
1278	1278	1277	—	—	—	—	—	—	
—	1265	1262		1264	1261		1262	—	$\nu_m(-C-O)$ or $\delta_m(-CH_2-)$
1237	1229	1237	1237	1237	1236	1237	1237	1234	
1161	1160	1160/ 1141	1160	1143/ 1159	1141/ 1160	1161	1142/ 1160	1160/ 1143	$\nu_{st}(-C-O)$ or $\delta_{st}(-CH_2-)$
1120	1119	1119	1119	1119	1119	1120	1120	1119	$\nu_m(-C-O)$
1097	1097	1096	1097	1096	1096	1097	1097	1097	
1066	1060	1061	1062	1062	1059	1060	1063	1060	$\nu_{m, vw}(-C-O)$
1030	1030	1030	1030	1031	1030	1030	1030	1031	
966	965	967	966	914/965	913/966	913/ 967	912/ 965	913/969 /949	$\delta_w(-HC=CH-, trans-)$ bending out of plane

913/ 867/ 851	911	912/ 867/ 849	848/ 868/ 913	–	870	689/ 867	694/ 869	791	$\delta_{vw}(-\text{HC}=\text{CH}-, \text{cis-})$ bending out of plane
766	767	765	765	–	766	770	765	765	
723	722	722	721	–	722	722	722	722	$\delta(-(\text{CH}_2)_n-$ and $-\text{HC}=\text{CH}-$ (<i>cis</i> -) bending (rocking)
–	689	691	694	–	687	662	664	690	

v – stretching, δ – deformation, s – symmetrical, as – asymmetrical, st – strong, vst – very strong, w – weak

PCA is a mathematical tool, which performs a reduction in data dimensionality and allows the visualization of underlying structure in experimental data and relationships between data and samples. FTIR spectra data of 7 oil samples were used in Principal Component Analysis (PCA). PCA of FTIR spectra using absorbencies at 16 wavenumber regions was carried out using Statistica 13 advanced statistics software. The PCA score plot (Fig. 3) was obtained from the correlation matrix of peak absorbencies at 16 frequency regions, namely 3007, 2921, 2853, 1744, 1654, 1461, 1418, 1373, 1235, 1161, 1119, 1096, 1031, 966, 913, 724/cm. In PCA, the first principal component (PC1) and the second principal component (PC2) account the largest and the next largest of variable variation. PC 1 explain 99,32% variance, meanwhile PC 2 accounted 0,62%; therefore the variance can be described by the first two PCs.

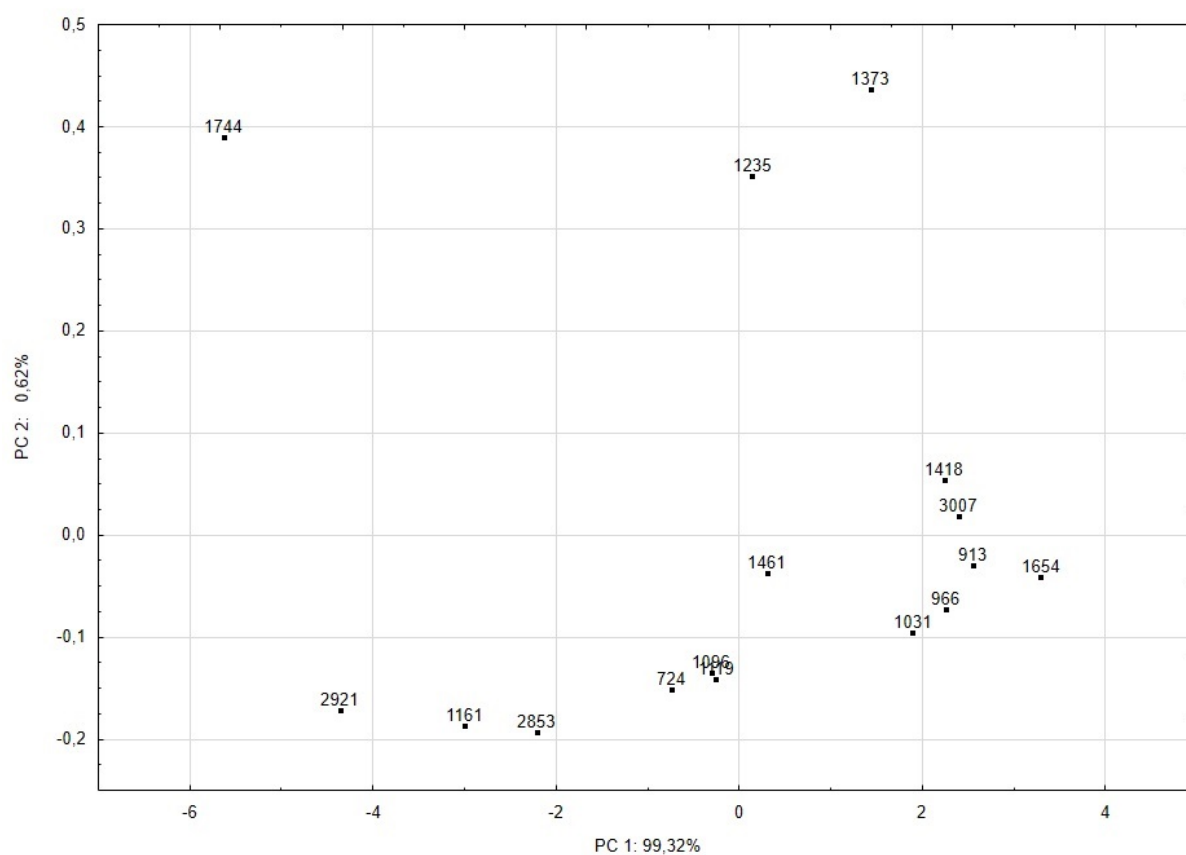


Figure 3. Projection of cases on the PC 1 and PC 2 plane.

Hierarchical cluster analysis (HCA) was performed in order to observe similarities or dissimilarities between the 'Bios', 'Markus' and 'Feliks' oil samples from spring plants grown with traditional NPK mixture fertilisation and digestate (D). The dissimilarity of different clusters was defined by Euclidean distance and calculated by single linkage method. The results obtained are presented in the dendrogram structure, showing the different groups. On the Fig. 4 we present the plot obtained from the 7 oil samples of 'Bios', 'Markus' and 'Feliks'. Considering the cut-off of 0.1 dissimilarity units we can distinguish three clusters (in red, green and blue colour, respectively). The first cluster, green coloured, is a small cluster, aggregated on the far left arm of the dendrogram. This cluster is formed by F-NPK oil of 'Feliks' sample. The second cluster, red coloured, is composed of all 'eco-digestate' oil samples including 'Bios', 'Markus' and 'Feliks' samples and samples of Control and of M-NPK. This result suggests that these oils have a physical-chemical properties more similar than the others. The sample of B-NPK oil is aggregated in third cluster (blue coloured) on the right arm of dendrogram.

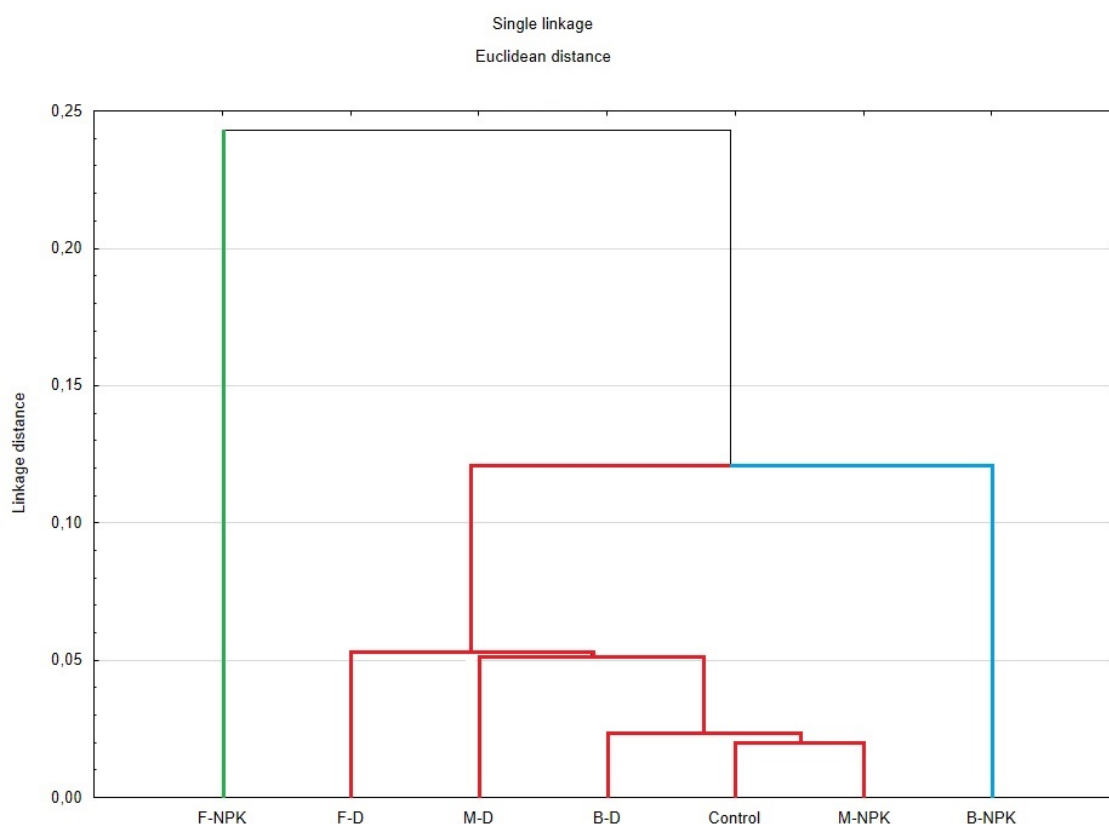


Figure 4. Dendrogram analysis (HCA) of all samples in the 3800-600/cm spectral region.

The PCA analysis was applied to fatty acid data. The obtained results are presented in Figs. 5-6. The greatest effect on PC 1 in PCA is positively correlated with the content of C18:1N9c + C18:1n9t (0.017), C16:0 (0.0127) and is negatively correlated with the content of PUFA (-0.0471). In turn, PC2 is negatively correlated with C18:2n6c + C18:2n6t and OMEGA 6 (-0,0091) and positively correlated with the content of C18:3n3 (alpha) and OMEGA 3 (0,0096).

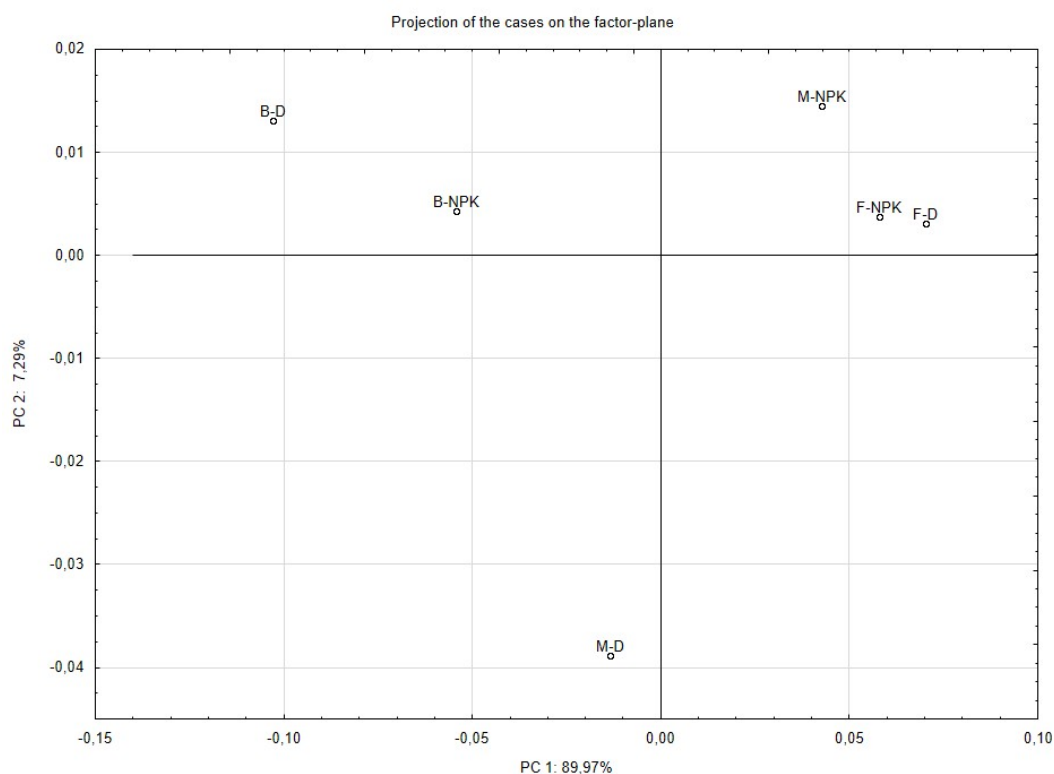


Figure 5. The plot of two first principal components after PCA analysis of fatty acid compositions. Projection of the cases on the PC 1 and PC 2 plane.

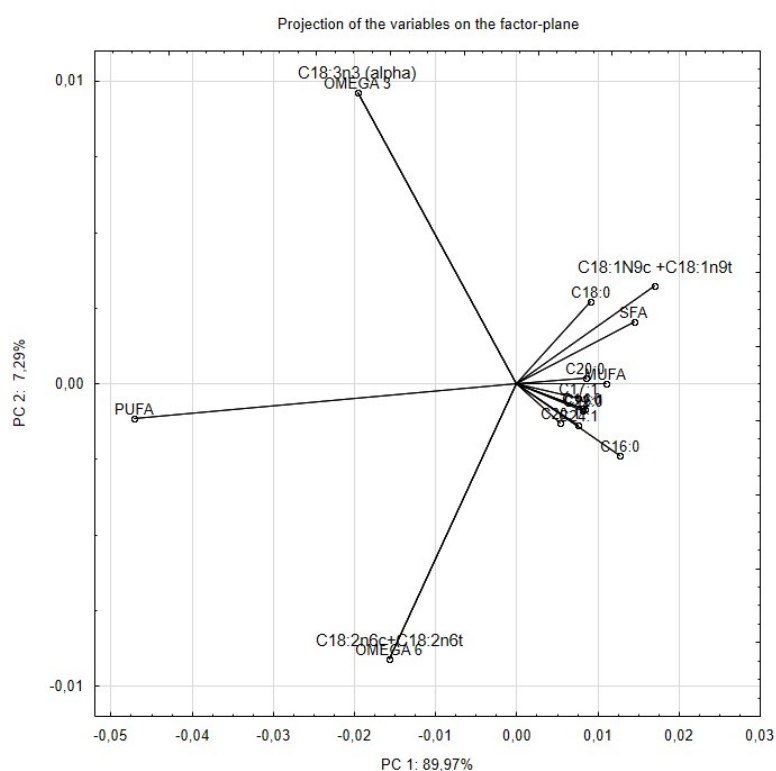


Figure 6. The plot of two first principal components after PCA analysis of fatty acid compositions. Projection of the variables on the PC 1 and PC 2 plane.

4. CONCLUSIONS

The obtained results reveal a correlation between the employed method of fertilisation, using digestate or a traditional NPK mixture, and the soil condition as well as the quality of the products, i.e. spring rapeseed and the oil cold-pressed therefrom.

Based on said results it can be concluded that statistically significant differences can be observed in terms of the content of nutrients available to plants between the control and fertilised plots. Statistically significant differences were also observed between the content of the analysed nutrients in the soil prior to sowing and after the harvest, with the exception of control plots related to the use of NPK fertiliser.

Analyses of the oil in terms of its oxidative stability revealed no direct correlation between the qualities of particular oils and the method of cultivating the seeds from which they were extracted, although the statistical analysis did reveal significant differences in terms of oil obtained from the 'Bios' cultivar, which was characterised by the lowest stability relative to digestate fertilisation. It is noteworthy that clear, although not particularly big, differences in terms of band intensity in the general cross-section of the FTIR spectrum were observed for samples fertilised with the mineral mixture, as compared to digestate. The same signify the possibility of a very efficient use of this method of field fertilisation. It is also noteworthy that FTIR spectroscopy techniques can be used for the purposes of rapid classification of oil samples without the need for sample preparation. FTIR spectroscopy techniques provide exquisite structural insights into the functional groups of oils, as required for discriminant analyses. FTIR spectroscopy has been found to be the most efficient method of oil discrimination and classification. To recapitulate, FTIR spectroscopy constitutes a simple, efficient and low-cost quantitative quality control tool available to the fat and oil industry.

ACKNOWLEDGEMENTS

Research supported by the Polish Ministry of Science and Higher Education as part of the statutory activities of the Department of Machinery Exploitation and Production Process Management, University of Life Sciences.

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Paper Received November 4, 2018 Accepted May 6, 2019

ANTIOXIDANT ACTIVITY AS WELL AS VITAMIN C AND POLYPHENOL CONTENT IN THE DIET FOR ATHLETES

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ABSTRACT

The aim of the study was to analytically evaluate the total content of vitamin C and polyphenols as well as the antioxidant potential of daily food rations planned for athletes. Chemical analyses showed that an average food ration for women (2,120.1 kcal, 90.8 g protein, 53.1 g fat and 354.0 g carbohydrates) contained 5.5 ± 2.6 mg vitamin C and 20.1 ± 4.1 mg polyphenols in 100 g fresh mass. An average food ration for men (2,648.8 kcal, 112.5 g protein, 63.1 g fat and 447.4 g carbohydrates) contained 5.6 ± 1.4 mg vitamin C and 22.9 ± 8.1 mg polyphenols in 100 g fresh mass. The antioxidant potential of an average ration for women expressed as reducing power (FRAP index) in 100 g fresh mass was 8.2 ± 0.7 mmol Fe²⁺, and for men, 8.9 ± 0.9 mmol Fe²⁺. The antioxidant potential of an average ration prepared for women and men expressed as antiradical activity against DPPH in 100 g fresh mass was respectively: 2.7 ± 0.2 mmol and 2.7 ± 0.4 mmol Trolox equivalent. Balanced food rations rich in products with high nutrient density can ensure the appropriate intake of vitamin C and polyphenols and high antioxidant potential of the diet.

Keywords: vitamin C, polyphenols, antioxidant potential, diet, athletes, chemical analyses

1. INTRODUCTION

The situation of intensive physical exercise leads to the disruption of body homeostasis, also in terms of prooxidant-antioxidant equilibrium (oxidative stress) as a result of intense metabolic processes and the influence of psychological and environmental factors. Intensive physical exercise induces the overproduction of reactive oxygen species, which cause oxidative damage to tissues as a result of peroxidation of lipids, proteins and DNA. By reducing the skeletal muscle contraction strength, speeding up fatigue and lowering immunity, they reduce athletes' performance (SUNG *et al.*, 2016; YAVARI *et al.*, 2015).

High oxidative stress occurring in athletes generates increased demand for antioxidant vitamins and polyphenols (HEATON *et al.*, 2017; MORILLAS-RUIZ *et al.*, 2006; ORLANDO *et al.*, 2018; SCHNEIDER *et al.*, 2018; YAVARI *et al.*, 2015). A rich source of dietary antioxidants is fruit and vegetables with high contents of bioactive substances, including vitamin C, carotenoids and polyphenols (phenolic acids and flavonoids with anthocyanins) (NADERI *et al.*, 2018; SIKORA *et al.*, 2008). The Swiss food pyramid for athletes recommends daily consumption of 5 portions of fruit and vegetables in all colors, ensuring a wide range of bioactive substances (WALTER *et al.*, 2007). In this context, planning balanced food rations, rich in products with high nutrient density, such as fruit and vegetables and other products with high nutrient density, is of special importance in athletes' diet (YAVARI *et al.*, 2015).

Literature review shows that many works present the nutritional value, including the content of antioxidant substances and antioxidant activity of selected products and dishes, such as e.g., honey (CIANCIOSI *et al.*, 2018), fruit (AIRES *et al.*, 2017; NADERI *et al.*, 2018; SHIN *et al.*, 2018; ZENTENO-RRAMÍREZ *et al.*, 2018), vegetables (JAISWAL *et al.*, 2012; SOTIROUDIS *et al.*, 2010), legumes (DURAZZO *et al.*, 2013), whole grains (DURAZZO *et al.*, 2015), several varieties of wheat and black barley (SIEBENHANDL *et al.*, 2007), different cereal grain species (VAN HUNG, 2016), sour cherry juice (FERRETTI *et al.*, 2010; MCCORMICK *et al.*, 2015), sprouts (HOTNOG *et al.*, 2017), as well as grilled chicken salad and spaghetti with tomatoes and parmesan cheese (FRĄCZEK and GACEK, 2013; GACEK *et al.*, 2012). Fewer works describe the nutritional value and antioxidant properties of daily food rations (ALIAKBARLU *et al.*, 2014; BEDOGNI *et al.*, 1999; KORÉISSI-DEMBÉLÉ *et al.*, 2017; MARCONI *et al.*, 2018; ZLOCH *et al.*, 2018). Evaluation of antioxidant potential of different products and dishes is one aspect of the innovative approach to research on food (DURAZZO, 2017).

Therefore, this study focused on vitamin C and polyphenol content as well as antioxidant properties of complete daily food rations planned for athletes with consideration of products and dishes they prefer, in accordance with qualitative and quantitative recommendations. The study aimed to answer the question whether it is possible to prepare food rations that meet the qualitative and quantitative recommendations for athletes (balanced in terms of energy and macroelements intake) and at the same time are rich in dietary antioxidants and have high antioxidant properties. The innovative approach to the issue is the verification through chemical analyses of the nutritional value of food rations based on theoretical databases. In this context, the aim of the study was to analytically evaluate the total content of vitamin C and polyphenols as well as the antioxidant properties of daily food rations planned for athletes (women and men) of disciplines that require them to maintain low body mass.

2. MATERIALS AND METHODS

2.1. Material

Daily food rations were prepared on the basis of dietary databases for Polish athletes (women and men) professionally doing disciplines that require them to maintain low body mass, with consideration of their dietary preferences. The criterion for the open selection of participants was doing the sport professionally for at least 3 years. The explored group of athletes for whom the menus were prepared included people aged 18-30 (22 ± 3.8), representing the following disciplines: long-distance running, middle-distance running, triple jump, race walking, ballet, artistic gymnastics, rhythmic gymnastics, ski jumping, Nordic combined, synchronised swimming and dancing.

The age and somatic indices of the participating athletes are presented in Table 1. Body composition (total fat mass TBF and total body water TBW) was measured using bio-impedance testing method (Body Comp MF from Akern).

Table 1. Statistical characteristics of anthropometric indices of the study subjects.

Group	Statistics	Age (years)	Body weight (kg)	Body height (cm)	TBF (%)	TBW (%)	BMI (kg/ m ²)
Overall	Mean	22.0	58.4	171.5	11.7	64.6	19.8
	SD	3.8	6.9	7.1	4.6	3.4	1.6
Men	Mean	21.3	62.6	175.3	9.1	66.5	20.0
	SD	3.7	5.3	6.4	3.3	2.4	1.5
Women	Mean	22.9	53.2	166.8	15.1	62.2	19.1
	SD	3.8	4.9	4.7	3.8	2.9	1.4

The athletes' dietary preferences were determined for 185 products and dishes using a 5-point hedonic scale (5 – like very much, 4 – like, 3 – neither like nor dislike, 2 – dislike, 1 – dislike very much). The proposed menus included products and dishes for which a high level of preference was obtained (mean values in the 4.64-4.00 range for 41 products, such as vegetables, fruit, cereal products, flour products, poultry, eggs and egg dishes). Products that were disliked were excluded from the diet (mean preference indices in the 2.50-2.99 range, such as pumpernickel, soy products, margarines, fish in cream sauce and in oil). Based on the analysis of dietary preferences, two weekly menus were prepared – 14 daily food rations (7 for women: W1-W7 and 7 for men: M1-M7).

The menus were prepared in accordance with qualitative (WALTER *et al.*, 2007) and quantitative recommendations for athletes (KREIDER *et al.*, 2010; POTGIETER, 2013) on the basis of data concerning products' and dishes' nutritional value in Polish "Tables of food ingredients and nutritional value" (KUNACHOWICZ *et al.*, 2005). The quantitative assumptions for the planned food rations are presented in Table 2. The nutritional value of dishes was calculated on the basis of the adopted recipes, taking into consideration raw products (and values for edible parts).

Table 2. Detailed assumptions for intended diet plans for women and men (CRP – daily food serving).

Energy/nutrient	Women	Men
Energy (kcal)	2162.0	2779.0
Protein (g/kg b.w.)	1.7	1.7
Protein (g)	90.4	106.4
Protein (% of energy)	16.7	15.3
Fat (g/kg b.w.)	1.0	1.0
Fat (g)	53.2	62.6
Fat (% of energy)	22.1	20.3
Carbohydrates (g/kg b.w.)	6.5	7.4
Total carbohydrates (g)	350.4	470
Indigestible carbohydrates (g)	315.4	430
Carbohydrates (% of energy)	61.2	64.4
Dietary fiber (g)	25-40	25-40

The planned daily food rations were prepared in a food laboratory and then sent for chemical analyses to the Malopolska Centre of Food Monitoring at the University of Agriculture in Krakow. Each food ration was prepared and analyzed twice in 2 samples (each ration was evaluated in 4 iterations). Tables 3 and 4 present the basic list of products and dishes in the weekly menus for athletes (women and men).

Table 3. Weekly menu prepared for women (the list of products / dishes).

	Breakfast	Lunch	Dinner	Supper
W1	Strawberry porridge	Chocolate pudding and a nectarine	Tomato soup with pasta, grilled chicken breast with rice and grilled vegetables	Rye bread with fish spread and vegetable salad
W2	Rye bread with honey, white cheese and vegetables	Banana shake (with buttermilk)	Tomato cream soup, beef chops with barley groats, raw salad (carrot, apple)	Vegetable salad with toasts
W3	Muesli with natural yoghurt, rye bread with white cheese and vegetables	Fruit salad with natural yoghurt	Cucumber soup with rice, tagliatelle with shrimp	Layer salad (vegetables, boiled eggs) with yoghurt sauce and white rye bread
W4	Scrambled eggs with rye bread, an orange	Steamed dumplings with shake (buttermilk, strawberries)	Vegetable soup with potatoes, spaghetti Bolognese	Rye bread with, white cheese and vegetables, fruit yoghurt
W5	Porridge with natural yoghurt, rye bread with chicken ham and vegetables	Yoghurt ice-cream	Zucchini cream soup, cod fillet with rice and boiled vegetables	Pancakes with strawberry jam, rye bread with cheese and tomato
W6	Rye bread with egg spread, natural yoghurt, lettuce, a nectarine	Pancakes with roasted apple	Beetroot soup with potatoes, farfalle with chicken and broccoli	Rye bread with white cheese spread and tomatoes
W7	Rye bread with strawberry jam and mozzarella, tomato, natural yoghurt	White rice with strawberry mousse	Tomato cream cheese, beef steak with pearl barley and lettuce	Lecsó with white rye bread

Table 4. Weekly menu prepared for men (the list of products/ dishes).

	Breakfast	Lunch	Dinner	Supper
M1	Scrambled eggs with rye bread, an orange	Steamed dumplings with strawberry shake (with buttermilk)	Vegetable soup, spaghetti Bolognese	Rye bread with, white cheese and vegetables, fruit yoghurt
M2	Porridge with natural yoghurt, rye bread with chicken ham and vegetables	Yoghurt ice-cream, sponge cake	Zucchini cream soup, roasted cod fillet with potatoes and vegetables	Pancakes with strawberry jam, rye bread with cheese and tomato
M3	Rye bread with egg spread and lettuce, a nectarine	Pancakes with roasted apple	Beetroot soup with potatoes, farfalle with chicken and broccoli	Rye bread with white cheese spread and tomato, cherry yoghurt with muesli
M4	Porridge with raspberry, a roll with strawberry jam	Banana shake (with buttermilk)	Tomato soup with pasta, chicken breast with rice and cabbage salad	Nice-style salad with rye bread
M5	Muesli with natural yoghurt, rye bread with Gouda cheese and vegetables, a banana	Strawberry dumplings	Tomato cream soup with toasts, risotto with vegetables	Rye bread with fish spread and vegetable salad
M6	Muesli with natural yoghurt, rye bread with white cheese, vegetables and strawberry jam, an apple	Yoghurt ice-cream, sponge cake	Leek cream soup with a baguette, beef steak with rice and rocket and tomato salad	Rye bread with chicken ham, egg and vegetables
M7	Muesli with natural yoghurt, rye bread with mozzarella cheese and tomato	Fruit salad with fruit yoghurt	Cucumber soup with rice, chicken pizza	Rye bread with white cheese spread and smoked salmon with vegetables

The mean mass of complete food rations for women was: 2,108.5 g (W1), 2,046.5 g (W2), 2,266.5 g (W3), 2,073.0 g (W4), 2,034.0 g (W5), 2,014.0 g (W6) and 2,085.5 g (W7), and for men: 2,359.5 g (M1), 2,572.5 g (M2), 2,454.5 g (M3), 2,824.0 g (M4), 2,566.5 g (M5), 2,507.0 g (M6) and 2,349.5 g (M7). The mean mass of an average food ration prepared for women (W1-W7) was: 2,090.42±92.29 g, and for men (M1-M7): 2,519.07±161.56 g.

2.2. Preparation of materials

The rations were homogenized and air-frozen using a freezing chamber (Feutron-type 3626-51, Germany) within 90 minutes in order to reach a temperature of -30°C in the thermal centre. The frozen material was then dried. Drying parameters: initial product temperature: -30°C, condenser temperature: -52°C, shelf temperature: 20°C; drying: 6 hours of total drying time at shelf temperature of 30°C. Drying was carried out for 24 hours.

2.3. Chemical analyses

Homogenates were used to determine dry mass content, and lyophilized samples were used to test the other indices. Dry mass content was determined in accordance with the AOAC procedure (2005, no. 930.04) with the weighing method, by drying up to solid mass at a temperature of 105 °C. Total nitrogen was determined in accordance with the AOAC

procedure (2005, no 978.04) with the Kjeldahl method using Distillation Unit B-324 (Büchi, Switzerland). Protein content was determined using the 6.25 rate of conversion, and fat content in accordance with the AOAC procedure (2005, no. 920.39) with the Soxhlet method using diethyl ether-based extraction. Total ash content was determined in accordance with the AOAC procedure (2005, no. 920.05) by incinerating the material at 485°C, and dietary fiber content in accordance with the AOAC procedure (2005, no. 991.43) enzymatic fermentation and drying the remnants at 105 °C. Total carbohydrates content and the energy value of the rations were determined in accordance with the guidelines of FAO (2003).

The vitamin C content in food rations was tested via the HPLC method according to EN 14130 (2003). The analysis was performed on a Thermo Scientific DIONEX ULTIMATE 3000 UPLC chromatograph with a DAD detector. The extract was injected onto an Onyx Monolithic C 18 column (100 x 4.6 mm). Elution was carried out using 0.1 M metaphoric acid at a flow rate of 1 ml/min. Absorbance measurement was carried out at a wavelength of $\lambda = 254$ nm. The sum of L-ascorbic acid and dehydroascorbic acid was determined after reduction with L-cysteine according to EN 14130 (2003). To determine antioxidant properties, 80% methanol extracts were made from lyophilized food rations using sonification.

The total polyphenol content was determined using the method described by SINGLETON *et al.* (1999). The appropriate amount of extracts from lyophilized food rations was collected and a reaction was carried out with the Folin-Ciocalteu reagent in the presence of Na_2CO_3 . After 60 minutes, absorbance was read on a Hitachi UV-VIS spectrometer, type U-2900 (Hitachi, Japan) compared to the blind sample at $\lambda = 675$ nm. The results were read on the basis of a standard curve prepared for gallic acid.

Antioxidant activity against DPPH radical (1,1-diphenyl-2-picrylhydrazole) was determined using the method described by PEKKARINEN *et al.* (1999). Extracts from lyophilized food rations were mixed with a DPPH radical solution, and after 10 minutes of reaction, the absorbance was measured on a Hitachi UV-VIS U-2900 (Hitachi, Japan) UV-VIS spectrophotometer at a wavelength of $\lambda = 516$ nm. The percentage of radical scavenging level (% RSA) was determined by referring the absorbance of extracts from lyophilized food rations to the absorbance of the blind sample. The value of antioxidant activity against DPPH radical is expressed in Trolox millimoles (water-soluble α -tocopherol analogue – 2-carboxyl-6-hydroxyl-2,5,7,8-tetramethylchromate).

Antioxidant activity using the FRAP method was determined according to the procedure described by BENZIE and STRAIN (1996). Extracts from lyophilized food rations were mixed with a TPTZ solution (2,4,6-Tripyridyl-S-triazine) and FeCl_3 in an acetate buffer. After 10 minutes of incubation at 37°C, absorbance was measured on a Hitachi UV-VIS U-2900 (Hitachi, Japan) UV-VIS spectrophotometer at a wavelength of $\lambda = 595$ nm against a blind sample. The value of antioxidant activity determined by the FRAP method was expressed in millimoles of Fe^{2+} ions.

3. RESULTS

The analyses showed that an average food ration prepared for women contained: 2,120.1 kcal, 90.8 g protein, 53.1 g fat and 354.0 g carbohydrates, and for men: 2,648.8 kcal, 112.5 g protein, 63.1 g fat and 447.4 g carbohydrates (Tables 5 and 6).

Table 5. Nutritional value of food rations planned for women (M±SD).

	W1	W2	W3	W4	W5	W6	W7	W1-W7
Energy value (kcal)	2066.0	2228.8	1969.8	2121.2	2135.0	2117.2	2203.0	2120.1±86.1
Protein (g)	86.8	88.5	84.7	93.3	92.0	95.3	94.9	90.8±4.1
Fat (g)	51.1	58.4	44.4	52.9	52.9	54.0	58.0	53.1±4.7
SFAs (g)	12.1	18.0	12.6	13.5	14.6	13.5	19.2	14.8±2.7
MUFAs (g)	27.5	29.1	22.6	27.6	28.1	28.9	27.2	27.3±2.2
PUFAs (g)	11.4	11.3	9.1	11.8	10.1	11.7	11.6	11±1.0
Carbohydrates (g)	346.6	380.9	336.7	349.3	351.4	349.2	363.8	354±14.3
Dietary fiber (g)	31.9	43.7	28.8	31.3	28.6	36.8	38.5	34.2±5.6

Table 6. Nutritional value of food rations planned for men (M±SD).

	M1	M2	M3	M4	M5	M6	M7	M1-M7
Energy value (kcal)	2585.7	2754.9	2578.9	2683.6	2714.8	2667.2	2556.2	2648.8±76.0
Protein (g)	116.7	114.4	114.5	100.4	110.7	112.5	118.7	112.5±6.0
Fat (g)	65.5	68.7	62.0	60.7	63.7	61.4	59.7	63.1±3.1
SFAs (g)	15.2	18.4	15.6	15.1	21.0	19.4	21.2	18±2.7
MUFAs (g)	34.9	38.2	32.7	32.9	28.0	29.5	30.1	32.3±3.5
PUFAs (g)	15.4	12.1	13.7	12.7	14.6	12.5	8.3	12.8±2.3
Carbohydrates (g)	420.7	460.0	426.9	484.3	466.5	452.9	420.4	447.4±25.1
Dietary fiber (g)	38.3	40.2	36.2	50.4	41.7	36.9	34.4	39.7±5.3

W1/M1 - first daily food serving, W2/M2 - second daily food serving, W3/M3 - third daily food serving, W4/M4 - fourth daily food serving, W5/M5 - fifth daily food serving, W6/M6 - sixth daily food serving, W7/M7 - seventh daily food serving

The chemical analyses showed that 100 g fresh mass of an average food ration prepared for women contained: 5.5±2.6 mg vitamin C and 20.1±4.1 mg polyphenols, and for men, respectively: 5.6±1.4 mg and 22.9±8.1 mg. The antioxidant properties of an average ration for women expressed as reducing power (FRAP index) in 100 g fresh mass was found to be 8.2±0.7 mmol Fe²⁺, and for men 8.9±0.9 mmol Fe²⁺. The antioxidant properties of an average ration developed for women and men expressed as antiradical activity against DPPH in 100 g fresh mass was respectively: 2.7±0.2 mmol and 2.7±0.4 mmol Trolox equivalent. The respective values for dry mass were higher (Tables 7 and 8).

Table 7. Vitamin C and polyphenol content as well as antioxidant potential of food rations planned for women (per 100 g fresh and dry mass) (M±SD).

Evaluated indices		W1	W2	W3	W4	W5	W6	W7	W1-W7
100g fm	Vitamin C (mg)	5.0±0.3	3.5±0.2	5.3±0.2	3.6±0.2	5.0±0.2	11.1±0.4	5.0±0.2	5.5±2.6
	Polyphenols as gallic acid (mg)	16.4±5.4	23.1±3.4	23.2±2.0	21.2±5.6	12.60.9	21.6±1.2	22.9±0.8	20.1±4.1
	Reducing activity (the FRAP method) (mmol Fe ⁺²)	7.5±0.4	9.2±0.4	7.6±0.4	7.9±0.2	7.9±0.8	7.9±0.4	9.1±0.8	8.2±0.7
	Antioxidant activity against DPPH (mmol Te – Trolox equivalent)	2.3±0.6	2.8±0.2	2.8±0.1	3.0±0.4	2.7±0.2	2.8±0.2	2.8±0.3	2.7±0.2
100g dm	Vitamin C (mg)	21.0±1.0	13.2±0.6	24.3±1.2	14.3±0.7	19.8±0.9	43.3±2.1	19.6±0.9	22.2±10.1
	Polyphenols (mg) as gallic acid	68.3±21.5	86.1±13.9	106.8±9.3	84.0±21.9	49.6±4.2	83.9±6.5	88.9±2.8	81.1±17.9
	Reducing activity (the FRAP method) (mmol Fe ⁺²)	31.3±1.1	34.4±1.4	35.1±2.1	31.6±0.6	31.1±3.8	31.0±1.8	35.5±3.7	32.9±2.0
	Antioxidant activity against DPPH (mmol Te – Trolox equivalent)	9.4±2.3	10.5±0.6	12.9±0.4	12.0±1.5	10.5±0.8	10.9±0.8	10.9±1.1	11.0±1.1
	Antioxidant activity against DPPH (%RSA for extract: 20 mg lyophilized food ration/g)	13.1±3.1	14.4±0.9	17.8±0.5	16.6±2.0	14.5±1.2	15.0±1.1	15.1±1.5	15.2±1.6

Table 8. Vitamin C and polyphenol content as well as antioxidant potential of food rations planned for men (per 100 g fresh and dry mass) (M±SD).

Evaluated indices		M1	M2	M3	M4	M5	M6	M7	M1-M7
100g fm	Vitamin C (mg)	4.9±0.3	5.9±0.3	4.2±0.3	4.8±0.3	5.1±0.3	8.6±0.5	5.8±0.3	5.6±1.4
	Polyphenols (mg) as gallic acid	26.2±1.0	19.4±0.8	20.1±0.2	18.7±1.3	16.9±2.8	19.4±1.7	40.2±6.2	22.9±8.1
	Reducing activity (the FRAP method) (mmol Fe ⁺²)	9.1±0.1	7.4±0.2	8.5±0.4	8.2±0.4	9.0±0.5	9.6±0.7	10.4±0.2	8.9±0.9
	Antioxidant activity against DPPH (mmol Te – Trolox equivalent)	2.8±0.1	2.4±0.2	2.5±0.2	2.6±0.1	2.8±0.9	2.3±1.2	3.6±0.3	2.7±0.4

100g dm	Vitamin C (mg)	18.4±1.2	22.8±1.4	16.4±1.0	20.2±1.3	19.8±1.2	33.1±2.1	21.8±1.4	21.8±5.4
	Polyphenols (mg) as gallic acid	97.7±3.4	74.8±3.5	78.8±1.9	78.8±5.7	65.2±11.1	75.1±8.2	151.4±2 5.2	88.8±29.2
	Reducing activity (the FRAP method) (mmol Fe ⁺²)	33.8±0.3	28.4±0.7	33.3±1.2	34.8±1.8	34.7±2.1	37.2±2.8	39.1±1.1	34.5±3.4
	Antioxidant activity against DPPH (mmol Te – Trolox equivalent)	10.3±0.1	9.4±0.8	9.7±0.9	11.0±0.5	10.8±3.8	8.9±4.7	13.6±1.5	10.5±1.6
	Antioxidant activity against DPPH (%RSA for extract: 20 mg lyophilized food ration/g)	14.2±0.2	13.0±1.0	13.4±1.3	15.2±0.6	15.0±5.2	12.3±6.4	18.8±2.1	14.6±2.1

4. DISCUSSION

Different groups of sports disciplines have different dietary requirements, connected with the kind of exercise and the dominant energy pathways, development of specific motor properties and the rigor of maintaining body mass and composition. One of special groups is athletes who train disciplines that require maintaining low body mass and low fat content, which is connected with planning a balanced diet with relatively lower energy intake but high nutrient density (rich in i.a., vegetables, fruit, wholegrain cereal products, legumes, fish and nuts) (THOMAS *et al.*, 2016).

The presented original study showed that food rations prepared for athletes (women and men) of disciplines that require maintaining low body mass, in accordance with qualitative and quantitative recommendations, balanced in terms of energy and basic nutrients intake, rich in fruit and vegetables and other products with high nutrient value and with high content of dietary antioxidants, including vitamin C and polyphenols, ensuring high antioxidant properties (with the energy intake of 2,120 kcal in female and 2,649 kcal in male athletes). High vitamin C and polyphenol content results from including in the prepared food rations an appropriate number of portions of products being natural sources of dietary antioxidants (i.a., fruit and/or vegetables in each meal).

The mean content of vitamin C found in the prepared food rations, i.e., 5.5 mg/100 g fresh mass (women) and 5.6 mg/100 g fresh mass (men), with the assumption of their average weight of 2,090.42 g for women and 2,519.07 g for men, corresponds to vitamin C content of 114.97 mg (women) and 141.07 mg (men). Comparing the content of vitamin C in an average (within a week) food ration to the norms of physiological demand for vitamin C (RDA: 75 mg/day for women and 90 mg/day for men) (KREIDER *et al.*, 2010), additionally increased in athletes (POTGIETER, 2013), shows that this diet can ensure its normative intake and the demand can be covered, also in the conditions of physical exercise. The described mean content of vitamin C in an average food ration corresponds to satisfying the RDA demand of 153.29% (in women) and 156.74% (in men). High vitamin C content in the prepared food rations results from high vitamin C content in products and dishes included in them, such as raw fruit and vegetables (KUNACHOWICZ *et al.*, 2005). Extremely high content of vitamin C occurs in red peppers, but it is also high in tomatoes and lettuce (KUNACHOWICZ *et al.*, 2005), which are the ingredients of many salads and (cream) soups planned in the analyzed food rations for athletes.

The total content of polyphenols in the analyzed food rations is also the product of their high content in the ingredients. The mean content of polyphenols found in the prepared food rations, i.e., 20.1 mg/100 g fresh mass (women) and 22.9 mg/100 g fresh mass (men), with the assumption of their average weight of 2,090.42 g for women and 2,519.07 g for men, corresponds to polyphenol content of 420.17 mg (women) and 576.87 mg (men). The recommended intake of polyphenols, promoting good functioning of the body, is estimated at 250-500 mg/day (SIKORA *et al.*, 2008). An average food ration, supplying this amount of polyphenols, covers the recommended intake. A study aimed to estimate the intake of phenolic compounds with the diet showed that a statistical Pole consumes approx. 440 mg polyphenols/day, and important sources of these antioxidants are vegetables (31%) and fruit (23%) (SIKORA *et al.*, 2008). It is estimated that Western societies consume on average 50-800 mg, and Eastern up to 2 g flavonoids/day. An average Mediterranean diet provides 100-1000 mg flavonoids/day (SIKORA *et al.*, 2008). In the Czech population the average intake of plant polyphenols was 426.6 mg/day (less than in other European as well as non-European, countries, including Spain, France, Ireland, Brazil etc.) (ZLOCH *et al.*, 2018). A population of elderly Japanese (mostly men) consumed 1492 mg/day of polyphenols on average, and coffee and green tea were the largest sources of polyphenols in their daily life (TAGUCHI *et al.*, 2015). Total polyphenol content in fruit and vegetables varies. In vegetables the content is the highest in: broccoli (290 mg/100 g), carrots (156 mg/100 g) and onions (150 mg/100 g) (CIEŚLIK *et al.*, 2006). In apples, peaches and mandarines the content of extracted polyphenols was between 18.8 and 28.0 mg/100 g fresh fruit (ARRANZ *et al.*, 2009). Out of the vegetables used to prepare the dishes in the analyzed food rations, the richest in polyphenols are red peppers (68.50 mg/100 g), onions (45.81 mg/100 g) and garlic (36.10 mg/100 g) (CIEŚLIK *et al.*, 2006). Foods' antioxidant properties are correlated with the content of substances with antioxidant properties, including vitamin C and polyphenols. The antioxidant potential of an average food ration determined in this study, expressed as reducing power (FRAP index) was: 8.2 mmol Fe²⁺/100 g ration fresh mass (for women) and 8.9 mmol Fe²⁺/100 g ration (for men), and expressed as antiradical activity against DPPH was: 2.7 mmol/100 g ration fresh mass (for women and men). Antioxidant properties of food rations prepared for athletes is the product of content of bioactive substances with antioxidant properties and antioxidant potential of individual products/dishes and results from including the recommended number of portions of fruit and vegetables with high antioxidant activity, including honey and wholegrain cereal products. It is worth pointing out that antioxidant capacity of vegetables is usually lower than that of fruit, especially berries, and higher than that of cereal products. Antioxidant activity of fruit varies from 1.02 (pears) to 3.91 mmol/100 g (strawberries), and of vegetables, mushrooms and legumes from 0.27 (cucumbers) to 6.91 mmol/100 g (beans). High potential (>2 mmol/100 g) also occurs in peas, dill, dock, red cabbage and beetroot. Especially high antioxidant potential expressed as DPPH scavenging activity has been described for Brussels sprouts and red peppers (EC₅₀ 7.8 mg and 11.9 mg, respectively) (CIEŚLIK *et al.*, 2006; SIKORA *et al.*, 2008). Significant antioxidant properties of fruit and vegetable snacks have also been reported (GRAMZA-MICHAŁOWSKA and CZŁAPKA-MATYASIK, 2011). With reference to antioxidant properties of wholegrain cereal products, studies have shown that the antioxidant potential of boiled wholegrain pasta (expressed as FRAP index) is from 3.26±0.08 to 19.52±1.28 μmol/g dry mass (DURAZZO *et al.*, 2014). Another study concerning dishes preferred by athletes showed high antioxidant properties of chicken salad (0.29 mmol/100 g) and tomato spaghetti (0.35 mmol/100 g) (GACEK *et al.*, 2012).

Antioxidant activity of the prepared and analyzed food rations, being the product of content of antioxidant substances, including vitamin C and polyphenols, proves they are useful in a rational diet, also for people engaging in intensive physical exercise, who need more antioxidants. Exogenous antioxidants have an impact on the total antioxidant capacity and physical fitness in athletes (MORILLAS-RUIZ *et al.*, 2006; SCHNEIDER *et al.*, 2018), so an important aspect of a rational diet is the appropriate intake of vegetable bioactive substances. Research has confirmed the importance of diet with high antioxidant properties for the restoration of athletes' antioxidant status. In this respect, it has been shown that antioxidant-rich diet improved the redox status of triathlon athletes (Schneider *et al.*, 2018), and high consumption of flavones from cocoa improved the total antioxidant capacity during workout and regeneration in professional cyclists (DECROIX *et al.*, 2017). It has been confirmed beyond doubt that satisfying higher nutritional demand, also in terms of vitamin C and other antioxidants, promotes the implementation of dietary strategies connected with muscle regeneration, glycogen replenishment, preventing fatigue, the improvement of immunity and preparation for further training and contests (HEATON *et al.*, 2017; MYBURGH, 2014). It is recommended to apply dietary strategies that improve diet antioxidant potential (so-called antioxidant food ration) (YAVARI *et al.*, 2015). When discussing the importance of antioxidants in the diet of physically active people, we may refer to a study that showed a positive influence of 6-week Nordic walking training on the improvement of blood antioxidant protection system in women over age 55 (CEBULA *et al.*, 2017).

Due to some cases of nutritional deficiencies and because of the importance of antioxidants for increasing antioxidant activity and protecting skeletal muscles from oxidation damage caused by physical exercise, researchers attempt to study the supplementation of athletes' diet with antioxidant substances, including melatonin (LEONARDO-MENDONCA *et al.*, 2017), coenzyme Q (ORLANDO *et al.*, 2018), vitamin C (YAVARI *et al.*, 2015), and L-carnitine (SUNG *et al.*, 2016) and whey protein (XU *et al.*, 2011). Some studies have also shown the risk of negative impact of supplementing high doses of vitamin C (1g/day) and E (≥ 260 IU/day) on disorders in skeletal muscles adjusted to long training sessions (MASON *et al.*, 2016). Functional drinks based on almonds and olive oil enriched with α -tocopherol and docosahexaenoic acid (DHA) can also be used to modulate oxidative stress and improve effort tolerance of athletes. They also help improve blood polyphenol concentration in older athletes and increase the expression of antioxidant enzyme genes in peripheral blood cells after exercise in young athletes (CAPO *et al.*, 2016). Supplementation with purple grape juice displayed ergogenic activity (by delaying exhaustion) and led to increasing antioxidant activity and decreasing the concentration of inflammation markers in recreational runners (TOSCANO *et al.*, 2015). Literature also includes other examples of supplementing athletes' diet with antioxidant substances (YAVARI *et al.*, 2015).

The presented results can be useful in planning diet and dietary strategies improving the antioxidant properties of the diet of people with high physical activity and increased nutritional needs. Regular consumption of fresh fruit and vegetables, whole grains, legumes and beans, sprouts and seeds is an effective and safe way to cover the antioxidant needs of physically active people.

5. CONCLUSIONS

Balanced food rations prepared for athletes (with the mean energy value of approx. 2,120 kcal for women and approx. 2,649 kcal for men included the normative amount of vitamin C (114.97 mg – women and 141.07 mg – men) and polyphenols (420.17 mg – women and 576.87 mg – men). Balanced food rations for athletes, including fruit and/or vegetables in each meal and the normative amount of vitamin C and polyphenols, have high antioxidant properties. Preparing balanced food rations for athletes, providing not only appropriate amounts of energy and macroelements but also bioactive substances (vitamin C and polyphenols) as well as high antioxidant properties is possible, though not easy. Chemical analyses confirmed the nutritional value of food rations planned on the basis of tables of products' and dishes' nutritional value.

RESERCH FUNDING

The study was supported from the European Union funds within the framework of the European Social Fund "Doctus - Lesser Poland Fund for Grants for PhD Students".

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Paper Received January 30, 2018 Accepted April 15, 2019

EFFECTS OF THERMOSONICATION ON WATERMELON RIND-HONEY BEVERAGE

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ABSTRACT

This study addressed the recent interest in utilizing waste as source of natural food product to build a safer environment. The sample used was watermelon rind, a by-product of an edible fruit, often ignored for its bland taste. Thermosonication is a method of treating food beverage to improve product quality, but its effects, specifically on rind beverage has not been extensively studied. This study determined the effects of temperature and time (optimization using Response Surface Methodology) on the physicochemical, vitamin C content and microbial load of rind beverage stored under different temperatures for a week. Thermosonication at 65 °C for 60 min significantly affected juice separation (stored under 4 °C in a week) (11.3%), TSS (12.9 °Brix), the total color difference value (1.7) and microbial load (6.2 log CFU/mL). Themsonicated rind juice can be stored longer at temperature below 4°C, which is beneficial to both the consumers and the country at large.

Keywords: physicochemical, storage, thermosonication, vitamin C, waste, watermelon rind

1. INTRODUCTION

The evaluation on fruit waste or fruit by-product has become a subject of interest in the food industry. The aim is to promote the use of natural waste in food application for a safer cause and a cleaner environment. Fruits such as watermelon, banana and papaya are examples of functional food with high fiber and nutrition value. Their intake helps to reduce the risk of cardiovascular disease such as coronary heart disease and stroke (WU *et al.*, 2015). Previous studies have reported that nutrient contents are higher in fruit peels and seeds than in the fruit pulp (MORAIS *et al.*, 2015; MOO-HUCHIN *et al.*, 2015; SANTOS *et al.*, 2014).

The unappealing taste of watermelon rind has been the main reason for discard despite its edibility and health-promoting content (AL-SAYED and AHMED, 2013). Recent studies claimed watermelon rind act as a good thickening, foaming and emulsifying agent, as it contains pectin and large quantity of antihypertensive and antioxidant properties due to its polysaccharides content (PETKOWITCZ *et al.*, 2017; ROMDHANE *et al.*, 2017). Watermelon rind was also used as wheat flour substitute in cake, pan bread, cookies and yellow noodles (AL-SAYED and AHMED, 2013; EL-BADRY *et al.*, 2014; NAKNAEN *et al.*, 2016; HO and DARCI, 2016). A mixture of 5% flour and 10% fat with watermelon rind powder slowed down staling and inhibited lipid oxidation and free fatty acids formation during storage (AL-SAYED and AHMED, 2013). The substitution had positive effects on human health including antioxidant activity that scavenges free-radicals (LEONG and SHUI, 2002; LEWINSOHN *et al.*, 2005), converts citrulline to arginine for boosting the immune system, circulatory system and heart, as well as relaxing blood vessels in cases of cardiovascular diseases and cancer (RIMANDO and PERKINS-VEAZIE, 2005). However, research on the application of watermelon rind as juice beverage seems limited.

Honey was chosen as sweetener for the rind juice because it has better antibacterial and medicinal properties compared to sugar (MANDAL and MANDAL, 2011) as well as its common use as preservative (BOGDANOV *et al.*, 2008). A milk sample that contains honey lasted longer and inhibited bacterial growth better than the sample without honey at 4°C storage (KRUSHNA *et al.*, 2006). The efficient antibacterial activity of honey in food was due to its hydrogen peroxide and polyphenol contents (WHITE, 1978; SNOWDON and CLIVER, 1996). Besides, high sugar-sweetened beverage intake contributes to heart complication, rise in blood pressure, weight-gain and cavities (CORLISS, 2016).

In beverage production, heat treatment is applied to maintain food stability and sensory quality. Pasteurization is the conventional method used to extend the shelf-life of fruit juices by applying heat that kills or inactivates certain enzymes and microorganisms (yeasts, molds and bacteria), which contribute to the juice's spoilage (POLYDERA *et al.*, 2003). However, the heating effect of pasteurization was found to detract the natural quality of fruit juices, resulting in flavor loss and other changes. The thermal application also decreases the product's physicochemical and nutritional values like vitamin C and E contents, polyphenols, pH and color (DUBROVIC *et al.*, 2011; GINER *et al.*, 2013; SANTHIRASEGARAM *et al.*, 2013). A study of the pasteurization effect on physicochemical properties of *Physalis* (*Physalis peruviana* L.) juice reported that pasteurization at 90°C for 2 min significantly improved the juice's organoleptic characteristics and reduced the ascorbic acid content from 38.90 to 30.20mg/100g during storage. The ascorbic acid content of the pasteurized juice was lower than the ascorbic acid content preserved in the fresh *Physalis* juice during storage (RABIE *et al.*, 2015). Thus, alternative technologies such as ultrasound and high hydrostatic pressure were developed to reduce the effects on product quality. Ultrasound is sound waves having frequency that

exceeds 20 kHz, which can affect the physical, mechanical and chemical properties of food (AWAD *et al.*, 2012). The application of ultrasound in food processing and food preservation can improve the food texture and flavor by enhancing heat and mass transfer processes. The treatment also serves as assistance to the existing thermal treatments to compensate for the loss of nutritional values caused by heat (KNORR *et al.*, 2011). Previously, low power ultrasound had been used in treating vegetables and fruits in pre- and postharvest applications (AWAD *et al.*, 2012).

Ultrasound treatment alone gives low germicidal effect since the process greatly depends on microorganisms' type, processing parameters and sonication medium in microbial destruction (CHENG *et al.*, 2007). The combination of ultrasound and moderate heating, known as thermosonication, inactivates heat-resistant enzymes and kills microorganisms at lower temperature within a shorter period (ABDULLAH and CHIN, 2014; ABID *et al.*, 2014). A study of the thermosonication effect on tomato showed that the treatment was effective in inactivating the enzyme pectin methylesterase, which degrades pectin and reduces the viscosity of tomato juice (ESKIN, 1979). There are also studies, which reported that thermosonication effectively inactivated various enzymes including polyphenoloxidase (CHENG *et al.*, 2007), peroxidase (ERCAN and SOYSAL, 2010; GAMBOA *et al.*, 2012) and polygalacturonase (TEREFE *et al.*, 2009). Instead of relying on sound waves alone to initiate bubble cavitation, thermosonication involves both sound waves and temperature, where the temperature is controlled to produce maximum cavitation bubbles for juice extraction (PATIST and BATES, 2008; HOLTUNG *et al.*, 2011). Thus, more polyphenols can be pertained (ABID *et al.*, 2014). Thermosonication treatment also increases microbial inactivation rate in fruit juice. The killing mechanism involved is caused by thinning of cell membranes, increasing localized heat and pressure as well as increasing the production of free radicals. The treatment with combination of heat and ultrasound resulted in extensive cell damage and breakage on *E. coli* K12 cells (LEE *et al.*, 2009). Examples of fruit and vegetable juices that have been studied using combined treatment include Kasturi, lime juice (BHAT *et al.*, 2011), apple juice (ABID *et al.*, 2013), soursop juice (DIAS *et al.*, 2015), grape juice (AADIL *et al.*, 2015) and carrot juice (ZOU and JIANG, 2016). They produced better results than the juice subjected to only heat treatment. Although, several studies have been done on the effect of ultrasound on fruit juices, none has been done on the optimization of thermosonication condition on watermelon rind-honey beverage.

The application of thermosonication treatment with a short processing time in juice preparation has categorized it as requiring minimal processing for freshness and health purposes. However, the study on thermosonication in improving the quality of fruit waste juice beverage is yet to be extensively conducted. The objective of this study is to determine the effect of different thermosonication condition (temperature and time) on watermelon rind beverage containing honey by evaluating the physicochemical, vitamin C content and microbiological properties of the beverage stored under different conditions for a week.

2. MATERIALS AND METHODS

2.1. Watermelon sample and honey

Approximately 2.5 kg of red seedless watermelon kept as a whole fruit at room temperature and honey were purchased from the local market in Sri Serdang, Selangor.

Both watermelon and honey were stored in a chiller of 4°C for 2 days and a week, respectively, before analysis.

2.2. Proximate analysis of raw watermelon rind

The proximate analysis was carried out on seeded red watermelon rind (SRWR) and seedless red watermelon rind (SLRWR) to determine which watermelon rind is more suitable in terms of high fiber content for the development of watermelon rind beverage containing honey. The proximate compositions include, crude fiber, crude fat, and ash content, crude protein, carbohydrate and moisture content (AOAC, 2006).

2.3. Preliminary experiment

A preliminary experiment was done to determine the acceptable sweetness of honey in watermelon rind (v/v %). A 12 mL honey was mixed in 100 mL watermelon rind juice to get a 12% watermelon rind- honey juice. A range of 12% to 17% of honey to watermelon juice mixture was prepared and distributed to untrained panelists. The result showed that more than 80% of participants selected 13% (v/v) as the most acceptable sweetness.

2.4. Preparation of watermelon rind juice

The watermelon was separated into respective parts of rind, flesh and skin using a stainless-steel knife and the rind was further cut into cubes. The rind was cleaned and washed before being put into the juice blender MIX-898M (Panasonic, Japan). After blending, the rind puree was obtained. The rind puree was then transferred to a cloth sieve and squeezed to obtain the juice. For control sample, 100 mL of watermelon rind juice was weighed using a weighing balance (Scaltec, UK) and was transferred into glass bottles with metal caps sterilized in boiling water (100°C). Such method was used for the control sample since watermelon rind juice is consumed without any heat treatment and there has been no prior study on the development of watermelon rind juice. As for sample undergoing treatment, watermelon rind juice and honey were weighed before honey was mixed manually into watermelon rind juice using a spoon with 13% (v/v) in 100 mL watermelon rind juice. Once completely mixed, the juice was transferred into sterilized glass bottles sealed with metal caps prior to thermosonication treatment. Analysis was conducted for a week but signs of mold growth was visible on the surface of the control sample on day 4 of storage.

2.5. Thermosonication process

Thermosonication treatment was applied at three different temperatures, 25, 45 and 65°C for 10, 35 and 60 min respectively, using an ultrasonic cleaner bath (DC150H; Delta, China). Thermosonication treatment is regarded as a better alternative method because it has better nutrient retention capacity than the conventional method (pasteurization at 71 to 82 °C) of treating orange and lime juice without altering or degrading the nutritional contents of the beverages (KHANDPUR and GOGATE, 2016).

The ultrasonic cleaner bath is a rectangular container (300 × 160 × 150 mm) with the maximal tank capacity of 7.5 L with frequency of 40 kHz and power W. These parameters 150 were chosen based on the study of thermosonication of grapefruit juice (AADIL *et al.*,

2015), which also used ultrasonic cleaner bath to carry out ultrasonic treatment. Six liters of distilled water was poured into the bath as transmission medium.

The ultrasound treatment sends acoustic waves that creates bubbles, whereby these bubbles induce either stable cavitation or transient cavitation phenomena (CHOWDHURY and VIRARAGHAVAN, 2009; THANGAVADIVEL *et al*, 2012). Watermelon rind beverage was kept sterilized glass bottles with metal caps at room temperature (25°C) and chill temperature (4°C) for further analysis.

2.6. Experimental design

Response Surface Methodology (RSM) was used to determine the effect of two independent variables; temperature (25 to 65°C) and duration (10 to 60 min) of ultrasound. These variables were chosen based on parameters of ultrasound on fruit and vegetable juices (BHAT *et al.*, 2011; AADIL *et al.*, 2015; ZOU and JIANG, 2016). In this experiment, 14 runs (Table 2) were generated from the central composite design (CCD) with two independent variables, each with three levels of low, center and high (Table 1).

Table 1. Level of independent variable using CCD.

Independent variable	Independent variable level		
	Low (-1)	Centre (0)	High (+1)
Temperature (°C)	25	45	65
Time (min)	10	35	60

Table 2. Generated experimental runs with variable combination obtained from CCD.

Experimental run	Blocks	Independent variable	
		Temperature (°C)	Time (min)
1	1	25	10
2*	1	45	35
3	1	65	60
4*	1	45	35
5	1	65	10
6*	1	45	35
7	1	25	60
8	2	45	10
9	2	25	35
10*	2	45	35
11	2	45	60
12*	2	45	35
13	2	65	35
14*	2	45	35

*Centre point.

2.7. pH

The rind beverage pH was determined using calibrated pH meter model Jenwah 305, (Keison, UK). Approximately 50 mL of beverage was placed in a 200 mL beaker and stirred continuously while inserting the meter rod into the beaker (AOAC, 2006). The pH value was taken and comparison was done between day 1, 4 and 7 of storage.

2.8. Separation (%)

The rind beverage separation was determined by thoroughly mixing the beverage and then transferring 100 mL of beverage into 100 mL graduated measuring cylinder. It was left to stand for 30 min at room temperature. Then, the volume of the top clear beverage serum was recorded by reading the level of measuring cylinder. The readings of separation were recorded and comparison was done between day 1, 4 and 7 of storage. The determination of separation was done using FoodTech Method 17 (FMC, 2005).

2.9. Total soluble solid (°Brix)

The total soluble solid of watermelon beverage was evaluated using handheld refractometer (0-32°Brix). A drop of watermelon beverage was placed and spread on the refractometer window and the °Brix value was determined. The total soluble solid values were taken and compared between day 1, and 7 of storage. The determination of total soluble solid was done according to AOAC (2006).

2.10. Color

The color of watermelon beverage containing honey was determined using Hunter Lab UltraScan PRO colorimeter (Hunter Associate Laboratory, International Commission, Reston, USA) with EasyMatch QC software. The measurement L^* (lightness), a^* (redness) and b^* (yellowness) color scale was taken with Regular Transmission (RTRAN) mode. The samples were placed in a transparent rectangular transmission cell unit until full to avoid air space inside the transmission cell that will interfere with the reading of color measurement. Analysis was done in triplicates to obtain accurate data analysis (PATHARE *et al.*, 2013; ASSAWARACHAN and NOOMHORN, 2010). Total color difference (TCD) value was also calculated using the following equation:

$$\sqrt{(L^*-L_o^*)^2 + (a^*-a_o^*)^2 + (b^*-b_o^*)^2} \quad (1)$$

where;

$L^* = L^*$ for sample

$L_o^* = L^*$ for control

$a^* = a^*$ for sample

$a_o^* = a^*$ for control

$b^* = b^*$ for sample

$b_o^* = b^*$ for control

2.11. Vitamin C content (ascorbic acid)

The analysis was conducted to estimate and compare the content of ascorbic acid between day 1, 4 and 7 of storage using the AOAC International method 967.21 (HORWITZ and LATIMER, 2006). Metaphosphoric acid-acetic acid solution was prepared by adding 100 mL deionized distilled water with 20 mL of acetic acid (Emsure, Germany). Then, 7.5 g of metaphosphoric acid (Fisher Scientific, UK) was added and stirred. Mixture was diluted to 250 mL with distilled water.

The mixture was filtered into an amber bottle with lid using a filter paper and refrigerated until further use. Ascorbic acid standard solution was prepared by weighing approximately 50 mg of ascorbic acid (Emsure, China). Thereafter, it was diluted to 50 mL with prepared metaphosphoric acid-acetic acid immediately before use.

In preparing indophenol solution-dye, 50 mL deionized water and 42 mg sodium carbonate were added in a 150 mL beaker and stirred. Then, 50 mg 2,6-dichloroindophenol sodium salt (Camlab, UK) was added. Mixture was diluted to 200 mL with deionized distilled water. The mixture was filtered into an amber bottle with lid and was refrigerated until further use. Two milliliters of standard ascorbic acid solution in 5 mL metaphosphoric acid-acetic acid solution was titrated against the dye solution until a light yet distinct rose pink color was obtained. The initial and final burette reading was recorded.

Five milliliters metaphosphoric acid-acetic acid solution and 2 mL of watermelon rind beverage was pipetted into 50 mL Erlenmeyer flask and it was titrated against the dye solution until a light yet distinct rose pink color was obtained. The initial and final burette reading was recorded. The amount of ascorbic acid was estimated using the following equation:

$$\text{mg of ascorbic acid / g or mL of sample} = (X-B) \times (F/E) \times (V/Y) \quad (2)$$

where;

X = average mL for test solution titration

B = average mL for test blank titration

F = mg ascorbic acid equivalents to 1.0 mL indophenol standard solution

E = sample weight (g) or volume (mL)

V = volume of initial test solution

Y = volume of test solution titrated

2.12. Microbiological analysis

Total plate count, yeast and mold count were determined to compare results between day 1, 4 and 7 of storage using the spread plate method. For total plate count, Plate Count Agar (PCA) was used. For yeast and mold count, Potato Dextrose Agar (PDA) was used. A series of dilutions (10^{-1} to 10^{-5}) were made from watermelon rind beverage sample in 0.1% peptone water. Then, 0.1 mL of dilution from the appropriate desired dilution series (10^{-3} to 10^{-5}) were pipetted onto the center of the agar plate surface. L-shaped glass spreader or hockey stick was dipped into alcohol and flamed over a Bunsen burner. The plates were incubated at 37°C for 24 h. The log CFU/mL value of the sample was calculated using Eq. (A.3). The determination of PCA and PDA was achieved 21 using FDA's standard method of Bacteriological Analytical Manual (FDA, 2001).

$$\text{CFU/ml} = \log [(\text{no. of colonies} \times \text{dilution factor of plate}) / \text{aliquot plated}] \quad (3)$$

3. RESULTS AND DISCUSSION

3.1. Proximate composition of SRWR and SLRWR

Researches on nutritional content of watermelon flesh have been done widely (SABEETHA *et al.*, 2017; NONGA *et al.*, 2014; TLILI *et al.*, 2011; YAU *et al.*, 2010), however, very few research has been done on the watermelon rind. The knowledge of nutritional content of watermelon rind can reassure consumers to accept the rind as a potential food product instead of it being considered as waste (FILA *et al.*, 2013). The proximate analysis for SRWR and SLRWR was conducted to investigate which type of rind is more suitable to be applied in this study. Table 3 shows the result of proximate analysis for SRWR and SLRWR.

Table 3. Proximate analysis of SRWR and SLRWR.

Proximate composition (%)	Red watermelon rind	
	Seeded (SRWR)	Seedless (SLRWR)
Moisture	90.69a±1.31	87.42b±0.49
Ash	5.03a±0.78	5.00a±0.40
Crude protein	0.39a±0.09	0.57a±0.10
Crude fat	0.49a±0.29	0.70a±0.26
Crude fiber	2.10b±0.76	4.48a±0.65
Carbohydrate	1.30a±0.31	1.83a±0.50

Data are mean±SD of three replicates. Values with the same letter within a row is not significantly different at 5% level ($p < 0.05$).

As shown in Table 3, there were no significant difference in ash, crude protein, crude fat and carbohydrate between SRWR and SLRWR. However, the moisture content in SRWR (90.69±1.31) was significantly higher than in SLRWR (87.42±0.49). Also, the crude fiber content in SLRWR (4.48±0.65) was significantly higher than in SRWR (2.10±0.76). Soluble fiber intake from juice makes it easier for absorption of vitamins, minerals and important phytonutrients (RUIZ-GUTIÉRREZ *et al.*, 2014). When compared with other studies, it was observed that the rind (0.30g/100g) does have higher content of fiber compared to the flesh (0.19g/100g) (FILA *et al.*, 2013). Therefore, SLRWR was selected to be used in the development of watermelon rind beverage containing honey since it has a significantly higher crude fiber content than SRWR.

3.2. Fitting the RSM to significant independent variable

The effects of temperature and time of thermosonication on the watermelon rind beverage containing honey was studied using central composite design (CCD). The response variables are pH and separation. Table 4 shows the experimental data obtained for both response variables. The linear, quadratic and interaction effects of time (x1) and

temperature (x2) on each response variables (yi) of watermelon rind beverage containing honey are shown in Table 4.

Table 4. The matrix of central composite design (CCD) and experimental data obtained for the response variables analysed; pH (Y1) and separation (Y2) (mean±SD).

Run Order	Blocks	Independent variables		Response variables	
		Temperature (°C)	Time (min)	pH (Y1)	Separation (Y2)
1	1	25	10	5.6±0.0	36.0±1.0
2*	1	45	35	5.7±0.0	29.0±6.0
3	1	65	60	5.9±0.0	5.0±0.0
4*	1	45	35	5.7±0.1	28.5±5.5
5	1	65	10	5.7±0.1	34.0±1.0
6*	1	45	35	5.7±0.1	35.0±0.0
7	1	25	60	5.6±0.0	29.5±6.5
8	2	45	10	5.7±0.1	37.0±0.7
9	2	25	35	5.6±0.0	35.0±0.7
10*	2	45	35	5.7±0.1	27.0±8.0
11	2	45	60	5.7±0.1	13.0±0.7
12*	2	45	35	5.6±0.1	36.0±2.0
13	2	65	35	5.9±0.1	17.5±5.5
14*	2	45	35	5.6±0.1	30.0±0.7

*Centre point.

The estimated regression coefficients for the response variables with their corresponding R1, R2 (adj), F-value and p-value of lack of fits are also included in Table 5.

Table 5. Regression coefficient, R2, adjusted R2, probability values and lack of fit for the final reduced models.

Regression coefficient	pH (Y1)	Separation (Y2)
b0	5.7	30.2
b1	0.1*	-7.3*
b2	0.1*	-9.9*
b21	0.0	-1.9
b22	0.0	-3.2
b12	0.1*	-5.6*
R2	0.9	0.9
R2 (adj)	0.8	0.9

*Significant level at $p < 0.05$ bi: the estimated regression coefficient for the main linear effects. bii: the estimated regression coefficient for the quadratic effects. bii: the estimated regression coefficient for the interaction effects. x1= time, x2= temperature

Table 5 shows the estimated regression coefficients of the final reduced model. The linear, quadratic and interaction effects of time (x1) and temperature (x2) on pH (y1) and separation (y2) were studied. The results showed that a high coefficient of determination ($R^2 > 0.8$) was achieved for all regression models. Hence, it is concluded that more than 80% of the variation of the response can be accurately explained as a function of time and temperature of thermosonication treatment.

The experimental and predicted values for both response variables at optimum and least optimum conditions showed no significant difference ($p > 0.05$). Hence, RSM was effective in estimating the effects of thermosonication conditions on pH and separation of watermelon rind beverage containing honey.

This was seen in another study of ultrasonic treatment of soursop juice, where RSM was demonstrated to be an effective technique for investigating the effects of ultrasound intensity and processing time on polyphenol oxidase residual activity, temperature increase, phenolic compounds, ascorbic acid content and total color difference (TCD) on soursop juice. The predicted and experimental values showed no significant differences (DIAS *et al.*, 2015). Similar result was observed in another study of enzymatic inactivation and antioxidant properties of blackberry juice using thermoultrasound, where time and temperature showed high correlation coefficients with the mathematical model of RSM. The predicted and experimental values for pectin methylesterase residual activity, polyphenol oxidase residual activity, diammonium salt, ascorbic acid, total phenolic compounds and anthocyanins of blackberry juice were not significantly different (CERVANTES-ELIZARRARAS *et al.*, 2017).

3.3. Thermosonication treatment conditions

In thermosonication for juice treatment, temperature and time are examples of factors that influence the fruit juice quality. This experiment employed preliminary treatment time from a previous study of ultrasound effect on grapefruit juice, with treatment time ranging from 0 to 90 min at 20°C to 60°C (AADIL *et al.*, 2015). The result showed that grapefruit juice sonicated for 90 min had significantly higher total carotenoids, lycopene, sugar contents and phenolic compounds with significant decrease in viscosity and microorganisms load (AADIL *et al.*, 2015).

From the RSM model, the optimum condition was determined based on the pH and lowest separation (%) of watermelon rind juice. From Tables 6 and 7, the experimental and predicted values for both response variables at optimum and least optimum conditions showed no significant difference. Hence, RSM is effective in estimating the effects of thermosonication conditions on pH and separation of watermelon rind beverage containing honey.

3.4. Thermosonication effect on physicochemical and microbiological properties of control and treated rind beverage

Acidity is usually correlated with the measure of pH (YAU *et al.*, 2010). The pH value is usually used to determine the processing requirements and for regulatory purposes (LIU *et al.*, 2012). As shown in Table 8, no significant difference was observed between the pH values of control and treated beverages, indicating that thermosonication does not alter the pH of beverages. A study of ultrasound done on blackberry juice also showed no significant effect on the juice's pH value and the treatment was able to maintain the juice's pH value at 3.27 to 3.83 (RAMÍREZ-MORENO *et al.*, 2017). The accepted range of pH for

commercial non-dairy beverages is from 2.1 (lime juice concentrate) to 7.4 (spring water) (SEOW and THONG, 2005). The acidity of beverage has to be controlled as it is one of the major causes of dental erosion amongst consumers (REDDY *et al.*, 2016).

Table 6. Values of response variables at good conditions (n=3).

Response	Watermelon rind beverage containing honey	
	Predicted	Experimental
pH	5.9a	5.0a
Separation (%)	5.2b	5.3b
Factors	Optimum condition	
Time (min)	60	
Temperature (°C)	65	

Values with different letters within a row is significantly different at 5% level ($p < 0.05$).

Table 7. Values of response variables at the least good conditions (n=3).

Response	Watermelon rind beverage containing honey	
	Predicted	Experimental
pH	5.4a	5.2a
Separation (%)	39.6b	40.5b
Factors	Least optimum condition	
Time (min)	10	
Temperature (°C)	25	

Values with different letters within a row is significantly different at 5% level ($p < 0.05$).

Separation is an important factor for consumer preference as it contributes to the physical appearance of juice. It measures the suspension degree of existing solid particles in the fruit juice to reduce layering. A comparison between juice separation (%) for treated and non-treated watermelon rind juice was done. The results in Table 8 shows that the juice separation (%) for control, optimum and least optimum are all significantly different from one another, showing temperature and time did influence juice separation (%). Watermelon rind juice treated under elevated temperature caused a significant decrease in juice separation (%). High temperature increases the kinetic movement of juice molecules which make it to collide with one another more rapidly. The mechanical forces developed from bubble cavitation disintegrate larger molecules into smaller molecules, hence, enhancing the solubility of juice molecules (DEMIRDOVEN and BAYSAL, 2008; SANTHIRASEGARAM, 2013). As thermosonication variables (time and temperature) increases, size of the particles in cantaloupe and grapefruit juice reduces, thereby providing better uniformity and stability which lead to reduced juice separation (AADIL *et al.*, 2015; FONTELES *et al.*, 2012).

Separation is one of the most common problems in fruit juice production, which makes it difficult to maintain the solids in suspension or dispersion in the beverage over a long period of time (DE-LEON and BOAK, 1984). It was shown that as temperature increases,

the cloudiness of juices also increases (CERVANTES-ELIZARRARAS *et al.*, 2017). However, another study stated that ultrasonic helps to increase clarity in juices. Ultrasonic has been shown to activate pectinesterase, which is capable of destabilizing colloidal pectin molecules suspended in a juice, making the juice clearer (CHENG *et al.*, 2007).

The total soluble solid (TSS) of fruits depends mainly on the type of cultivar, fruit species, maturity status, agricultural practices and seasonal changes (NAYAK *et al.*, 2017; TASNIM *et al.*, 2010). As shown in Table 8, TSS of control is significantly lower than beverages treated under optimum and least optimum conditions. The significantly different TSS value may be due to the addition of honey in the treated beverages. However, there is no significant difference between the TSS value of beverage thermosonicated at optimum condition and beverage thermosonicated at least optimum condition. This indicates that thermosonication did not significantly affect the TSS of beverage. A study done on purple cactus pear juice using thermosonication reported that the treatment had minimum effect on the juice TSS (CRUZ-CASINO *et al.*, 2015). A study carried out by Abid *et al.* (2014) using thermosonication on apple juice also reported insignificant difference in the TSS of treated and non-treated apple juice. A combined treatment of thermosonication and pulse electric field on orange juice also resulted in no significant changes in TSS (WALKING-RIBEIRO *et al.*, 2009).

For color evaluation, there is no significant difference for L^* value between control sample and sample thermosonicated at the least optimum conditions, but there is a significant difference for a^* and b^* values. The comparison between control sample and sample treated at optimum conditions showed no significant difference for b^* value, whereas, there is a significant difference in L^* and a^* values. These results indicate that thermosonication greatly influenced the beverage color (lightness and redness).

The study shows that there is no significant difference ($p > 0.05$) in vitamin C content of beverage between control and the treated sample at the least optimum condition, while there is a significant difference ($p < 0.05$) between the vitamin C content of beverage treated under optimum condition with control beverage and beverage treated under least optimum condition. The statistic indicates that beverage treated with thermosonication at optimum conditions have lower vitamin C compared to untreated and treated beverages at least optimum conditions of thermosonication.

Vitamin C (ascorbic acid) is an important antioxidant that possess protective properties against some types of cancers (ADEKUNTE *et al.*, 2010). As shown in Table 8, there was no significant difference ($p > 0.05$) between control and thermosonicated sample at the least optimum condition. This result is similar with results obtained from studies on thermosonication of apple, watermelon and tomato juice (ABID *et al.*, 2013; RAWSON *et al.*, 2011; WALKING-RIBEIRO *et al.*, 2009). However, there was significant difference ($p < 0.05$) between control and thermosonicated sample at optimum condition similar to studies on thermosonication of orange and strawberry juice (TIWARI *et al.*, 2008; HART and HENGLEIN, 1985). Sample that was thermosonicated at optimum condition and stored at 25°C experienced complete loss of vitamin C compared to other treatment methods and storage conditions.

Microbiological analysis showed that the log CFU/mL of TPC and PDA for both sonicated samples reduced significantly compared to the control sample. This result is similar with the sonication of orange, strawberry, carrot and mango juice (ZOU and JIANG, 2016; SANTHIRASEGARAM *et al.*, 2013; TIWARI *et al.*, 2008; TOMADONI *et al.*, 2017). It was concluded that with the increase in ultrasound treatment time, destruction of microbes was greater (ZOU and JIANG, 2016). This was similar with the storage study of strawberry juice (TOMADONI, *et al.*, 2017). Ultrasound has also shown to have destructive

effect on microorganisms in honey (PUTTONGSIRI and HARUENKIT, 2010). Reduction in microbial rate is a probable effect from total polyphenols increment, contributed by the release of free amino acids through the decomposing of cell structure, caused by ultrasound and thermal treatment (D'ARCY, 2017). The microbial inactivation by power ultrasound occurred due to cavitation, localised heating and free radical formation. Free radicals were formed during the application of ultrasound to liquids due to sonolysis of water, and these free radicals had a bactericidal effect.

Table 8. Effect of thermosonication conditions on physicochemical and microbiological properties of control beverage compared with treated beverage.

Properties		Treatment		
		Control	Optimum condition	Least optimum condition
Physicochemical	pH	5.47a±0.07	5.24a±0.01	5.49a±0.02
	Separation (%)	51.0a±1.0	12.0c±2.7	32.7b±2.5
	Total soluble solid (°Brix)	4.0b±0.0	13.0a±0.0	13.0a±0.0
	Color values	L*	69.0b±2.0	72.7a±0.6
		a*	2.1b±0.2	7.2a±0.1
		b*	30.1a±0.5	29.8ab±0.5
		TCD	-	6.5a±1.0
	Vitamin C content (mg/100 mL)	0.70a±0.00	0.00b±0.00	0.70a±0.10
Microbiological	TPC (log CFU/mL)	7.2a±0.3	4.6b±0.7	5.3b±0.5
	PDA (log CFU/mL)	6.5a±0.3	4.1b±0.1	4.2b±0.1

Data are mean±SD of three replicates Values with different letters within a row is significantly different at 5% level ($p < 0.05$).

3.5. Thermosonication effect on physicochemical and microbiological properties of control and treated rind beverage stored at 25°C and 4°C for a week

As shown in Table 9, all sonicated samples treated at optimum and the least optimum condition stored at 25°C and 4°C showed significant decrease in pH within a week of storage. This is due to the increase in lactic acid bacteria and the possible formation of hydroxyl methyl furfural throughout the storage period as well as the increase in titrable acidity (BHARDWAJ *et al*, 2005; KHANDPUR & GOGATE, 2016; WANG *et al.*, 2005). The values of pH for stored watermelon rind beverage containing honey after being treated with thermosonication ranges from 3.77 to 5.49 and it has already been stated that most beverages or juice have their pH ranges between 3.5 and 5.5 during storage (PEARSON, 1995). Therefore, from the results, the pH of sonicated watermelon rind beverage containing honey is still in the acceptable range even for a week storage.

A comparison for juice separation (%) between juices stored under 25 and 4°C for 7 days was done. The separation (%) of rind juices thermosonicated at 65°C for 60 min showed significant gradual increase from day 1 to day 7 when stored under 25°C. Fruit juices have the same taste, color and aroma as their whole fruit because mechanical separation exerts minimal effect on the juices' physicochemical properties. Thus, they still contain colloids

(pectin, proteins and polyphenols) and fibers, contributing to the cloudy appearance of juice. Presence of pectin hinders the formation of aggregation of juice particles and prevents floating substances from settling (HUI *et al.*, 2006). During storage under ambient temperature, the increase in separation may be due to the breaking down of pectin by pectinase into simple sugars. Hence, the juice separation is harder to maintain when stored under ambient temperature. At storage temperature of 4°C, the low temperature and high relative humidity inhibits enzyme activities, thereby, minimizing the increase of separation in juice (SINGH and MATHUR, 1983). The separation (%) of rind juice thermosonicated at optimum condition and rind juice thermosonicated at least optimum condition differed significantly, however, the increase in separation (%) through day 1 storage until day 7 storage is still temperature dependent.

The TSS of beverages for both treatment showed no significant decrease throughout the storage period under both storage conditions. A similar result was obtained from storage study of strawberry ultrasonicated at 40 kHz for 10 and 30 min, where control (untreated juice), thermally treated juice (pasteurization at 90°C for 60 s) and ultrasonicated juice showed no significant difference in TSS after 10 days of storage at 5°C (TOMADONI *et al.*, 2017).

For color analysis, the a^* value significantly decreased from day 1 to day 7 for all sonicated samples. The beverage thermosonicated at optimum conditions and stored at 25 and 4°C showed decreasing TCD value during a week of storage, while beverage thermosonicated at least optimum condition and stored at 25 and 4°C showed inconsistent change in TCD value during a week of storage. This shows that low temperature and short duration of sonication and storage at 25°C has the highest negative impact on the beverage color.

Among the primary factors of consumers' evaluation on the freshness of fruits and vegetable products is the color attribute. Better color characteristics will give better appeal to the consumers (KAYS, 1999). The increase in L^* was similar with studies of ultrasonic treatment for orange, strawberry, soursop, watermelon and mango juice (DIAS *et al.*, 2015; RAWSON *et al.*, 2011; SANTHIRASEGARAM *et al.*, 2013; TIWARI *et al.*, 2008; TOMADONI *et al.*, 2017). The TCD value of beverage was thermosonicated at optimum and the least optimum condition was more than 2, which indicated that there were visually noticeable differences in the sonicated samples compared to control. However, on the seventh day of storage, the color difference between the beverage thermosonicated at optimum condition and the control cannot be visually perceived.

A study done on purple cactus pear showed that the color of juice thermoultrasonicated at 80% for 25 min was not visually different from control at the first day of storage only, but afterwards, the color of thermosonicated juices were visually different from control (CRUZ-CASINO *et al.*, 2015). The cavitation caused by ultrasonic may accelerate chemical reactions, increasing diffusion rate, dispersion, aggregates formation and particles breakdowns which leads to color changes in treated beverages (SALA *et al.*, 1995). Increase in L^* value was also observed in sonicated honey, which were due to the reduction in crystal size (QUINTERO-LIRA *et al.*, 2017). The L^* value of sample sonicated at the least optimum condition and stored at 25°C had significantly increased, similar to the result of sonicated orange juice after storage (TIWARI *et al.*, 2008), whereas the sample sonicated at least optimum temperature and stored at 4°C showed a decrease in day 4 and an increase in day 7. Studies showed that an increase in L^* value was due to the partial precipitation of unstable suspended particles, whereas, a decrease is due to oxidative darkening (GENOVESE *et al.*, 2006).

This result is similar with thermosonicated strawberry juice (HERCEG *et al.*, 2013). Studies show that a decrease in a^* and b^* values might be attributed to the development of

browning degree in the beverage (IBARZ *et al.*, 2005). The results show that low temperature and short duration of sonication and storage at 25°C has highest negative impact on color. Based on the study of thermosonication on purple cactus pear juice, changes in color may be due to acceleration of chemical reactions by the increase in temperature, dispersion, particles breakdown and formation of aggregates by cavitation of thermosonication (CRUZ-CASINO *et al.*, 2015).

The degradation of vitamin C as shown in Table 9 could be due to the formation of free radical by sonication reaction, associated with the oxidative process (AGUILAR *et al.*, 2017).

Table 9. Effect of thermosonication conditions on physicochemical properties of beverage stored at 25°C and 4°C for a week.

Physicochemical properties		Storage (Day(s))	Storage condition			
			25°C		4°C	
			Treatment			
			Optimum	Least optimum	Optimum	Least optimum
pH		1	5.24aA±0.01	5.49aA±0.02	5.24aA±0.01	5.15aA±0.02
		4	4.39bB±0.01	4.30bB±0.01	4.91bB±0.01	4.98bB±0.01
		7	3.77cC±0.01	3.78cC±0.01	4.14cC±0.01	4.32cC±0.01
Separation (%)		1	21cC±2	36cA±1	7bD±1	31bB±1
		4	53bB±1	61bA±1	10aD±2	36aC±2
		7	84aB±1	91aA±1	11aD±1	39aC±1
Total soluble solid (°Brix)		1	13.0aA±0.0	13.0aA±0.0	13.0aA±0.0	13.0aA±0.0
		4	13.0aA±0.1	13.0aA±0.1	13.0aA±0.1	13.0aA±0.1
		7	12.9aA±0.1	12.9aA±0.1	12.9aA±0.1	12.9aA±0.1
Color	L*	1	73.0aA±0.5	72.8bA±2.5	72.4aA±0.4	73.4bA±0.1
		4	69.9bC±0.2	78.1abA±2.2	72.0aB±0.2	71.1cB±0.8
		7	69.0bC±0.7	79.0aA±1.9	68.9bC±0.1	75.6aB±0.6
	a*	1	8.2aAB±0.1	6.4aB±2.0	8.0aAB±0.0	9.2aA±0.0
		4	4.9bA±0.1	5.2aA±0.2	6.3bA±1.1	5.7bA±0.2
		7	3.1cA±0.1	0.3bB±0.2	3.1cA±0.1	0.1cB±0.1
	b*	1	30.1bAB±0.1	27.4aB±2.3	30.7cA±0.1	30.4cA±0.1
		4	23.3cC±0.2	20.0bD±0.1	32.9aB±0.1	33.2aA±0.1
		7	31.7aA±0.2	28.3aB±0.8	31.3bA±0.2	32.3bA±0.1
	TCD	1	7.9aA±0.9	6.9bA±1.3	6.8abA±0.2	8.4aA±0.0
		4	7.5aB±0.2	13.9aA±1.4	5.9aBC±0.8	5.3cC±0.2
		7	2.0bC±0.3	10.3abA±1.9	1.7bC±0.2	7.3bB±0.6
Vitamin C (mg/100 mL)		1	0.00aB±0.06	0.67aA±0.02	0.67aA±0.02	0.67aA±0.02
		4	0.00aA±0.00	0.00bA±0.00	0.00bA±0.00	0.00bA±0.00
		7	0.00aA±0.00	0.00bA±0.00	0.00bA±0.00	0.00bA±0.00

Data are mean±SD of three replicates. Values with different lower case letters within a column is significantly different at 5% level ($p<0.05$) between storage day(s). Values with different capital letters within a row is significantly different at 5% level ($p<0.05$) between storage conditions.

The vitamin C content for beverage treated at least optimum condition were retained for at least a day of storage under both temperatures (25°C and 4°C). Studies also showed that the degradation of vitamin C in juices is influenced greatly by the presence of air; therefore, juice should be degassed prior to treatment for better retention of ascorbic acid (OMS-OLIU *et al.*, 2009). In some cases, degradation of vitamin C occur in order to protect other compounds such as carotenoids, phenolic compounds or anthocyanins (TIWARI *et al.*, 2008). This shows that ascorbic acid is an unstable compound, making it very easy to degrade. Therefore, for better retention of vitamin C, milder processing conditions should be used (BASMALI, 2017).

As shown in Table 10, all sonicated samples showed an increase in log CFU/mL of TPC and PDA within a week of storage. For all samples, on PDA, there is only growth of yeast with no growth of fungi. From this experiment, the control sample would not be compatible for human consumption starting from day 0, since the maximum limit of microorganisms in minimally processed food is 7 log CFU/mL (TOMADONI *et al.*, 2017). As for sample sonicated at optimum condition, stored at both 25 and 4°C, the watermelon rind beverage containing honey were still fit for consumption even after reaching the 7th day of storage. The application of ultrasound treatment at high temperature can regulate microbial growth in watermelon juice containing honey at a level whereby the juice shelf life could be prolonged.

Table 10. Effects of optimum and least optimum sonication conditions on microbiological properties of sample stored at 25 and 4 °C for a week.

Microbiological properties	Storage (Day(s))	Storage condition			
		25°C		4°C	
		Treatment			
		Optimum	Least optimum	Optimum	Least optimum
TPC (log CFU/mL)	1	4.6bA±0.5	5.3bA±0.7	4.6bA±0.4	5.3bA±0.4
	4	5.5bA±0.5	6.2abA±0.3	5.0abA±0.6	6.2abA±0.8
	7	6.8aA±0.3	7.4aA±0.5	6.3aA±0.6	7.0aA±0.2
PDA (log CFU/mL)	1	4.2bA±0.5	5.2bA±0.3	4.1bA±0.3	5.2bA±0.6
	4	5.1bA±0.4	6.1bA±0.2	5.0abA±0.6	6.1abA±0.7
	7	6.8aA±0.7	7.2aA±0.5	6.2aA±0.5	7.2aA±0.4

Data are mean±SD of three replicates. Values with different lower case letters within a column is significantly different at 5% level ($p < 0.05$) between storage day(s). Values with different capital letters within a row is significantly different at 5% level ($p < 0.05$) between storage conditions.

4. CONCLUSIONS

This study shows that the optimum condition for thermosonication process for watermelon rind-honey beverage is at 65°C for 60 min. Thermosonication has no significant effect on the pH and TSS of beverage. However, throughout the storage period, the pH of beverage decreased, depending on the storage temperature but it is still safe for consumption. The % separation, color, vitamin C content and microbial load of beverage were all significantly affected by thermosonication. The changes in beverage pH, % separation and color during storage period were temperature dependent. All the results

showed that the beverage treated with thermosonication can be preserved all through the storage period of a week. Therefore, it can be concluded that watermelon rind beverage containing honey can be better treated using thermosonication method rather than thermal treatment alone to obtain longer beverage shelf life. However, further enhancement study should be done for better vitamin C retention in fruit juices using thermosonication method.

ACKNOWLEDGMENTS

This research was supported by Faculty of Food Science and Technology, Universiti Putra Malaysia.

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Paper Received July 31, 2018 Accepted April 8, 2019

NEW CONSUMER TARGETS TOWARDS A TRADITIONAL SPIRIT: THE CASE OF GRAPPA IN PIEDMONT (NORTHWEST ITALY)

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ABSTRACT

A choice experiment was conducted in Piedmont, Italy, to define purchasing preferences and behaviours of Grappa consumers. A total of 667 individuals were interviewed at different points of Grappa purchase/consumption. The most important attributes considered during Grappa purchase by consumer were defined using the Best-Worst Scaling methodology. The Latent Class Analysis was employed to identify consumer clusters characterized by different Grappa preferences and consumption styles. For Piedmont consumers, Grappa choice was related to previous experience, product knowledge and origin. Conversely, consumers considered "alcohol content" and "packaging" the two least important factors to be considered during purchase. The lClass analysis allowed the identification of four clusters of Grappa consumers that were described in function of socio-demographic variables.

Keywords: Best-Worst scaling, cluster analysis, choice factors, Italian distillate, socio-demographic variables

1. INTRODUCTION

1.1. Grappa history: from illegal product to national symbol

European Union Regulation 110/2008 recognises the intrinsic value of Grappa, in particular as a liquor of unique geographical origin and as the only Italian product derived from the distillation of pomace, according to the production regulations (Ministry of Agriculture, Food and Forestry, Decree 5389, 2011).

Grappa is a traditional and historical Italian alcoholic distillate that became a product to drink around 700, although the first methods of distillation date back to the seventh and sixth centuries BC (VACCARINI and PILLON, 2017; ONOFRI and BOATTO, 2015). However, a precise date of the first Grappa distillation process is still to be defined (ANTONINETTI, 2011). Originally, the pomace distillation for liquor production was considered an illegal operation (BEHRENDT and BEHRENDT, 2000) and the distillation process was usable only in medicine and applied sciences. This could explain the mystery that still surrounds the initial date of this liquor. Various literature proclaims the official birth of the pomace distillation method for Grappa production is attributed to Jesuit studies dating back to the mid 1600s, which refined and improved the practice and tools for this production process¹. From there on, Grappa could be legally consumed as it became a symbol for intellectuals during the Italian Renaissance (MCCRACKEN, 1988), the period when the distillation process received the official academic recognition (ANTONIETTI, 2011).

Today, the social image of this product has evolved over the decades from a product consumed exclusively in local taverns or restaurants, from a defined target of consumers, in a liqueur to be enjoyed on several occasions by all (ANTONIETTI, 2011). Grappa became a phenomenon linked to different social classes, consolidating its presence in northern Italy, but opening up to the channel of mass distribution and advertising.

However, Grappa has suffered a sharp decrease in consumption in recent decades. In 1974, there were 39 million litres consumed in Italy, then dropped to 21 million in 1999. Consumption increased to 30 million by 2008 and then plunged back down to 23 million in 2017 (PIGOZZO, 2018; GALLETTTO and ROSSETTO, 2005). In the national context, Grappa showed a negative trend in production of 29% from 2006 to 2016, and a drop from 117,000 to 82,000 in anhydrous alcohol (ASSODISTL, 2017). From 2017 Grappa production increased recording a significant change in value (from 42.9 to 44.2 million euros, + 5.9%) with recorded quantity from 27,935 to 30,919 anhydrous alcohol (FEDERVINI, 2017). Nielsen data from 2018 revealed a new positive trend in the Italian market (+ 0.8% in volume) (FEDERVINI, 2018). The exportation remained however limited: the quantities of product exported in 2017 fell by 12% compared to the previous year (from 28.9 to 25.3 in thousand anhydrous alcohols). The most important markets are Germany, which imported bottled Grappa for a value of almost 19 million euro, followed by Switzerland with 6.4 million euro (FEDERVINI, 2018).

1.2. Research background and aims

In several literature researches the consumption occasions, as well as the cultural and socio-demographic variables were analysed as drivers for consumer preferences and behaviour definition regarding agro-food and oenological products (DAL VECCHIO *et al.*, 2018; CARSANA and JOLIBERT, 2017; DEKHILI *et al.*, 2011; MERLINO *et al.*, 2017; MU *et al.*, 2017; SCHÄUFELE and HAMM, 2017; BRUWER *et al.*, 2017 BORRA and

TARANTOLA, 2015). Even in the case of spirits such as gin, whisky and vodka, researchers have investigated consumer preferences in recent years, including the characteristics and behaviour during purchasing (CLARKE and KOPTEV, 1992; DUBININA and ALIEVA, 2015; GUY *et al.*, 1989; GAUTHIER and MAZIÈRES, 2013). However, in the case of Grappa, few studies in literature investigate the consumer preferences and buying behaviours for this product.

In general, consumer preferences about liquors, as well as, for Grappa, has been evolved meaning that purchases were no longer based exclusively on objective attributes (such as price or economic availability), but also considering emotional and irrational attributes during the product selection (i.e. brand, place of purchase, link with the territory, packaging, certifications, indications or designations of origin) (LOCK *et al.*, 2006). Moreover, unlike food and other beverages, the nutritional and health aspects of alcoholic beverages lose considerable importance for consumer in favour of other more important aspects (aroma, colour, place of production/origin). In addition to preferences, also the characteristics of the typical prototype of Grappa consumer, mainly low-middle income and exclusively male, have evolved during the '80s, thanks to the enhancement programs and marketing strategies applied by Italian producers, restaurateurs and bars. Some quality aspects of Grappa have been modified and improved to allow the consumer to choose from a wider range of products. For example, producers improved the alcohol content, which has led to a "softer spirit" with an alcohol content of about 40% instead of the usual 50% or 60% alc. This latter aspect has expanded the opportunities for conventional consumers to enjoy Grappa on several occasions (WILSON, 2009; ANTONIETTI, 2011), transforming this distillate into a more relevant product even for non-experts not used to higher alcohol levels and with a strong flavour. To date, grappa has a new identity and is appreciated by a greater demographic variety, even by young people (DEMOSKOPEA, 2003). To this end, the aim of our research, conducted in north-west Italy, was to investigate on the purchasing preferences and behaviour of Grappa's consumers, also to understand if young people were included in the consumers target. At this purpose, the sampling phase was also addressed towards places typically frequented by young individuals.

The Best-Worst Scaling (BW) methodology (Finn and Louviere, 1992; Marley and Louviere, 2005), already used to analyse consumer preferences in the agri-food sector (LOCKSHIN *et al.*, 2015; MERLINO *et al.*, 2018; GIRGENTI *et al.*, 2016), as well as in other areas (REZAEI *et al.*, 2016), was employed in our study. The paper results are structured as follows: firstly, the Grappa consumers characteristics and habits are described; then, is defined the importance of 12 quality attributes of Grappa expressed by consumers in the decision-making phase; finally, the Cluster analysis results and the differences in terms of socio-demographic variables between the consumer targets are analysed.

2. Materials and methods

A total of 667 individuals were involved in the research to assess Grappa's consumer preferences. Face-to-face interviews were conducted through a paper questionnaire from March to June 2018 at various Grappa purchasing or consumption points (bars, wineries, supermarkets) distributed in Cuneo city and in the metropolitan areas of Turin (northwest Italy) and on the campus of the University of Turin.

The paper questionnaire consisted of three sections: the first contained questions to investigate the socio-demographic characteristics of respondents; the second part focused

on the survey of Grappa consumption habits and styles; the third section focused on the analysis of consumer preferences on the twelve attributes of Grappa selected for the Best-Worst scaling application. Socio-demographic characteristics of the involved sample are described in Table 1.

Table 1. Respondents characteristics in terms of gender, age, number of family components, educational level, annual average income and employment (%).

Sample (N= 667)			
Gender		Educational level	
Male	53%	Primary school	1%
Female	47%	Lower secondary school	11%
Age		Upper secondary school	71%
18-24 years	40%	Master's degree	17%
25-35 years	21%		
36-50 years	21%	Annual average income	
51-65 years	13%	<25,000 €/year	29%
over 65	5%	25,000 - 35, 000 €/year	45%
		> 35,000 €/year	26%
Family composition		Employment	
One member	11%	Student	37%
Two members	13%	Employed	38%
Three members	26%	Self-employed	11%
Four members	40%	Retired	7%
More than 4 members	10%	Unemployed	4%
		Housewife	3%

2.1. Best-Worst scaling methodology and Cluster analysis

The BW method model helps to identify the most and least relevant attributes for consumers within a designed set of features that describe and characterize a product (SECCIA *et al.*, 2012). Data collection takes place through interviews, during which respondents are asked to indicate between a defined number of attributes organized in sets (from three to five elements), the one that is the most important (BEST) and the least important (WORST) during the purchase and/or selection of the product. The BW method has several advantages compared to traditional methods of discrete choice. Firstly, it appears to be easier to understand by consumers and quick to fill out. There is also greater consistency in the options chosen by the consumer, especially when these are the contrary or extreme. Finally, the BW method helps to obtain a good amount of information about consumer preferences through a classification of the same (MARLEY and LOUVIERE, 2005).

Using this methodology, respondents evaluate all the attributes present in each set as if they were pairs, consequently choosing the most representative pair for each set that corresponds to the maximum difference pair. For this reason, the BW method is also known as a maximum difference scale (MaxDiff) that provides a more efficient evaluation of the coupled data, thereby obliging the respondent to make a discriminating choice

between attributes compared to traditional systems of comparison (AUGER *et al.*, 2007; Cohen, 2003; FINN and LOUVIERE, 1992; MARLEY and LOUVIERE, 2005). According to ORME (2005) it is advisable to have between four or five attributes per set because a greater number would determine a minimum increase in the information obtained. In our research, 12 Grappa attributes have been selected and organized in nine sets of four attributes each, allowing single item to appear three times in the questionnaire. This was feasible by using the appropriate software (Sawtooth Software v.2.0.2, Orem, UT, USA; <http://www.sawtoothsoftware.com/>). The Sawtooth software has also created four different versions of the questionnaire in order to minimize the differences (both subjective and cultural) in the way of personal classification and create greater diversification in the presentation of items.

The level of importance for each Grappa attribute was evaluated by the average raw Best-Worst score analysis (CASINI *et al.*, 2009; COHEN, 2003; GOODMAN *et al.*, 2005). This score is a numerical value calculates dividing the BW score (number of BEST minus number of WORST) to the number of respondents and the frequency with which each attribute appears in the set of choices. The confidence limit used for BW score calculation was set equal to 95% and the standard deviation was used to evaluate sample variability. The 12 attributes of Grappa selected through a literature research and used in the BW analysis are reported in Table 2.

Table 2. The twelve attributes of Grappa selected and used for the Best-Worst analysis.

Grappa attributes	References
Taste/flavour	DIAMANTIDOU <i>et al.</i> , 2018; LOUW and LAMBRECHTS, 2012; VIOLONI, 2008; FINZI, 2007; UBIGLI, 2001; UBIGLI and CASTINO, 1992; DA PORTO, 2012; APOSTOLOPOULOU <i>et al.</i> , 2005.
Packaging/bottle format	DIAMANTIDOU <i>et al.</i> , 2018; VIOLONI, 2008; DA PORTO, 2012.
Brand	DEMOSKOPEA, 2003; PRENTICE and HANDSJUK, 2016; CARSANA and JOLIBERT, 2017; LOUW and LAMBRECHTS, 2012.
Price	FINZI, 2007; GALLETTTO and ROSSETTO, 2005; MU <i>et al.</i> , 2017; DA PORTO, 2012.
Grapevine	DIAMANTIDOU <i>et al.</i> , 2018; BORSA <i>et al.</i> , 2008; GALLETTTO and ROSSETTO, 2005; DA PORTO, 2012.
Aging	DIAMANTIDOU <i>et al.</i> , 2018; VIOLONI, 2009; SOUFLEROS <i>et al.</i> , 2004.
Information on the label	VIOLONI, 2009; DA PORTO, 2012.
Origin/place of production	CHANDRA <i>et al.</i> , 2017; SCHÄUFELE and HAMM, 2017; DA PORTO, 2012; LOUW and LAMBRECHTS, 2012.
Adding aromas/flavour	DIAMANTIDOU <i>et al.</i> , 2018; VIOLONI, 2009; ASIOLI <i>et al.</i> , 2017; DUBININA and ALIEVA, 2015; DA PORTO, 2012; LOUW and LAMBRECHTS, 2012.
I know/already tried	MULLER <i>et al.</i> , 2010; HARRINGTON, 2007.
It was recommended to me	AGNOLI <i>et al.</i> , 2011; HARRINGTON, 2007.
Alcohol content	DIAMANTIDOU <i>et al.</i> , 2018; DUBININA and ALIEVA, 2015; PIGOZZO, 2018; LOUW and LAMBRECHTS, 2012; APOSTOLOPOULOU <i>et al.</i> , 2005.

The Latent Class (IClass) Analysis was used to divide the whole sample of individuals into homogeneous groups (clusters) according to their purchasing behaviour and expressed preferences. The Sawtooth software automatically created five clusters, each of which is characterized by different values of the following indicators: the Akaike Constant Information Criterion (CAIC), the Log-Likelihood (LL) and the Bayesian Information

Criterion (BIC). In our research, the most appropriate segmentation was chosen as the one with the lowest BIC value, which, in our case, was corresponding to four clusters, also in accordance with MERLINO *et al.* (2018) and DEKHILI *et al.* (2011) (Table 3).

Table 3. Values of BIC of the lClass analysis results: the lowest value was used for clusters number choice.

Groups	Replications	BIC
2	5	5845.89
3	4	5726.20
4	2	5639.28
5	4	5640.28

3. RESULTS

3.1. The consumers of Grappa: socio-demographic characteristics

The 31% of the total sample (n=207 individuals) declared to consume Grappa. Among those who have declared that they do not consume Grappa "non-consumers", the main reason was linked to the organoleptic aspect ("I do not like it"), leaving out other reasons such as health or religious aspects.

The sub-sample of Grappa consumers was represented mainly by men (77%) with respect to women (23%), and by individuals belonging to the age groups of the youngest (under 35 years), while a minority of over 65 consumed Grappa (Table 4).

The distribution of the genders proportion in the different age groups is described in Fig. 1.

Table 4. Age ranges of Grappa consumers.

Age ranges	Consumer sample (n=207)
18-24	38%
25-35	22%
36-50	21%
51-65	14%
over 65	5%

From data reported in Fig. 1 emerged a majority of men among Grappa consumers (77%) compared to women (23%). However, when analysing the distribution between women and men in the different age groups, a majority of women among the youngest consumers emerge, while an evident numerical superiority of men is highlighted in the other considered age ranges. In the over 65 consumers women represented the 40%.

Grappa consumers differed in level of education and occupational characteristics compared to the whole sample (Table 5). Regarding the family composition, 43% of consumers represented four-member families, 22% with three members, 16% with more

than two children, 13% belonged to families with two members and only 6% to single-parent families.

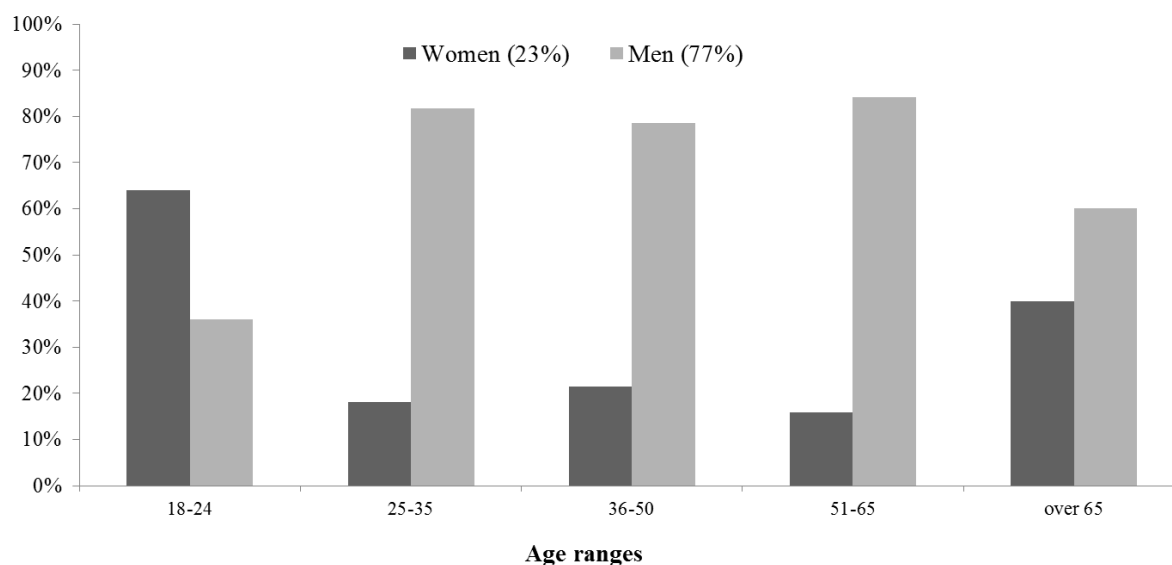


Figure 1. Genders distribution among the considered age groups and in the sub-sample of Grappa consumer.

Table 5. Educational level, family composition, annual average income and employment of Grappa consumers.

Grappa consumers (n = 207)			
Educational level		Family composition	
Primary school	0%	One member	15%
Lower secondary school	8%	Two members	14%
Upper secondary school	68%	Three members	23%
Master's degree	23%	Four members	34%
		More than 4 members	13%
Annual average income		Employment	
<25,000 €/year	24%	Student	51%
25,000 - 35, 000 €/year	44%	Employed	24%
> 35,000 €/year	42%	Self-employed	14%
		Retired	7%
		Unemployed	7%
		Housewife	0%

Households with several members (four and five members) had a prevalence of affirmative answers to the question, "someone in the family drinks Grappa". On the contrary, by analysing the family composition of respondents who said they did not consume Grappa, the data revealed that 33% belonged to single member families and 20% to two member families.

3.2. Habits and styles of Grappa consumption

The majority of Grappa consumers involved in this study (69%) stated that they drink Grappa occasionally, while the 16% consume Grappa once or twice a month and 12% once or more a week. Only 2% of consumers drink this liquor every day (Table 6). The study found that majority of consumers drink Grappa habitually inside their home (35%), followed by 20% of individuals who consumed it in restaurants/pizzerias, special events (17%), in pubs and bars (17%) and at social tastings (10%). Only the 2% answered to drank Grappa in unspecified occasions. Different results emerged from the analysis of answers about the frequency of Grappa purchase (the bottle); among consumers, only 21% of those declared to never buy Grappa throughout the year, while 42% said they bought it occasionally. The 16% of consumers who bought Grappa two to four times a year, while 14% of respondents bought annually. Those who bought several times a year represented 8% of the whole sample. About the reasons to purchase Grappa, the main expressed by respondents was the convivial consumption with friends (44%), followed by purchase as a gift (31%), and for personal consumption (25%). In the latter case, there was a clear difference in behaviour between products in the case of personal consumption, highlighting a clear prevalence of men in this category.

Table 6. Frequency of Grappa consumption and purchase declared by interviewees.

Frequency of Grappa consumption		Frequency of Grappa (bottle) purchase	
Everyday	2%	Several times a year	8%
More than 2 times a week	2%	2-4 times a year	16%
1-2 times a week	10%	Annually	14%
1-2 times monthly	16%	Occasionally	42%
Occasionally	69%	never	21%

3.3. The importance of Grappa attributes

The numbers of selected BEST and WORST and the BW average raw score for single Grappa attributes for Piedmont consumers are reported in Table 7.

The most important attributes considered during the decision-making process of Grappa choice and purchase were "I know it/already tried" with an average raw score of 2.13, followed by "It was recommended to me" (average raw score equal to 1.68), "brand" (raw score equal to 1.71), and "origin/place of production" with a raw score of 1.08. On the contrary, among the attributes that not influence grappa purchase there was the "alcohol content", with the lowest average raw score (-2.30), followed by the "packaging/bottle format" (raw score of -1.71), by "addition of aromas/flavours" and by "information on the label" with average raw score values, respectively, of -1.21 and -0.79. The attributes considered least important by consumer at the time of purchase all present negative raw scores.

3.4. Latent Class Analysis of Grappa consumers

Clusters of consumers defined in function of their expressed preferences for Grappa attributes are described in Table 8. The same table shows the dimensions of the different clusters, as well as the BW raw score values for each attribute that define their importance within the single consumer group. Each of the four clusters has been named according to their expressed preference and perception defined in function of the importance given to the individual factors by consumers.

Table 7. Number of BEST, number of WORST and B-W average raw score for each attributes of Grappa.

Attribute	Number of selected BEST	Number of selected WORST	B-W average raw score
Taste/flavour	83	102	-0.40
Packaging/bottle format	55	181	-1.71
Brand	162	30	1.71
Price	75	105	-0.40
Grapevine	101	86	0.31
Aging	109	87	-0.10
Information on the label	66	130	-0.79
Origin/place of production	146	48	1.08
Adding aromas/flavour	51	150	-1.21
I know/already tried	188	51	2.13
It was recommended to me	163	43	1.69
Alcohol content	25	211	-2.30

Table 8. Average BW raw score for the four clusters representative of considered consumers sample: Nonexpert, Price Sensitive, Experts and Quality Sensitive.

	Clusters			
	Nonexpert	Price Sensitive	Experts	Quality Sensitive
Cluster dimension	27.4%	15.9%	30.7%	26.0%
Attributes	Average Raw Score			
Taste/flavour	1.37	3.60	-0.01	0.96
Packaging/bottle format	0.73	2.84	-0.36	-0.20
Make/brand	2.01	2.16	2.70	3.02
Price	1.59	3.51	0.15	0.64
Grapevine	-0.41	3.31	1.87	2.29
Aging	-0.51	1.72	2.23	2.48
Information on the label	1.18	1.19	0.09	1.79
Origin/place of production	0.99	3.62	2.21	2.43
Adding aromas/flavour	0.83	0.36	0.64	0.97
I know/already tried	2.74	1.68	4.13	1.36
It was recommended to me	2.75	2.85	3.45	0.78
Alcohol content	0.00	0.00	0.00	0.00

Important differences in attributes preferences evaluation between the four groups of consumers emerged from cluster analysis. The main group (30.7% of the entire sample), called *Experts*, was represented by respondents who considered their consolidated knowledge of Grappa, the recommendations on the product, as well as the brand, as the most important factors in the purchasing process. In some aspects, the *Experts* had similarities with the *Nonexpert* group (27.4%). In fact, even for these two types of consumers, the attributes "I know/already tried", "It was recommended to me" and "brand" were the most important factors for the product choice. However, these latter individuals differed from the *Experts* on the least important attributes. For *Experts* consumers, packaging and taste/flavour were irrelevant for Grappa selection, whereas the *Nonexpert* considered qualitative aspects such as the grape variety and the aging of the product unimportant factors in the Grappa selection.

The third group was named of *Quality Sensitive* and represented the 26% of the entire sample. Respondents that considered discriminant during Grappa purchase the grape variety, the product aging and the origin/ place of production, characterized this cluster. Among the four groups, *Quality Sensitive* was the only one that emphasized the intrinsic qualities of the product, with high raw score values for the information on the label. This group also considered aspects such as price and packaging irrelevant in the decision-making process.

Price sensitive individuals (15.9% of the entire sample) represented the fourth cluster. Respondents who considered the price the most important attribute during Grappa purchase, followed by the origin/place of production and the taste/flavour, characterized this group.

The respondents' profiles were also analysed considering the consumers socio-demographic characteristics. In particular, if the *Nonexpert* group was characterized by a slight majority of women (58%) compared to men (42%), the *Price Sensitive* and *Expert* clusters presented the same distribution with a minority of men (32%) compared to women (68%), while the *Quality Sensitive* clusters were represented by 83% of men and only 17% of women. The percentages of individuals divided by age group in the different clusters are shown in Table 9. In general, the majority of young people emerge among the expert drinkers of Grappa, while the *Nonexperts* were mainly more mature individuals.

Table 9. Age differentiation of respondents belonging to the selected clusters.

Cluster	Age ranges (years old)				
	18-24	25-35	36-50	51-65	>65
Price sensitive	41%	0%	27%	23%	9%
Nonexpert	46%	23%	15%	12%	3%
Expert	21%	0%	25%	23%	12%
Quality sensitive	38%	9%	26%	27%	0%

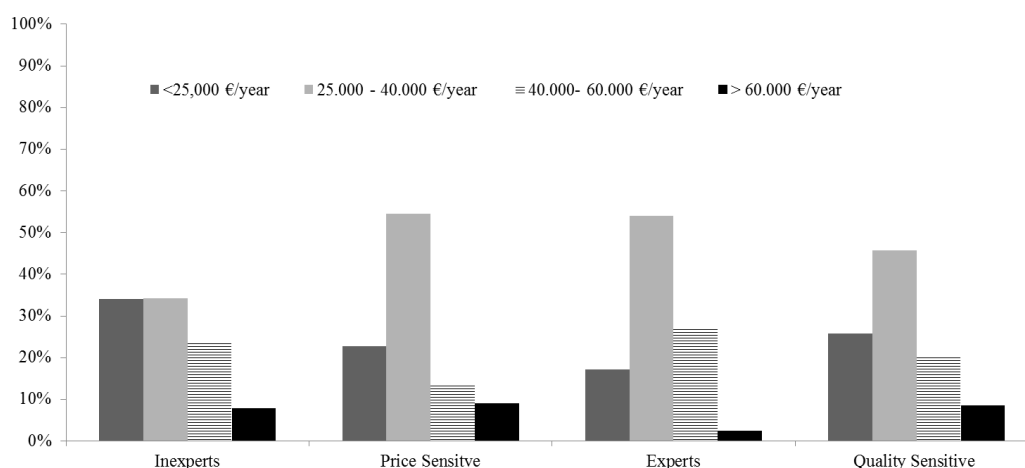


Figure 2. Clusters characterization in function of the annual average income.

Fig. 2 highlights clusters characterization in function of the annual average income range of respondents. The *Price sensitive*, *Expert* and *Quality sensitive* groups were mostly represented by consumers with a mean yearly income between 25,000 to 40,000 €/year. In general, from our results the intermediate income level emerged as widespread among all the considered clusters, constituting at least one third of each cluster in all cases, excluding the *Nonexpert* group.

The clusters composition was then analysed based on the expressed level of Grappa knowledge (low, medium, high) declared during the interviews (Table 10).

The behaviour of the four groups was analysed according to their willingness to spend (in euros) for a bottle of Grappa purchase (Table 11).

Table 10. Level of Grappa knowledge expressed by the four defined clusters of consumers.

Cluster	Level of knowledge		
	Low	Medium	High
Nonexpert	89%	11%	/
Quality sensitive	54%	46%	/
Expert	65%	27%	8%
Price sensitive	70%	21%	9%

Table 11. Willingness to pay (Euros ranges) for a bottle of Grappa purchase declared by the different clusters of consumers (Nonexpert, Price sensitive, Expert and Quality sensitive).

Cluster	Price ranges				
	less than 10€	10-20 €	21-40 €	41-60€	more than € 61
Nonexpert	16%	59%	22%	3%	0%
Price sensitive	0%	33%	48%	10%	5%
Expert	0%	48%	42%	0%	6%
Quality sensitive	3%	35%	55%	3%	3%

On average, the sample was willing to pay between 21 and 40 euros for Grappa purchase. Only *Nonexpert* consumers were willing to spend less than 10 euros on Grappa, while *Price sensitive* consumers showed a clear price sensitivity by focusing on product value for money.

4. DISCUSSION

This study analysed Grappa consumer characteristics, buying and consumption habits in Piedmont. In particular, the preference degree of 12 Grappa attributes was measured by dividing the considered sample into four clusters of individuals. Subjects with similar behaviours, attitudes and preferences towards Grappa product characterized each cluster. The socio-demographic analysis helped with describing the sample of Grappa consumers who represented the 31% of the total of interviewed. These individuals were especially men and young subjects under 35 years. This latter result underlines how young people, in particular women, are joining the target group of Grappa consumers, and further confirming the evolution of Grappa from a product associated with a specific category of consumers to a product for all individuals (ANTONIETTI, 2011).

The probable correlation between the personal consumption of Grappa and a more or less habitual drinking within the family emerged from our analysis. Although focused only on Grappa, this latter result is also confirmed by literary research conducted on the overall consumption of alcohol, confirming the influence of alcohol consumption by family components on the individual behaviour. Both SCAFATO *et al.* (2004) and ISTAT (2016) clearly show how the influence of consumption patterns of parents and, in particular, the head of the family, is a key element in determining behaviour, especially in the younger age group.

Concerning the consumption habits, the profiled consumer in our study tends to be an occasional consumer who buys Grappa infrequently in association with special events, perhaps for convivial consumption with friends or as a gift. These attitudes show differences, however, depending on the gender, revealing a greater propensity of women to buy Grappa on specific occasions to taste it in company, in opposition to men respondents that had a greater tendency to buy it for personal consumption.

In general, however, while other alcoholic beverages (wine or beer) are consumed more easily due to their alcoholic range, as shown in literature, emerged the tendency of Grappa consumers to choose this spirit carefully to taste it on special occasions, without almost never abusing it. The exploration of Grappa consumption habits highlighted how consumers prefer their home as a place to taste this traditional distillate. Grappa is often and traditionally drunk after meals, such as lunch or dinner, served as a “digestive” or “to correct a cup of espresso” (ANTONIETTI, 2011). These latter results underline that the social factor is extremely related both to the reasons for buying and to the occasions of consumption of Grappa. In the case of buying Grappa as a gift, the two genders seem to be very similar during purchasing behaviour. The identification of Grappa as a gift to give to friends or relatives symbolizes a recognition in this product by the consumer of an added and symbolic value, appreciable as a gift.

The results belonging to the Best-Worst scaling methodology application highlighted as the choice of Grappa is driven by the memories arising from a previous tasting experience, a known product and a specific brand, putting the product quality aspects in second place. Although Grappa producers are focusing their marketing strategies on product enhancing and differentiating through the improvement of aspects such packaging originality,

elegance and communicative power⁴, in our study both packaging and bottle size were among the less important attributes considered during Grappa purchase. The lesser importance of the attribute "adding aromas/flavour", on the contrary, is not a surprising result because also literature have confirmed the tendency of Grappa consumers to prefer the pure product version, appreciating the taste, aroma, transparency, in particular the "white" colour of grappa (KOCH, 2008; BELLONE, 2011; ONOFRI and BOATTO, 2015, ONOFRI and KOCH, 2006).

Clusters analysis allowed the entire sample division into four groups of consumers characterized by homogeneous features and behavioural preferences. The most represented group was that of *Experts*, composed mainly by young men, with a medium-high income bracket and a willingness to pay an intermediate price for the purchase of Grappa.

These consumers reflect the trend of the entire sample by relying on their previous experiences and product image during purchasing. In this case, the experience is accompanied by a greater product knowledge, paying less attention to attributes such as the packaging and the taste/flavour of Grappa.

The second most represented group was of *Nonexpert*. It is interesting to note that the groups of *Experts* and *Nonexpert* have given importance to the same attributes in the grappa choice in relation to product knowledge and recommendations provided by others. However, the same preferences expressed probably need to be interpreted differently. In fact, for *Experts*, product knowledge and experience give the subject confidence in their own knowledge, which gives them certainty in the choice and reassurance during the purchase phase.

The *Nonexpert* group was represented by consumers who rely on their previous experience during the choice, perhaps to make a safe choice of product, and also, not having sufficient knowledge of the product, rely on recommendations for fear of making a mistake in buying. In this group, mainly represented by young people, age has played a key role as it leads to greater inexperience. Their willingness to pay for a bottle of Grappa was associated with a low-medium price, perhaps in correlation with their lower income bracket. Finally, this group was characterized by a higher percentage of women.

The group of *Quality sensitive* were mainly mature men who showed greater attention to the intrinsic factors of Grappa at the time of purchase, such as aging, the grape variety and geographical origin. Probably these consumers are connoisseurs who do not give importance either to price or packaging. *Quality Sensitive* probably belong to those expert consumers, enhancers of the gustatory quality of the product that conceive the tasting of Grappa as a ritual or a moment to appreciate all the unique connotations conferred by the aging process of a specific producer.

These consumers were probably connoisseurs for whom the brand image becomes important again because it is associated with an intrinsic quality of the product, without paying attention to aspects not directly related to the product itself, such as price and packaging. In this category there was a strong prevalence of men, who were willing to pay an average price for the purchase of Grappa, as well as characterized by a good knowledge of the product.

Price sensitive, the last cluster by size, considered price and taste/flavour important attributes during their decision-making process of Grappa purchase. Their willingness to pay for a bottle of Grappa has never fallen below 10 € indicating a lack of confidence in products too cheap and looking for a good value for money. In this case, knowledge plays a fundamental role because no consumer of this group relied on past experience or even on the image of the product because otherwise it doesn't guarantee quality. In addition,

there has been an increase in the average age for this category along with an increase in the middle-income bracket, which is in line with the positive perception of the attribute "price". However, these consumers were looking for the best value for money, also paying attention to the production areas. The combination of price, origin and taste can probably be associated with a consumer who assesses the price as an indicator of superior quality of the Grappa product. On the other hand, the information on the label and the addition of flavours/aromas are irrelevant for this category.

5. CONCLUSIONS

This study identified four different profiles of Grappa consumers: despite the differentiation in term of preferences and socio-demographical variables, in general, a good part of involved consumers stated to have a medium-low level of knowledge towards Grappa, except for a few passionate connoisseurs.

The heterogeneity between clusters preferences defines the importance of studying consumer attitudes, especially for products linked to tradition, but whose consumption is limited to special occasions or convivial moments such as Grappa. Consumer preferences must be interpreted and seen by the producers as a tool and an indicator to deal with marketing and production decisions. Grappa has enormous potential; the last twenty years has witnessed the production sector undergoing an evolution that has affected the product, the structure and the organisation of the production chain. The goal of reaching new and younger targets is being realized; however, the intrinsic potential of this product could allow it to expand even further products, opportunities and ways of consumption. The operators of the sector, in collaboration with the points of sale and consumption of Grappa, could envisage this objective.

A limitation of this research lies in the characteristic of the sample in terms of circumscription in a single geographical area (single region in the northwest of Italy), and in the sampling method that could represent a limitation in this type of research. In the future, it could be considered to expand to more areas at the national level and to involve individuals interviewed only in point of Grappa consumption. In addition, it would be interesting in future work to assess the level of knowledge of Grappa in other areas at the international level and to provide a tool of enhancement to companies with the intention of expanding their market.

NOTES

¹www.istitutograppa.org/ita/cosa-e-la-grappa.html/ available at 1/01/2019

²www.istitutograppa.org/ita/stampa/la-grappa-tra-passato-presente-e-futuro-da-vinitaly-buone-prospettive-per-il-distillato-di-bandiera.html Available at 10/12/2018

³www.grappa.com/ita/grappa_dettaglio.php/titolo=chi_beve_la_grappa/idpagina=10/idnews=1/idsezione=6 Available at 10/12/2018

⁴www.anag.it/premio-design-il-vestito-della-grappa-alla-grappa-clessidra-ma-vince-tutto-il-mondo-della-distillazione/ Available at 12/12/2018

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Paper Received October 10, 2018 Accepted March 18, 2019

CHEMICAL-NUTRITIONAL COMPOSITION, MICROBIOLOGICAL ANALYSIS AND VOLATILE COMPOUND CONTENT OF FOSSA CHEESE RIPENED IN DIFFERENT PITS

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ABSTRACT

Fossa cheese samples were ripened for 90 days in two different pits and analysed to evaluate the influence of the environment on the chemical and nutritional characteristics.

The significant changes were recorded only for certain parameters, particularly the contents of fatty acids and volatile molecules. In the fatty acid profiles, the sum of monounsaturated fatty acids showed a significant decrease in the mature cheeses due to a strong decrease in oleic acid. Even the sum of polyunsaturated fatty acids and the ratio between the sums of saturated fatty acids and polyunsaturated fatty acids decreased after ripening in both pits.

HP-SPME-GC/MS analysis allowed the identification of 77 volatile compounds that increased in the cheese samples during ripening.

The results of this study indicated that there are substantial differences between the chemical and chemical/physical parameters, and certain fatty acids of the just curdled cheese samples and the cheese ripened in the two pits showed different geological-geochemical parameters.

Keywords: Fossa cheese, microclimate conditions, cheese composition, microbiological analysis, volatile compounds

1. INTRODUCTION

Fossa cheese, literally pit cheese, is a typical Italian product of the Montefeltro area, specifically from Talamello, Sogliano al Rubicone and other towns located in a small geographical area (the Emilia Romagna and Marche regions of central Italy) (GOBBETTI *et al.*, 1999; BARBIER *et al.* 2012).

The cheese is variously produced from sheep, bovine or a mix of sheep and bovine milk; it is a hard cheese, produced in limited quantities (approximately 200 tons/year), and it has great economic importance in its market niche. The first phase of maturation takes place at dairy farms for a period of approximately 60 days at 6-14°C and a relative humidity of 75-92 %, after which it is aged for a period of 90-100 days in pits dug in tuffaceous rock sanitized by fire and smoke. Wooden boards are laid on the bottom of the pit forming a floor, and the pit walls are lined with a 15-20-cm-thick layer of straw before the cheeses are placed inside. The pits are then filled with the cheeses and hermetically sealed from August to November (AVELLINI *et al.*, 1999). The denomination of protected origin "Formaggio di Fossa di Sogliano al Rubicone and Talamello" is reserved for cheese that meets the requirements of this specification.

When put on the market, the "Fossa cheese from Sogliano al Rubicone and Talamello" DOP has the following characteristics: the colour of the outer part of the finished product varies from ivory white to amber yellow. At the end of ripening, the cheese exhibits irregular forms, with typical bumps and depressions. The cheese surface is primarily wet and greasy and, in some cases, may be covered by butterfat and mould that is easily scraped off. The presence of small cracks and possible yellow ochre stains, more or less intense, on the surface, fits all the characteristics of the product. A skin is absent or barely visible.

The internal consistency is easily friable, with a white or slightly yellow amber colour. The smell is typical and lingering, sometimes intense, with a rich aroma reminiscent of woodland undergrowth with hints of mould and truffles. The aging process gives the product its unique, highly appreciated flavour, which is different from that of cheeses not aged in pits.

The geological characteristics of the pits play a key role in the process of cheese ripening and affect the quality.

The present study aimed to test the influence of the geological-geochemical nature of two different pits on the chemical, microbiological, nutritional and olfactory properties of a product in the Italian culinary tradition, very peculiar in its organoleptic characteristics, which are intimately related to the process of ripening (GOBBETTI *et al.*, 1999).

The two geographical areas in which cheese ripening took place were those of Talamello (Province of Rimini, Emilia-Romagna Region) and Cartoceto (Pesaro-Urbino Marche Region).

The first area is characterized by soils composed of bipolar cross-laminated sandstone-type medium- to coarse-grained "fishbone" material, belonging to the formation of "Arenarie di Monte Perticara" (Pliocene).

The second area is characterized by soils composed of very thick arenaceous layers intercalated with thin pelitic layers belonging to "Formazione a Colombacci" (Miocene superiore).

2. MATERIAL AND METHODS

2.1. Cheese samples

The Fossa cheese was produced in a single cheesemaking process (Valmetauro Fattorie Marchigiane - Amandola, FM, Italy) at the same dairy farm and ripened for 60 days. After this period, two Italian companies located in Emilia Romagna (Talamello) and Marche (Cartoceto) provided the cheese ripening environment as imposed by the Consortium of Fossa cheese regulations (GOBBETTI *et al.*, 1999); the samples previously ripened at the dairy farm were equally distributed in the companies' respective pits for 90 days. The analyses were performed in triplicate on each different batch consisting of three distinct samples. In this paper, the following samples were analysed: sheep milk, cheese after curdling, cheese ripened at the dairy farm for sixty days and after ripening in two different pits for ninety days.

All samples were homogenized with a laboratory mixer (MSM87160 MaxoMixx, Bosch GmbH, Germany) before being subjected to the chemical analyses. All analyses were performed in triplicate.

2.2. Microclimate measurements

Microclimate measurements were carried out using relative humidity and temperature sensors (WM33 and 52, Michell Instruments) located at the surface and the bottom of each pit. A digital signal converter and a PC transformed the sensor signal to be compatible with a specific software program developed to acquire and store data. To analyse the microclimate dynamics inside the pits regularly, the downloaded data were controlled weekly using a UMTS/GPRS modem and the remote control software "TeamViewer".

2.3. Chemical-physical analysis

2.3.1 Dry matter method

The free water content in the samples was determined by drying an aliquot (5 g) of the sample to a constant weight in an oven at 105°C. The weight loss corresponded to the loss of moisture. The result was expressed as a percentage (AOAC, 1990).

2.3.2 Ash methods

The sample (approximately 2 g) was dried in an oven at 100°C and thereafter calcinated in a muffle at 525°C; the weight obtained after calcination was the ash content (AOAC, 1990).

2.3.3 pH determination

The pH determination was performed by potentiometric analysis. The pH of the milk was measured without dilution; regarding the cheese samples, 100 mL of distilled water previously brought to a boil was added to 10 g of cheese, and the mixture was vortexed on a magnetic plate for 15 min.

The mixture was centrifuged for 5 min and left to decant to separate the supernatant. Finally, the pH of the supernatant was measured (AOAC, 1990).

2.4. Determination of the total protein content

The total nitrogen content of the samples was determined with the Kjeldahl method (AOAC, 1990).

One gram of homogenized sample was digested inside an appropriate reaction tube with 10 mL of sulfuric acid (96 %), 5 mL of hydrogen peroxide (30 %) and a catalyst based on copper sulphate pentahydrate and heated at a high temperature (250°C) to destroy all the organic material. Afterwards, 50 mL of distilled water and 50 mL of sodium hydroxide (30 %) were added. Adding an excess of sodium hydroxide solution, the ammonium ions were released in the form of ammonia, distilled and added to a boric acid solution. The ammonia content was determined with a volumetric acid solution or by back titration with a sodium hydroxide solution of a known concentration. The percentage of total protein was calculated using a conversion factor of 6.38.

2.5. Determination of the sodium chloride content (the Mohr method)

Approximately 2 g of the dried (in an oven at 105°C until constant weight) sample (cheese and milk) was added to 40 mL of bi-distilled water for 2 h under stirring at room temperature, followed by centrifugation for 10 min at 2683 g, and finally filtered.

The pH of the solution was adjusted with 0.1 N sodium hydroxide up to a value of 8.0. Twenty millilitres of distilled water containing 5 drops of a 5 % K_2CrO_4 indicator was added to the mixture, which was then titrated with 0.1 N silver nitrate until the colour changed (from white to brick red).

Twenty millilitres of sample with a few drops of indicator was titrated with silver nitrate until the colour changed from yellow to red brick (Johnson and Olson, 1985). When the silver chloride was completely precipitated, the excess of titrant formed a silver chromate precipitate, which indicated the end point.

2.6. Extraction of the total lipids from milk

After vortexing for 90 seconds, 300 mL of a solution composed of a dichloromethane:ethanol mix in a 2:1 ratio (v/v) was added to 30 g of sample, and the mixture was centrifuged for 10 min at 2683 g. The supernatant was removed, and the extraction was repeated twice (STEFANOV *et al.*, 2010).

The lower organic phase was recovered and filtered into a round bottom flask, and the dichloromethane was removed using a rotary evaporator at 35°C (model Hei-VAP Value; Heidolph, Schwabach, Germany). The residue was placed in a drier and regularly weighed until a constant value was reached.

2.7. Extraction of the total lipids from cheese

Forty millilitres of hydrochloric acid (25 %) and 40 mL of ethanol (95 %) were added at approximately 12 g of sample. The mixture was stirred for 30' in a water bath at 50°C. After cooling, 100 mL of a solution of n-heptane:diethyl ether (1:2, v/v) was added, and the mixture was maintained under stirring for 15 min at room temperature.

Then, the mixture was allowed to decant, and the supernatant (organic phase) was recovered. The extraction procedure was carried out three times, and the supernatant was gathered. The solvent was removed by a rotary evaporator (model Hei-VAP Value;

Heidolph, Schwabach, Germany) (ROMANO *et al.*, 2011). The residue was placed in a drier and regularly weighed until a constant value was reached.

2.8. Fatty acid methyl ester analysis

Fatty acid methyl esters (FAMES) were prepared according to AOAC 996.06 (2011). Briefly, to 200 mg of a lipid extract, 2 mL of a 1.25 M HCl/CH₃OH solution was added and the mixture was heated for 60 min at 90°C. Then, the methyl esters were extracted with 1 mL of *n*-hexane and 1 µL of the methyl esters was injected in a TRACE GC Ultra gas chromatograph (Thermo Scientific, Waltham, MA, USA) equipped with a flame ionization detector (FID) and SP-2560 capillary column (100 m × 0.25 mm × 0.20 µm, Supelco, Bellefonte, PA, USA).

Helium was used as the carrier gas with a constant flow rate of 1.5 mL min⁻¹. The samples were introduced with a split-splitless injection system in split mode (ratio 1:100) using an AS 3000 autosampler (Thermo Scientific, Waltham, MA, USA). The operating conditions that were followed corresponded to those observed by SIANO *et al.* (2016).

The ramp started at a temperature of 140°C, which was stabilized for 5 min; the temperature was increased at a rate of 4°C per minute up to a temperature of 240°C for 15 min. The run lasted 45 min. The temperature of the injector and detector was 260°C.

To perform the qualitative and quantitative analysis, the retention times of the fatty acids detected in the cheese samples were compared with those of a mixture of fatty acid methyl esters (FAME Mix-37, Supelco, Bellefonte, PA, USA).

2.9. Microbiological analysis

A microbial analysis of the milk used in the Fossa cheesemaking process was not performed because, according to the production regulations, the milk had been pasteurized at 72°C for 15 min, cooled, inoculated with the selected starter and added to the rennet calf powder.

Under sterile conditions, 10 g of each cheese sample was placed in sterile stomacher bags, 90 mL of a sterile peptone-saline solution (bacteriological peptone 0.1 %; NaCl 0.85 %) was added, and the mixture was homogenized in a Stomacher apparatus (Lab-Blender 400, PBI International, Italy). The homogenates were serially diluted 10-fold. To count the bacterial population, the following media, and temperature and time conditions of incubation were used.

One millilitre of each dilution was inoculated on MRS agar and M17 agar plates (Oxoid, Thermo Fisher, Italy), incubated under anaerobic conditions (Anaerogen, Oxoid, Thermo Fisher, Italy) at 28°C for 72 h. *Lactobacillus* and *Lactococcus* spp. in Plate Count Agar (PCA) (Oxoid, Thermo Fisher, Italy) and incubated at 28°C for 72 h, to enumerate the total microbial mesophilic bacteria (TMC). The sulphite-reducing clostridia (SRCs) on SPS agar plates incubated under anaerobic conditions at 28°C for 5 days were counted; the total and faecal coliforms were evaluated on Violet Red Bile Glucose agar and Violet Red Bile Lactose Agar (Oxoid, Thermo Fisher, Italy) after incubation at 36 and 44°C, respectively, for 48 h. In addition, 100 µL of the diluted solution was streaked onto mannitol salt agar plates (Oxoid, Thermo Fisher, Italy) and incubated at 28°C for 3-5 days to count *Micrococcaceae* and on YPD plates (yeast extract 1 %; bacteriological peptone 2 %; dextrose 2 %; and agar 2 %), incubated at 28°C for 3-5 days, for the detection of yeasts and moulds. To evaluate the bacterial load of the Fossa cheese, the plates that contained between 15 and

300 colonies were counted. The values obtained were expressed as the colony forming units of a gram of sample (CFU/g). The microbial counts were carried out in triplicate.

2.10. Determination of the profile of volatile molecules

The extraction of volatile compounds was carried out using the headspace solid-phase microextraction technique (HS-SPME) combined with gas chromatography paired with mass spectrometry (HS-SPME-GC/MS). Five grams of sample (pre-equilibrated to 45°C for 10 min) was weighed in vials of 20 mL containing 5 µL of 3-octanol (internal standard, 100 mg/L standard solution) and the volatile compounds (VOCs) were extracted from the samples by a fibre DVB/CAR/PDMS; the compounds were held for 45 min and block heated to 45°C in the headspace of the sample. The analysis of the volatile compounds was performed using an Agilent 7890A/5975C GC/MS with a Gerstel MPS2 autosampler, using an INNOWax capillary column (30 m × 0.25 mm × 0.50 µm) and the following temperature programme: 40°C for 2 min, 5°C/min to 230°C for 10 min. The injector, quadrupole, source and transfer line temperatures were 240, 150, 230 and 200°C, respectively. The electron ionization mass spectra in full-scan mode were recorded at an electron energy level of 70 eV in the range of 20-400 amu (2 s/scan). The volatile molecules were identified by comparing the recorded values to the mass spectra present in the Wiley 07/NIST 98 libraries and the retention index present in the database or in the literature. Afterwards, to calculate the RI value of the compounds, the *n*-alkanes (C5-C25) were also analysed under the same conditions using GC-MS (VAN DEN DOOL and KRATZ, 1963). The results were expressed as the relative peak area (RAP) with respect to the internal standard.

2.11. Statistical analysis

The experimental data (the moisture content, ash, pH, lipid content and sodium chloride concentration) were statistically analysed using Statistica software version 10 (Statsoft, Tulsa, USA). One-way repeated measures analysis of variance (RM ANOVA) was used to estimate the significant differences during the manufacture and ripening of the cheeses. To isolate the group or groups that differed from the others, multiple comparisons versus control group (the Holm-Sidak method) were used. ANOVA followed by Kruskal-Wallis one-way analysis of variance on ranks was used to estimate the differences in the fatty acid content ($P < 0.05$). The averages and the standard deviations were calculated with Microsoft Office Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Microclimate measurements

Microclimate measurements were performed during the cheese ripening in both pits (78 days, in the *Talamello* pit and in *Cartoceto* pit). The results are shown in Figs. 1 and 2. Different microclimate characteristics were found between the pits. In *Talamello*, the pit temperature remained constant over time at both depths (18°C at the surface and 20-21°C at the bottom), while in the *Cartoceto* pit, the temperature increased over time (increasing from 12°C to 23°C at the surface and from 17°C to 27°C at the bottom). On the other hand, the relative humidity was variable at all depths and in both pits. In the *Talamello* pit, the

surface values were in the range of 88 to 99 % and the bottom values varied between 85 and 90 %, whereas in the *Cartoceto* pit, the relative humidity ranged between 90 and 99 % at the surface and between 80 and 88 % at the bottom.

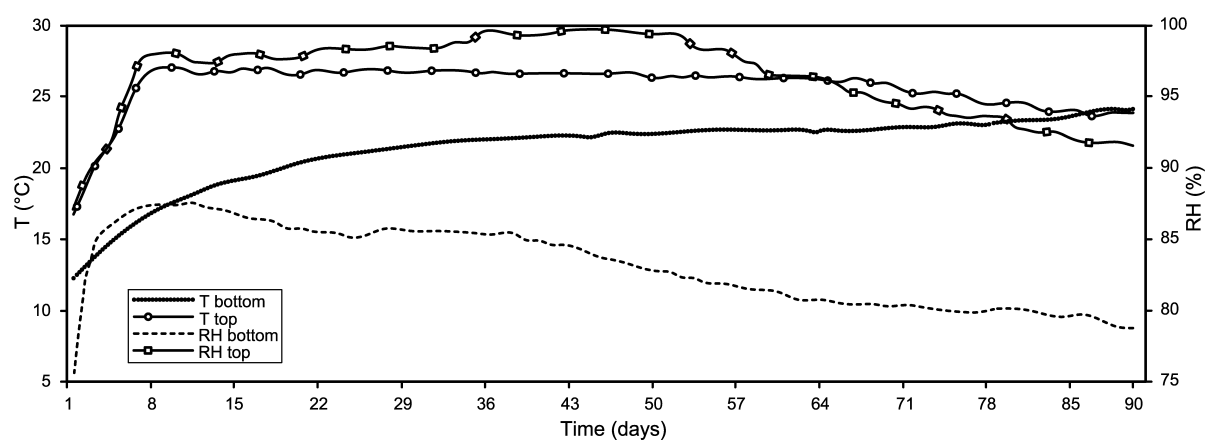


Figure 1. Trend of the temperature and humidity inside the Talamello pit.

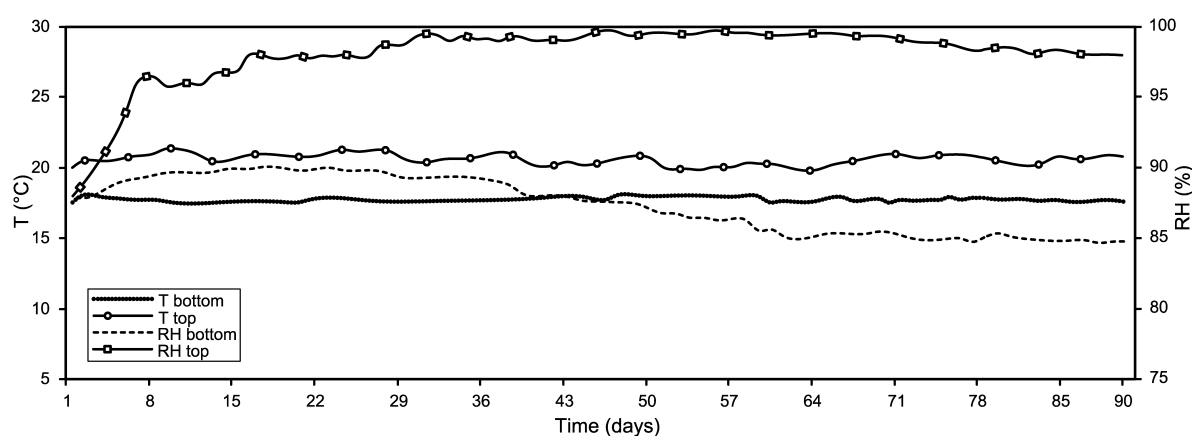


Figure 2. Trend of the temperature and humidity inside the Cartoceto pit.

3.2. Chemical-nutritional composition

The average values ($\pm$ SD) of the pH, the moisture, lipid, and protein content, and the ash and salt concentration of the Fossa cheese samples are shown in Table 1. RM ANOVA showed significant differences between treatments ($F = 18.642$ with 3 degrees of freedom, $P = 0.004$). Multiple comparisons versus control group (the Holm-Sidak method) showed significant differences in the comparison of “cheese ripened at the dairy farm for sixty days” versus “cheese from the *Talamello* site” ($P = 0.004$) and “cheese ripened at the dairy farm for sixty days” versus “cheese from the *Cartoceto* site” ($P = 0.027$); in contrast, there was no statistically significant difference in the comparison between “cheese from the *Talamello* site” versus “cheese from the *Cartoceto* site” ($P > 0.05$).

The pH of the milk was 6.51, while the pH of the cheese curds decreased to 5.43, a value similar to that of the Talamello-ripened cheese and slightly different from that of the Cartoceto-ripened cheese (5.19).

The protein, lipid, ash and NaCl content of the cheese after ripening in the two pits increased significantly with respect to the cheese curd values, but no significant differences were recorded between the ripened cheese in the two pits and the cheese ripened at the dairy farm for sixty days.

Table 1. The gross composition of the Fossa cheese during production and ripening.

	Milk	Cheese after curdling	Cheese ripened at the dairy farm for sixty days	Cheese from the Talamello site	Cheese from the Cartoceto site	Statistical significance
pH	6.51±0.02	5.43±0.04	5.42±0.01	5.42±0.27	5.19±0.15	ns
Moisture (%)	83.02±4.98	42.33±1.52 ^a	35.46±1.28 ^b	34.33±6.71 ^b	32.59±3.81 ^c	**
Lipid ¹ (%)	33.92±5.71	49.26±2.75 ^a	52.13±4.19 ^b	51.25±2.40 ^b	51.10±2.20 ^b	*
Protein ¹ (%)	32.27±0.59	32.35±5.77 ^a	42.10±3.95 ^b	40.61±6.58 ^c	40.32±3.50 ^c	*
Ash ¹ (%)	2.35±1.88	5.10±0.78 ^a	5.77±1.08 ^b	8.14±0.61 ^c	8.58±0.67 ^c	*
NaCl ¹ (%)	0.00±0.00	2.25±0.00 ^a	4.91±0.01 ^b	6.85±0.56 ^c	6.67±0.50 ^c	*

a, b, c, d: The different letters in the same row indicate the statistically significant differences ($P < 0.05$).

ns = not significant, * $P < 0.05$; ** $P < 0.01$. ¹The concentrations are expressed in terms of the dry matter.

The fatty acid contents in the cheese samples during the production and ripening, together with the results of the variance analysis, are shown in Table 2. Significant differences between the free fatty acid contents of the Fossa cheese samples during the production time and the ripening phase ($F = 81.093$ with 20 degrees of freedom, $P \leq 0.001$) were observed.

For all types of cheese, the major saturated fatty acids (SFA) were myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acid; all the cheese showed highly significant different values ($P < 0.001$) during the production time. Moreover, cheese ripening in the *Talamello* pit resulted in high values of SFA compared to cheese from the *Cartoceto* pit.

Three monounsaturated fatty acids (MUFA) were identified: myristoleic (C14:1), palmitoleic (C16:1), and oleic (C18:1 ω -9,*cis*) acid. The most important differences observed in the content of MUFA were the strong decreases during ripening, up to values of approximately 15%.

Among the polyunsaturated fatty acids (PUFA), only linoleic (C18:2 ω -6,*cis*) and linolenic (C18:3 ω -3) acid were identified, but only the C18:2 ω -6,*cis* content was significantly different during ripening ($P < 0.001$).

In particular, the fatty acid profile of the cheese after ninety days of ripening in the pits showed a percentage increase of the butyric, caproic, caprylic and capric acid content compared to the cheese after sixty days of ripening at the dairy farm, with respect to just the cheese curds, confirming the fact that the short-chain fatty acids are produced by lipolysis phenomena; in parallel, a decrease in the percentage of long chain fatty acids (oleic, linoleic and linolenic acids) in the cheese after sixty days at the dairy farm was observed compared to the cheese after ninety days of ripening in the pits, with respect to

just the cheese curds. The values of the long chain fatty acid content were almost constant except for oleic acid and palmitic acid, as suggested by various authors (OLMEDO and COLL-HELLIN, 1976; NAJERA *et al.*, 1993), highlighting the higher values in linoleic and linolenic acids.

The sum of saturated fatty acids showed no substantial differences between the milk and mature cheese, even if there was a decrement measured in the cheese curds.

Instead, the sum of MUFA showed a significant decrease from 21.39 % in the milk to approximately 16.70 % in the ripened cheeses. According to ALEWIJN *et al.* (2005), this decrease is due to the conversion of fatty acids into aldehydes, ketones, and lactones as a result of β -oxidation followed by decarboxylation. The most significant decrease was observed for the oleic and stearic acids, the main components of the total fatty acids in the analysed samples.

Even the sum of PUFA and the ratio between the sums of PUFA and SFA decreased after ripening in the pits at both sites.

Table 2. Concentration of fatty acids in the milk and Fossa cheese expressed in %.

	Milk	Cheese after curdling	Cheese ripened at the dairy farm for sixty days	Talamello Cheese	Cartoceto Cheese	Statistical significance
Butyric C4:0	1.80±0.12	0.68±0.08	1.33±0.10	2.10±0.14	1.76±0.10	**
Caproic C6:0	1.92±0.15	1.04±0.12	1.61±0.09	2.17±0.11	1.77±0.12	**
Caprylic C8:0	1.96±0.09	1.42±0.10	1.92±0.12	2.51±0.13	2.10±0.10	**
Capric C10:0	6.06±0.25	5.13±0.26	6.24±0.26	8.10±0.22	6.86±0.24	**
Lauric C12:0	3.49±0.16	3.13±0.12	3.56±0.15	4.39±0.20	3.89±0.18	**
Myristic C14:0	10.89±0.24	10.19±0.28	10.96±0.28	11.28±0.22	11.33±0.24	**
Myristoleic C14:1	0.16±0.06	0.14±0.02	0.55±0.05	0.20±0.08	0.22±0.09	*
Pentadecanoic C15:0	1.25±0.18	1.28±0.14	1.22±0.10	1.23±0.11	1.38±0.14	**
Palmitic C16:0	26.38±0.16	25.74±0.08	26.42±0.36	25.49±0.32	25.10±0.48	**
Palmitoleic C16:1	1.06±0.10	1.39±0.05	0.99±0.08	0.76±0.10	0.79±0.12	*
Heptadecanoic C17:0	0.78±0.08	0.87±0.04	0.83±0.08	0.73±0.04	0.83±0.10	*
Stearic C18:0	11.83±0.66	11.56±0.78	11.56±0.65	9.98±0.88	10.29±0.87	**
Oleic C18:1 ω -9, <i>cis</i>	20.17±0.95	21.89±0.86	21.03±0.88	15.43±0.98	15.92±0.77	**
Linoleic C18:2 ω -6, <i>cis</i>	2.24±0.22	2.44±0.42	2.37±0.36	1.89±0.42	1.80±0.37	**
Arachidic C20:0	0.34±0.04	0.40±0.08	0.39±0.09	0.28±0.02	0.33±0.07	*
Linolenic C18:3 ω -3	1.47±0.21	1.54±0.23	1.44±0.26	0.89±0.23	1.15±0.18	ns
Σ -SFA	66.70±1.14	61.44±1.37	66.04±1.28	68.26±1.77	65.63±1.98	
Σ -MUFA	21.39±1.51	23.42±1.22	22.57±1.29	16.39±1.61	16.93±1.01	
Σ -PUFA	3.71±0.15	3.98±0.38	3.81±0.32	2.77±0.41	2.95±0.28	
Σ -PUFA/ Σ -SFA	0.06	0.06	0.06	0.04	0.04	
Oleic C18:1 ω -9, <i>cis</i>	9.00	8.97	8.87	8.18	8.83	
Linoleic C18:2 ω -6, <i>cis</i>						

Note: *, ** indicate the significant differences during ripening, with $P < 0.05$ and $P < 0.001$; ns = not significant.

3.4. Microbiological analysis

The data on the microbial population of the different Fossa cheese samples are shown in Fig. 3.

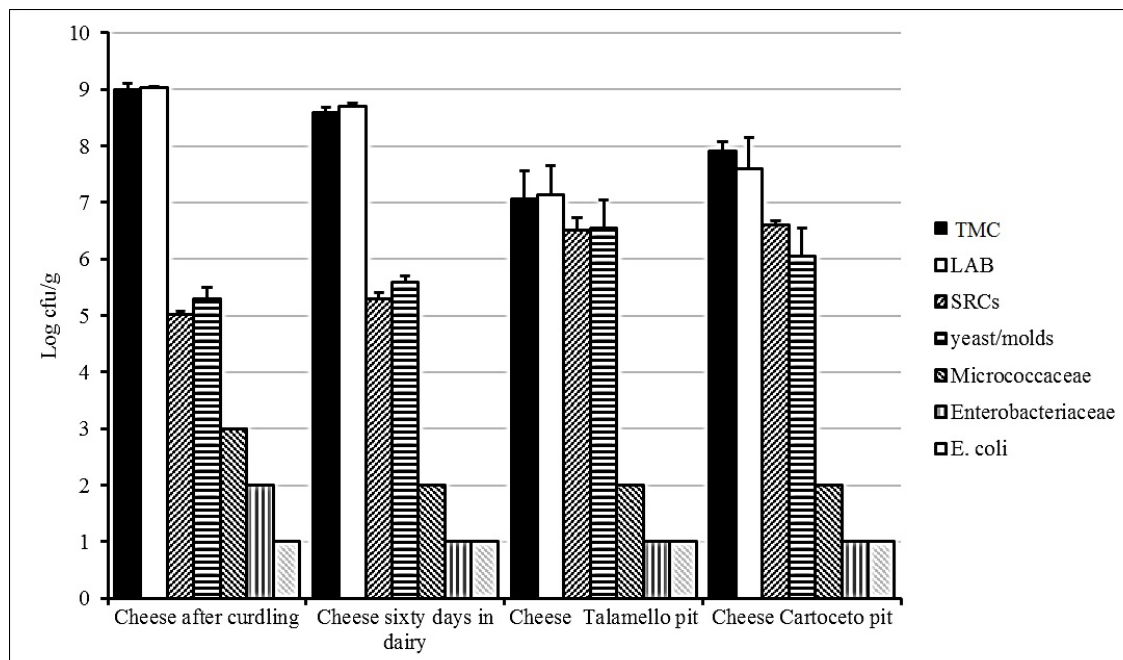


Figure 3. Bacterial dynamics of the principal microbial groups in the Fossa cheese during the manufacturing and ripening process in the pits located in different geographical areas. Data are the means $\pm$ SD of the three cheese samples.

In summary, in the cheese ripened for 24 h and sixty days, the total microbial count (TMC) was between 10^9 and 10^8 CFU/g. However, the mesophilic lactic microflora count was approximately 10^9 and 10^7 CFU/g; in this case, the higher load of lactic acid bacteria (LAB) and, consequently, the higher total bacterial count were related to the addition of the starter during cheesemaking, and the content of sulphite-reducing clostridia (SRCs) was approximately 10^5 CFU/g. Instead, the contents of the yeast and moulds grown during ripening were approximately (on the order of) 10^4 and 10^5 CFU/g, respectively. A very low load of *Micrococcaceae* was detected in the Fossa cheese, as well as coliforms and *Escherichia coli*. Moreover, in the cheeses that were ripened for 3 months in underground pits (fossa) located at two different sites (*Talamello* and *Cartoceto*), the microbial population showed certain changes in the order of magnitude relative to the total mesophilic microbial count, which dropped from 10^9 to 10^7 CFU/g in the cheese of the Talamello pit, while the count decreased from 10^9 to 10^8 CFU/g in the Cartoceto cheese; the same trend was recorded for the lactic microflora (lactobacilli and lactococci).

The high relative humidity, fairly high temperature and size of the pit environment may influence the oxygen pressure during ripening and may affect the metabolic pathways of cheese microflora that characterize the finished product. Through enzymatic processes, the

elimination of fat and residual moisture occurs, and at the same time the drying of the product is limited (POZZETTO, 2000).

Conversely, the content of sulphite-reducing clostridia increased up to one order of magnitude, and the same trend was shown by yeast and mould contents, probably because of the cheese ripening process and the pit habitat benefitting their growth compared to the other microbial components. Finally, Enterobacteriaceae and *Escherichia coli* were not detected in the 10 g cheese samples. These results suggest that the content of Fossa cheese microflora during ripening decreased because the chemical-physical parameters changed. In fact, during maturation, the cheeses undergo a considerable decrease in weight (approximately 20 %) and take on irregular shapes. The surface of the shapes is wet and greasy, and in some cases, the surface is covered by mould; a skin is absent (GOBBETTI *et al.*, 1999). The microflora involved in the Fossa cheese-making process were composed of starter cultures and native microflora that played important roles during the manufacturing of the cheese at the dairy farm and during its ripening in the pit. In particular, the starter microbiota carried out a rapid acidification by the production of lactic acid but also produced enzymes that are important for flavour development during ripening (LEROY and DE VUYST, 2004). Furthermore, non-starter lactic acid bacteria (NSLABs), which are complex mixtures of bacteria, yeasts and moulds, play an important role, together with environmental factors, in achieving the specific characteristics of cheese varieties (FOX and WALLACE, 1997; BERESFORD *et al.*, 2001). Additionally, filamentous fungi may reach the cheeses in the environment of the natural caves during ripening (LOPEZ-DIAZ *et al.*, 1996; BUDAK *et al.*, 2016). The content of non-starter lactic acid bacteria usually increases from a low number in fresh curds to eventually dominate the microflora in the mature cheese because the bacteria tolerate the hostile environment during the cheese ripening well. Furthermore, the heterogeneity of the NSLAB strains together with a pool of enzymatic activities, such as proteolytic and lipolytic activities, may determine a higher complexity in cheese flavour (FOX *et al.*, 1998; MCSWEENEY and SOUSA, 2000; DE ANGELIS *et al.*, 2001).

3.5. Volatile compounds

The HP-SPME-GC/MS analysis of the cheese samples allowed the identification of 77 compounds belonging to seven groups of volatile compounds. In this work, we quantified 5 aldehydes, 16 ketones, 15 esters, 16 alcohols, 12 acids, 7 terpenes, 3 lactones and 3 S-compounds. The relative amounts of the individual compounds were expressed in terms of their relative peak area (RAP) (Table 3). The total quantities of the volatile compounds typically increase in almost all cheeses during ripening while the profile changes (MASSOURAS *et al.*, 2006). Additionally, the addition of spice plants during cheese manufacturing enhances the volatile compounds both qualitatively and quantitatively (CAKIR *et al.*, 2016). Aliphatic aldehydes (hexanal, heptanal and nonanal) are transitory compounds and do not accumulate in cheese because they are rapidly transformed to alcohols or to the corresponding acids (HAYALOGLU *et al.*, 2007). Branched chain aldehydes are normally found in cheese, 3-methylbutanal is formed by the Strecker degradation of Leu amino acid (URBACH, 1995) and has been found to be a potent odour compound in different cheese varieties (CURIONI and BOSSET, 2002; HAYALOGLU *et al.*, 2007). Benzaldehyde, mainly derived from the metabolism of phenylalanine in cheese, which has the aromatic note of bitter almond, is commonly found in cheese and is formed by the oxidative reactions of cinnamic acid or phenylacetaldehyde (MOLIMARD and SPINLER, 1996).

Table 3. Relative peak area (area of the compound/IS area)×100±the standard deviation for the volatile compounds of the Fossa cheese (n = 3).

		Cheese after curdling	Cheese ripened at the dairy farm for sixty days	Cheese from the <i>Talamello</i> site	Cheese from the <i>Cartoceto</i> site	Odour description*
RI	<i>Aldehydes</i>					
1165	hexanal	13.7±0.5 ^b	38.7±0.2 ^a	nd	nd	Herbaceous
1264	heptanal	20.3±0.9 ^b	73.8±3.8 ^a	nd	nd	Sour milk
1466	nonanal	26.0±0.03 ^a	10.5±0.07 ^b	nd	nd	Floral, citrus
1009	butanal, 3-methyl	nd	nd	2.7±0.2 ^b	4.2±0.1 ^a	Mild, oil
1589	benzaldehyde	nd	nd	44.9±4.9 ^a	7.9±0.5 ^b	Sweet
	<i>Ketones</i>					
872	2-propanone	208.3±6.7 ^b	240.9±2.3 ^a	101.7±7.2 ^d	189.0±5.2 ^c	Apple, pear
993	2-butanone	20.8±0.1 ^a	44.5±0.5 ^c	33.4±0.5 ^b	41.3±2.0 ^c	Chemical, fruity
1066	2-pentanone	39.3±0.9 ^d	965.5±31.3 ^a	263.3±4.6 ^c	501.7±35.2 ^b	Sweet, floral
1069	2,3-butanedione	100.1±7.2 ^a	82.2±1.5 ^b	nd	nd	Fruity, buttery
1165	2-hexanone	nd	nd	12.2±0.4 ^a	7.6±0.2 ^b	Fruity, fungal
1261	2-heptanone	68.5±0.7 ^d	645.8±27.1 ^c	1254.7±19.1 ^a	1034.9±29.5 ^b	Blue cheese
1277	2-heptanone, 3-methyl	nd	nd	2.0±0.2 ^a	2.3±0.2 ^a	-
1303	5-hepten-2-one	nd	nd	13.1±0.4 ^a	2.9±0.1 ^b	-
1359	2-octanone	nd	nd	22.3±1.7 ^b	28.1±0.9 ^a	Dairy, waxy
1360	acetoin	342.8±18.6 ^a	159.1±7.1 ^b	4.7±0.2 ^c	7.3±0.03 ^d	Cream, dairy
1410	6-methyl-5-hepten-2-one	nd	2.7±0.04 ^a	1.1±0.1 ^b	2.5±0.1 ^a	Citrus
1461	2-nonanone	31.3±1.6 ^d	438.4±16.6 ^c	1139.6±81.4 ^a	1294.4±80.4 ^a	Fruity, floral
1497	5-nonen-2-one	nd	nd	2.1±0.2 ^b	3.8±0.2 ^a	Fruity
1512	8-nonen-2-one	nd	nd	67.6±3.6 ^a	94.9±2.6 ^b	Fruity, baked
1556	2-decanone	nd	nd	7.2±0.2 ^a	7.3±0.2 ^a	Orange, fatty
1663	2-undecanone	nd	17.9±0.1 ^c	56.8±1.1 ^a	43.5±0.8 ^b	Waxy, fruity
	<i>Esters</i>					
975	ethyl acetate	63.5±3.5 ^a	45.3±0.2 ^b	nd	3.2±0.2 ^c	Fruity
1047	propanoic acid, ethyl ester	13.7±0.2 ^a	7.3±0.08 ^b	nd	nd	Fruity
1137	butanoic acid, 2-methyl, methyl ester	2.5±0.1 ^a	3.7±0.1 ^b	nd	nd	Fruity
1124	butanoic acid, ethyl ester	19.3±0.4 ^d	23.7±0.8 ^c	63.7±0.8 ^a	45.1±0.6 ^b	Fruity, cheesy
1135	butanoic acid, 2-methyl, ethyl ester	6.7±0.5 ^a	6.5±0.4 ^a	nd	nd	-
1156	acetic acid, butyl ester	12.7±0.6 ^a	12.3±0.3 ^a	nd	nd	Fruity
1253	butanoic acid, 2-propenyl ester	nd	nd	0.9±0.1 ^a	1.1±0.03 ^a	Fruity, green
1286	butanoic acid, 1-methyl, butyl ester	nd	nd	1.0±0.1 ^a	0.8±0.01 ^a	Fruity
1310	hexanoic acid, ethyl ester	8.2±0.03 ^d	20.7±0.1 ^c	28.0±1.8 ^a	25.1±0.6 ^b	Sweet, pineapple
1390	butanoic acid, 3-methyl, butyl ester	2.8±0.02 ^b	3.4±0.1 ^a	nd	nd	Apple, fruity
1505	octanoic acid, ethyl ester	nd	7.2±0.06 ^c	24.1±0.8 ^a	9.7±0.4 ^b	Sweet, fruity
1701	decanoic acid, ethyl ester	nd	nd	33.8±1.8 ^a	13.6±0.3 ^b	Waxy, fruity
1423	formic acid, hexyl ester	4.0±0.3 ^a	4.1±0.1 ^a	nd	nd	Ethereal, sweet
1348	acetic acid, hexyl ester	11.6±0.5 ^a	16.3±0.1 ^b	nd	nd	Fruity, green
1942	butanoic acid, propyl ester	nd	nd	10.2±0.5 ^b	19.5±0.9 ^a	Sweet, fruity

<i>Alcohols</i>						
1023	ethanol	440.9±13.3 ^a	343.8±1.7 ^b	169.8±17.0 ^c	204.9±13.0 ^d	Alcoholic
1226	1-butanol	8.7±0.1 ^a	7.5±0.1 ^b	1.3±0.1 ^d	3.5±0.2 ^c	Banana, fusel
1230	isobutanol	nd	nd	3.4±0.3 ^b	9.4±0.1 ^a	Fusel
1208	2-pentanol	nd	nd	44.2±2.7 ^b	88.1±4.0 ^a	Mild, green
1284	3-methyl-1-butanol	12.0±0.1 ^b	42.3±0.2 ^a	2.07±0.1 ^c	1.9±0.1 ^d	Fusel, fermented
1297	1-hexanol	nd	nd	1.5±0.1 ^a	1.6±0.1 ^a	Green, fruit
1324	1-pentanol	25.6±0.9 ^a	21.3±0.5 ^b	2.6±0.2 ^c	0.9±0.02 ^d	Fusel, fermented
1391	2-heptanol	nd	nd	71.1±3.4 ^a	58.7±2.6 ^b	Fresh, lemon
1392	3-methyl-2-buten-1-ol	15.9±1.3 ^a	9.4±0.1 ^b	nd	nd	-
1518	1-octen-3-ol	7.7±0.03 ^b	11.2±0.1 ^a	nd	nd	Mushroom, earthy
1556	2-ethyl-hexanol	6.8±0.2 ^a	5.1±0.01 ^b	nd	nd	Sweet, fatty
1638	2,3-butandiol	18.4±0.9 ^b	68.5±0.4 ^a	nd	nd	Fruity, creamy
1640	2-octanol	nd	nd	1.5±0.1 ^b	5.6±0.3 ^a	Fresh, woody
1647	2-nonanol	nd	nd	51.4±3.9 ^a	41.9±1.2 ^b	Waxy, green
1721	2-furanmethanol	nd	nd	1.6±0.01 ^b	4.1±0.2 ^a	Burnt, sweet
1016	2-propanol	nd	nd	7.3±0.8 ^b	18.2±0.4 ^a	Must, woody
<i>Acids</i>						
1524	acetic acid	141.4±4.9 ^c	249.3±16.5 ^a	95.9±3.2 ^d	154.0±5.8 ^b	Pungent, sour
1610	propanoic acid	nd	2.7±0.1 ^c	4.8±0.3 ^b	16.1±0.3 ^a	Acidic, dairy
1634	propanoic acid, 2-methyl	nd	nd	nd	5.5±0.2 ^a	Sour, cheese
1695	butanoic acid	132.2±8.9 ^d	1022.9±58.5 ^c	1386.9±78.5 ^b	1902.1±69.6 ^a	Sharp, cheese
1735	butanoic acid, 3-methyl	nd	nd	7.8±0.3 ^a	6.3±0.2 ^b	Cheesy, dairy
1736	pentanoic acid	nd	11.6±0.3 ^c	20.4±0.6 ^a	19.6±0.3 ^b	Sweet, rancid
1909	hexanoic acid	120.8±3.8 ^d	584.3±35.3 ^c	1337.4±72.9 ^b	2028.3±100.1 ^a	Sickening, sour
2012	heptanoic acid	5.9±0.03 ^c	6.3±0.05 ^c	12.3±0.6 ^b	15.1±0.4 ^a	Rancid, cheese
2117	octanoic acid	24.3±0.6 ^d	44.3±0.6 ^c	346.1±8.5 ^b	444.3±14.9 ^a	Fatty, waxy
2220	nonanoic acid	nd	nd	6.1±0.2 ^a	5.7±0.3 ^a	Waxy, dirty
2326	decanoic acid	nd	7.5±0.06 ^c	115.7±6.8 ^a	74.6±1.5 ^b	Fatty
2384	9-decenoic acid	nd	nd	3.1±0.03 ^a	2.7±0.03 ^a	Waxy, green
<i>Terpenes</i>						
1119	dihydromyrcene	22.7±1.5 ^a	21.4±0.1 ^a	2.1±0.1 ^c	2.4±0.1 ^b	Citronellol, herbal
1178	p-menth-4(8)-ene	43.8±1.4 ^a	45.4±0.7 ^a	nd	2.7±0.2 ^b	-
1238	α-phellandrene	11.4±0.3 ^a	6.4±0.1 ^b	1.5±0.1 ^c	1.4±0.03 ^c	Citrus, lime
1272	limonene	152.6±12.7 ^b	360.6±7.6 ^a	6.3±0.2 ^c	2.3±0.1 ^d	Pine, peppery
1107	α-pinene	20.4±0.1 ^a	20.5±0.1 ^a	1.2±0.1 ^c	1.9±0.1 ^b	Woody, pine
1319	γ-terpinene	3.7±0.02 ^a	4.7±0.1 ^b	nd	nd	Citrus, lime
1195	sabinene	2.2±0.1 ^b	3.4±0.1 ^a	nd	3.7±0.04 ^c	Woody, citrus
<i>Lactones</i>						
1766	γ-caprolactone	nd	5.4±0.5 ^c	7.0±0.3 ^b	13.0±0.5 ^a	Herbal, coconut
2246	δ-decalactone	nd	nd	2.6±0.3 ^a	4.2±0.2 ^a	Coconut, sweet
1974	γ-octalactone	nd	nd	nd	2.4±0.1 ^a	Sweet, coconut
<i>S-compounds</i>						
761	dimethyl sulphide	35.9±0.1 ^b	44.1±0.2 ^a	nd	nd	Vegetable, dairy
1308	methyl propyl disulphide	7.4±0.03 ^b	12.8±0.5 ^a	nd	nd	Onion, radish
1958	dimethyl sulfone	10.3±0.3 ^a	6.6±0.1 ^b	0.03±0.00 ^c	2.2±0.1 ^c	Sulphurous, burnt

Mean data for the three batches of Fossa cheese, cheeses analysed in triplicate.

a, b, c, d: The different letters in the same row indicate the statistically significant differences (P< 0.05).

nd = not detected; RI= retention index; * www.thegoodscentscompany.com/

Ketones are formed by the enzymatic oxidation of fatty acids to keto-acids and their consequent decarboxylation to methyl ketones (MCSWEENEY and SOUSA, 2000). Moreover, the content of longer chain methyl ketones increases during cheese ripening. The ketones have distinctive odours and low perception thresholds. According to

GIOCCHINI *et al.* (2010), in Fossa cheese, the major ketones are 2-heptanone and 2-nonanone, which contribute to the aroma with blue cheese notes.

Different esters were detected in the volatile fraction of the Fossa cheese, namely, 7-ethyl ester, 1-propyl ester, 3-butyl ester, 2-hexyl ester, 1-propenyl ester and 1-methyl ester. Esterification reactions may occur between the short- to medium-chain fatty acids and the alcohols. Nevertheless, the esters might also be synthesized directly from triglycerides and alcohols via an alcoholysis reaction. In particular, the proliferation of esters after ripening is due to the presence of ethanol and the abundance of short-chain FFA. These compounds are probably the result of microbial metabolism of the fatty acids. They play an important role in the formation of the fruity feature and characterize the flavour of certain Italian cheeses (PANSERI *et al.*, 2008). They also contribute to the balance of the flavour by minimizing the sharpness imparted by the free fatty acids. We observed a variability in the level of esters during ripening. The more representative esters in the Fossa cheese samples that increased were butanoic acid ethyl ester, hexanoic acid ethyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester and butanoic acid propyl ester. Ethyl esters, due to their high content, probably contributed to the overall flavour of Fossa cheese because they have low detection thresholds (DELGADO *et al.*, 2010).

The alcohol class showed higher variability during ripening, and the different patterns could be associated with the different metabolic pathways involved in the formation of alcohols in cheese, namely, lactose metabolism, methyl ketone reduction, amino acid metabolism and degradation of linoleic and linolenic acids (DELGADO *et al.*, 2010).

Twelve acids were identified in the samples, and they had a positive contribution to the typical flavour in a majority of the cheeses (PANSERI *et al.*, 2008). During the ripening of cheese, carboxylic acids could originate from three main biochemical pathways: (i) lipolysis (hydrolysis of the triglycerides into free fatty acids), (ii) proteolysis (cracking of the caseins into peptides and amino acids) and (iii) lactose fermentation (CURIONI and BOSSET, 2002). Based on this information, we have found acids with a microbial origin (acetic acid and propanoic acid), acids with an origin of lipolysis (butanoic, pentanoic, hexanoic, heptanoic, octanoic, nonanoic and decanoic acid), and acids with an origin in amino acids (propanoic acid, 2-methyl- and 3-methylbutanoic acid).

Finally, the trend of the acids increased during the ripening process of the Fossa cheese. Hexanoic and butanoic acids were the most abundant acids identified. Due to their low aroma thresholds, they are considered important contributors to the flavour profile in a wide variety of cheeses (MOIO and ADDEO, 1998; DELGADO *et al.*, 2010; DELGADO *et al.*, 2011). The branched-chain fatty acids (propanoic acid, 2-methyl- and 3-methylbutanoic acid) are characteristic odour-active compounds that have an impact on goat and sheep milk cheeses.

Terpenes are important volatile compounds with origins in plants that constitute the forage mixture of pastures (DELGADO *et al.*, 2011).

Three lactones, namely, γ -caprolactone, δ -decalactone, and γ -octalactone, were detected in the cheese ripened for 90 days, but γ -octalactone was only detected in the cheese from the Cartoceto pit. The lactones have fruity sweet, creamy, fermented notes, and they could contribute pleasant odour notes to the aroma of Fossa cheese (DELGADO *et al.*, 2010). Moreover, three S-compounds were identified (dimethyl sulphide, methyl propyl disulphide and dimethyl sulfone), and these compounds decreased during ripening.

Finally, the cheese from the Cartoceto pit contained more volatile compounds than the cheese from the Talamello pit, in particular for the alcohol, acid and lactone classes.

The results of this study showed that there were substantial differences between the chemical and chemical/physical parameters, and many fatty acids of the just curdled

cheese samples and the cheese ripened for 90 days in the two pits were characterized by different geological-geochemical parameters.

Slight differences in the nutritional parameters between the cheeses ripened in the different pits could be identified in certain components of the fatty acids and in the content of certain groups of volatile molecules.

In particular, the cheese ripened in the Talamello pit showed high values of SFA compared to the cheese from the Cartoceto pit, while the sum of PUFA in the Cartoceto cheese was higher. The cheese from the Cartoceto pit had more volatile compounds than the cheese from the Talamello pit, in particular for the alcohol, acid and lactone class.

In conclusion, although the cheeses ripened in the two different pits had been prepared with the same milk, the pedo-climatic environment in the pits significantly influenced only certain nutritional parameters.

ACKNOWLEDGEMENTS

The authors thank Prof. Francesca Rosati (Sworn Translator) for her support in checking the manuscript for English form.

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Paper Received December 18, 2018 Accepted March 14, 2019

INVESTIGATION ON 'FREISA' RED GRAPE VARIETY: PHYSICO-CHEMICAL PROPERTIES OF GRAPES FROM FIVE PIEDMONT GROWING AREAS AND OF THE PRODUCED WINES

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ABSTRACT

This study aimed to investigate the physico-chemical characteristics of "Freisa" red winegrapes from five growing areas at three ripeness degrees. Wines were produced with grapes from each growing area. Results highlighted that "Freisa" grapes have relatively hard skins (break force > 0.8 N), medium anthocyanin content (> 800 mg/kg grapes), and high flavanol content (PRO > 2500, FVA > 1200 mg/kg grapes). Skin thickness, total anthocyanin and flavanol contents were significantly influenced by environmental conditions and ripeness. Grapes from Monferrato area showed the thickest skins and highest contents of anthocyanins but also of seed flavanols. Coherently, wine color characteristics and phenolic composition depended on growing area.

Keywords: phenolic composition, texture parameters, "Freisa" grapes, growing areas, ripeness, minor varieties

1. INTRODUCTION

Vitis vinifera L. cv. “Freisa” is an Italian native and historic red grape variety included in the National register of vine varieties from 1970 (GU 149-17/06/1970). This variety grows mainly in the central part of the Piedmont hills (North-West Italy), in the municipalities of Albugnano (AT), Castelnuovo Don Bosco (AT), Pino d’Asti (AT), and Moncucco Torinese (AT) (AJASSA *et al.*, 2015), although vineyards of this grape variety are present also in other zones of the Piedmont region. The first citation of “Freisa” grapes is dated in 1517 in a customs document of Pancalieri town (TO), while the first vine-plantation was near Neive (CN) in 1692 (MAINARDI, 2003). Since then, in the centuries “Freisa” grapevines spread throughout all Piedmont because of its good yield, resistance to late frosts and diseases, *Plasmopara viticola* in particular (PECILE *et al.*, 2018). Outside Piedmont, this variety has a little spread in other three regions of Italy (Valle d’Aosta, Lombardia, and Veneto), while abroad some vines are planted in Argentina and in the USA state of California (SCHNEIDER *et al.*, 2013).

The Italian National Institute of Statistics (ISTAT) in 1970 reported in Italy 7,410 hectares planted with “Freisa” variety, while the last census in 2010 recorded a decrease of planted surface to 1,041 hectares. Of those, 80% of “Freisa” planted area is located in the Piedmont region, and about 420 hectares in the province of Asti (REGIONE PIEMONTE, 2018). Nowadays, the Italian nursery production of “Freisa” vines is active with a production over 150,000 vine plants in 2017 vintage (PECILE *et al.*, 2018).

The strong linkage between this variety and its Piedmont growing area is evidenced by five Designations of Controlled Origin (DOC, *Denominazioni di Origine Controllata*): Freisa d’Asti DOC, Freisa di Chieri DOC, Colli Tortonesi DOC Freisa, Monferrato DOC Freisa, and Langhe DOC Freisa (ROSSI, 2012). Furthermore, from 2014 three of these Designations (Freisa d’Asti DOC, Langhe DOC, and Monferrato DOC) are included in a major wine-producing area of Piedmont that became a UNESCO World Heritage Site (UNESCO ITALIA, 2019).

Over the years, some investigations on “Freisa” variety were done. Regarding viticultural aspects, LISA *et al.* (2005) showed that the most suitable training system for “Freisa” variety is the lateral cordon trellis system, able to satisfy quality and consistent yields. Moreover, ASTEGIANO and CIOLFI (1974), CRAVERO and DI STEFANO (1992), GERBI *et al.* (2005), and ROLLE *et al.* (2008a) studied some aspects of the phenolic composition of “Freisa” grapes and showed that the content of tannins was generally high (> 3500 mg/kg) and of anthocyanins in the skins was satisfactory (> 800 mg/kg). ROLLE *et al.* (2008a) also observed a difficult extraction of anthocyanins from the skin into the must and, on the contrary, a relevant contribution of tannins from the seeds. Furthermore, GERBI *et al.* (2005) and ROLLE and GUIDONI (2007) showed that the anthocyanin profile of “Freisa” grapes was characterized by preponderance of di-substituted forms, cyanidin-3-O-glucoside (about 20%) and peonidin-3-O-glucoside (about 50%), similarly to “Nebbiolo” grape variety. Indeed, SCHNEIDER *et al.* (2004) and LACOMBE *et al.* (2013) studied the genetic profile of “Freisa” variety and established that it is an offspring of the Italian red grape varieties “Nebbiolo” and “Avanà”. Furthermore, SCHNEIDER *et al.* (2004) found that there is a genetic relationship between “Freisa” variety and the white grape variety “Viognier”.

Wines produced from “Freisa” grapes have had supporters and critics in the past. Generally, the wine made from ripe “Freisa” grapes has smooth and lightly astringent tannins. However, when the grapes do not reach the optimal ripeness degree, the resulting wines have a high level of acidity and tannicity, leading to sensory perceptions of

excessive astringency and bitterness (SCHNEIDER *et al.*, 2013). In order to mitigate this fact and to produce quality wines, both for wines to be consumed young or after an ageing period, the winemaking of “Freisa” grapes often involves the partial removal of the seeds from the fermenting must after at least 48 hours from the beginning of alcoholic fermentation (ROLLE *et al.*, 2008a).

Although some technical information on “Freisa” grapes is already present in scientific literature, to our knowledge, no comprehensive physico-chemical characterization of grapes and wines has already been reported. Therefore, the main aim of this work was to investigate the mechanical properties of berry skin and the total extractable phenolic composition of berry skins and seeds from “Freisa” grapes, taking into consideration five different growing areas in the South-East of Piedmont region and three different grape ripeness levels. Indeed, the study aimed to assess the real impact of the production area and/or ripeness degree on grape characteristics. Finally, monovarietal wines were produced from “Freisa” grapes of each production area in order to propose appropriate enological techniques according to the grape features.

2. MATERIALS AND METHODS

2.1. Vineyards

Grape samples of *Vitis vinifera* L. cv. “Freisa” were collected from different vineyards located in five South-East Piedmont growing areas (North-West Italy): Astigiano (San Paolo Solbrito, AT), Collina Torinese (Chieri, TO), Langhe (Barolo, CN), Tortonese (Monleale, AL), and Monferrato (Casorzo, AT). The production zone was delimited by the following geographical coordinates: 44.374–45.023 N latitude and 7.500–8.974 E longitude, including the five Designations of Controlled Origin linked to “Freisa” wines: Freisa d’Asti DOC, Freisa di Chieri DOC, Langhe DOC Freisa, Colli Tortonesi DOC Freisa, and Monferrato DOC Freisa, respectively. The five mentioned vineyards, chosen each as representative of the respective growing location, were planted for commercial use and met the designation rules: the vines were at least 10 years old, planted on medium slope hills with exposure South (Astigiano, Collina Torinese, and Langhe locations), East (Tortonese location) or South-East (Monferrato location), vertical growth by lateral cordon trellis system, and Guyot pruned. From each production area, “Freisa” grapes were harvested in plastic boxes (maximum capacity of 20 kg to avoid grape crushing during transport) and about 20 kg were randomly selected and transported to the laboratory, while 150 kg were brought to the experimental cellar of the University of Turin for the winemaking process.

2.2. Grape samples and density class selection

Once in the laboratory, the berries were manually separated from the stalk with harvest shears and then placed on paper trays. About 200 berries were randomly taken for the determination of standard chemical parameters and other 200 berries were used for the evaluation of phenolic extractability indices. All the other berries were classified according to their density (i.e., total soluble solid content) by flotation as described by FOURNAND *et al.* (2006) and ROLLE *et al.* (2011b), in order to improve the physiological homogeneity inside the density class, to permit the comparative evaluation between ripeness levels at harvest, and to assess growing area effects.

In brief, for each growing area, the berries were floated and separated using saline solutions ranging from 130 to 190 g/L of sodium chloride, with densities spread between 1088 and 1125 kg/m³. After flotation, all berries were washed with water and dried using absorbent paper. The three most represented density classes (by weight) were chosen: 1100 kg/m³ (lower), 1107 kg/m³ (middle), and 1115 kg/m³ (higher). For each one, 20 berries were randomly selected for the determination of skin mechanical properties, and three sub-samples of 10 berries were used for the determination of skin and seed phenolic composition.

2.3. Mechanical properties of grapes

For the Texture Analysis test, a Universal Testing Machine (UTM) TAxT2i Texture Analyzer (Stable Micro Systems, Godalming, Surrey, UK), equipped with a HDP/90 platform and a 5 kg load cell, was used. The determination of the skin hardness parameters was carried out, on the whole berries placed in a horizontal plane on the metal plate of the UTM, by a puncture test using a SMS P/2 N needle probe (LETAIEF *et al.*, 2008). Berry skin break force (N, as F_{sk}), berry skin break energy (mJ, as W_{sk}), and berry skin Young's modulus (N/mm, as E_{sk}) were measured. Then, berry skin thickness (μm , as Sp_{sk}) was determined, on a portion of skin (ca. 0.25 cm²) removed by a razor blade from the lateral side of each berry, by a compression test using a 2-mm SMS P/2 flat cylindrical probe (RÍO SEGADÉ *et al.*, 2011a). For each sample, 20 berries were individually analyzed for each test.

2.4. Phenolic compounds extractability trials

For each replicate ($n = 3$), 10 berries were weighed and peeled. The skins and the seeds were manually removed from the pulp using a laboratory spatula, counted, weighed, and quickly immersed separately in 50 mL of a hydroalcoholic buffer solution at pH 3.20 containing 5 g/L of tartaric acid, 2 g/L of Na₂S₂O₅, and 12% v/v of ethanol. The skin and seed samples were then placed in an oven at 25°C for 12 h and one week, respectively (DI STEFANO and CRAVERO, 1991; RÍO SEGADÉ *et al.*, 2014). The skins into the buffer solution were homogenized at 8000 rpm for 1 min with an Ultra-Turrax T25 high-speed homogenizer (IKA Labortechnik, Staufen, Germany), centrifuged for 5 min at 3500 × g at 20°C using a PK 131 centrifuge (ALC International, Milano, Italy), and the supernatant was collected for analysis. In the case of seeds, they were removed from the buffer solution while the extract was used for the determination of the seed phenolic fraction.

2.5. Wine production

In brief, the “Freisa” grapes harvested in each location were separately destemmed, crushed, and the mash was added with 40 mg/L of potassium metabisulfite. After about 8 h, selected yeasts (Lalvin BRL97, Lallemand Inc., Montreal, Canada) were inoculated at a dose of 20 g/hL. Alcoholic and malolactic fermentation were carried out at controlled temperature of 27±2°C and 19±1°C, respectively. At the end of the fermentations, 60 mg/L of potassium metabisulfite were added and wines were cold-stabilized at 0 °C for 2 weeks, filtered (Seitz K300 grade filter sheets, Pall Corporation, Port Washington, NY, USA), and then bottled in glass bottles of 0.75 L with cork stoppers.

2.6. Chemical analysis

2.6.1 Reagents and standards

Solvents of HPLC-gradient grade and all other chemicals of analytical-reagent grade were purchased from Sigma-Aldrich (Milan, Italy). The solutions were prepared in deionized water produced by a Milli-Q system (Merck Millipore, Darmstadt, DE). Chemical standards of malvidin-3-O-glucoside chloride and cyanidin chloride were supplied by Extrasynthèse (Genay, France), whereas (+)-catechin was purchased from Sigma-Aldrich.

2.6.2 Standard chemical parameters of grapes and wines

Standard chemical parameters of grape musts, obtained by manual crushing and centrifugation, and wines were determined according to OIV (2016) methods. In particular, the following parameters were determined: grape potential alcohol degree (% v/v; OIV-OENO 466:R2012), must pH (OIV-MA-AS313-15:R2011), total acidity (g/L as tartaric acid; OIV-MA-AS313-01:R2015), wine alcohol content (% v/v; OIV-MA-AS312-01A:R2016), and wine dry net extract (g/L; OIV-MA-AS2-03B:R2012 and OIV-MA-AS311-02:R2009). The contents (g/L) of reducing sugars (as sum of glucose and fructose), tartaric acid and malic acid in grape musts were determined by HPLC (TORCHIO *et al.*, 2010).

2.6.3 Phenolic extractability indices and phenolic composition of grapes and wines

Phenolic extractability indices in grape berries were assessed for each sample in accordance with the procedure described by GLORIES and AUGUSTIN (1993), modified by CAGNASSO *et al.* (2008). The extractant solution at pH 1 was prepared just before use by mixing equal volumes of 1.0 M of hydrochloric acid and 2 g/L of potassium metabisulfite, while the extraction at pH 3.2 was carried out using a buffer solution containing 5 g/L of tartaric acid. The parameters obtained at pH 1 and pH 3.2, namely total phenolic content (A_{280}) and total anthocyanin content (TA_1 and $TA_{3.2}$), were used for the determination of the following extractability indexes: cellular maturity (EA%) and seed maturity (Mp%). The latter index was assessed by taking into consideration the average ratio (TAR) between the skin contents of total phenols (A_{280}) and total anthocyanins (expressed as g/L), equal to the value of 40 (CAGNASSO *et al.*, 2008). The EA% and Mp% indexes were calculated as follows: $EA\% = [(TA_1 - TA_{3.2})/TA_1] \times 100$; $Mp\% = \{[A_{280} - ((TA_{3.2}/1000) \times TAR)] / A_{280}\} \times 100$.

On berry skin and seed extracts, and on resulting wines, spectrophotometric assessments were done in order to evaluate their phenolic composition. Total anthocyanins index (mg malvidin-3-O-glucoside chloride/kg grape or L wine, as TA) was evaluated in berry skin extracts and wines (DI STEFANO and CRAVERO, 1991; TORCHIO *et al.*, 2010), while monomeric anthocyanins index (mg malvidin-3-O-glucoside chloride/L wine, as MA) was determined only in wines previous isolation on polyvinylpolypyrrolidone (PVPP) and elution with an ethanol:water:HCl 37% (70:30:1) solution (DI STEFANO *et al.*, 1989). For both skin and seed extracts, and wines, total flavonoids index (mg (+)-catechin/kg grape or L wine, as TF), flavanols vanillin assay (mg (+)-catechin/kg grape or L wine, as FVA), and proanthocyanidins assay based on Bate-Smith reaction (mg cyanidin chloride/kg grape or L wine, as PRO) were evaluated (DI STEFANO and CRAVERO, 1991; TORCHIO *et al.*, 2010). A UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used for all analysis. The skin anthocyanin profile was determined by HPLC-

DAD after purification on a 1-g Sep-Pak C18 SPE cartridge (Waters Corporation, Milford, MA, USA) according to the protocol described by RÍO SEGADÉ *et al.* (2014). The chromatographic separation was performed on a LiChroCART analytical column (25 cm × 0.4 cm i.d.) purchased from Merck (Darmstadt, Germany) and packed with LiChrospher 100 RP-18 (5 µm) particles supplied by Alltech (Deerfield, IL, USA), using formic acid/water (10:90, v/v) and formic acid/methanol/water (10:50:40, v/v) as mobile phases. The different individual anthocyanin forms were expressed as area percentage of total forms.

2.6.4 Wine color parameters

Wine color was evaluated by the CIEL*a*b* parameters including lightness (L*), red/green color coordinate (a*), and yellow/blue color coordinate (b*) according to the method OIV-MA-AS2-11 (OIV, 2016). Furthermore, the color intensity (on 10 mm optical path) and the color hue were calculated using the method OIV-MA-AS2-07B (OIV, 2016). A UV-1800 spectrophotometer (Shimadzu Corporation) was used with 2-mm path length cuvettes.

2.7. Statistical analysis

Statistical analyses were carried out using R Statistics software version 3.4.0 (R Core Team, 2017). Levene's and Shapiro-Wilk's tests were used for assessing the homogeneity of variance and analysis of variance (ANOVA) residuals normality, respectively. In case of heteroscedasticity, we used the ANOVA with Welch's correction, followed by Tamhane's T2 post-hoc test when null hypothesis was rejected. In the case of homoscedasticity, we used one-way ANOVA and the Tukey HSD test for $p < 0.05$ to assess significant differences between groups.

3. RESULTS AND DISCUSSION

3.1. Grape chemical composition at harvest and berry sorting

In all five "Freisa" grape samples harvested from the representative vineyards of each growing area studied, the content of sugars at harvest was suitable to meet the minimum potential alcohol degree [% v/v] indicated in the disciplinary rules of each Designation of Controlled Origin (Table 1).

Regarding the phenolic maturity of unsorted grapes, the cellular maturity index (EA%) and the seed maturity index (Mp%) varied between 35-45% and 77-82%, respectively, depending on the growing area. These values agreed with those previously reported by ROLLE *et al.* (2008a) and confirmed particular varietal characteristics, such as the difficulty for releasing anthocyanins from the skin during maceration (EA% >30) and a high contribution of tannins from the seeds (Mp% >30) (RIBÉREAU-GAYON *et al.*, 2006). Moreover, in the present work, the Astigiano and Tortonese growing areas showed the lowest contents not only of potential anthocyanins (extracted at pH 1) but also of extractable anthocyanins (extracted at pH 3.2), the grapes from Tortonese area also showing the lowest anthocyanin extractability (EA% =45).

Table 1. Grape must standard chemical parameters and phenolic extractability indices of “Freisa” grapes harvested in five Piedmont growing locations.

Parameter <i>Designation of Origin</i>	Location				
	Astigiano <i>Freisa d’Asti DOC</i>	Collina Torinese <i>Freisa di Chieri DOC</i>	Langhe <i>Langhe DOC Freisa</i>	Tortonese <i>Colli Tortonesi DOC Freisa</i>	Monferrato <i>Monferrato DOC Freisa</i>
Minimum potential alcohol content [% v/v]	10.5	10.5	10.5	11.0	10.0
Density at 20°C	1.102	1.106	1.097	1.110	1.106
Sugars content [g/L]	239	249	227	258	247
Potential alcohol degree [% v/v]	14.2	14.8	13.5	15.3	14.6
pH	3.57	3.44	3.40	3.61	3.59
Total acidity [g/L as tartaric acid]	5.51	6.30	6.65	6.27	6.43
Tartaric acid [g/L]	5.54	6.81	6.61	6.76	5.18
Malic acid [g/L]	2.33	2.00	2.35	2.23	3.31
EA%	37	35	40	45	40
Mp%	82	78	77	82	77
TA _{3.2} [mg/kg malvidin-3- <i>O</i> -glucoside chloride]	605	762	758	571	766
A ₂₈₀ on extract at pH 3.2	132	137	130	125	135
TA ₁ [mg/kg malvidin-3- <i>O</i> -glucoside chloride]	959	1169	1269	1032	1273

EA% = cellular maturity index; Mp% = seed maturity index; TA_{3.2} = total anthocyanins extracted at pH 3.2; A₂₈₀ = absorbance at 280 nm; TA₁ = total anthocyanins extracted at pH 1.

The distribution percentages of all “Freisa” grape berries in different density classes at harvest were determined for the five vineyard locations. In agreement with data reported in scientific literature (KONTOUDAKIS *et al.*, 2011; ROLLE *et al.*, 2012), bell-shaped distributions were found (data not shown). In general, as already reported by FOURNAND *et al.* (2006), the difference in the total sugar content of the berries belonging to two consecutive density classes was ~17 g/L (i.e. 1% v/v potential alcohol). For all the growing areas, the three more representative density classes were 1100 kg/m³, 1107 kg/m³, and 1115 kg/m³. More than 80% of the berries were belonging to these three density groups. In Table 2 some berry physical traits are shown for the three considered density groups. In order to evaluate the real impact of the growing area and ripeness degree, two comparisons were done by statistical analysis: the first among the five locations considering the same density class, the second among the three density classes inside each growing area. The first comparison showed that the grapes from Astigiano location presented, in two density classes out of three (the middle and the higher), the highest values of both berry and skin weight while, on the contrary, the grapes from Collina Torinese had the lowest berry weight values. For these two parameters, the second comparison showed that the ratio of skin and berry weight increases with increasing the sugar contents. This ratio ranged between 7.3 and 9.1% in the low density class, 8.2 and 8.9% in the middle density class, and 9.6 and 12.2% in the high density class.

Table 2. Physical parameters of “Freisa” grapes harvested in five Piedmont growing locations and sorted according to density.

Density class	Location	Berry weight (g)	Skin weight (g)	Seeds (n)	Single seed weight (g)
1100 kg/m ³	Astigiano	2.21 ^{ab,a}	0.17 ^{ab,a}	2.0 ^a	0.04 ^{ab}
	Collina Torinese	1.93 ^{a,ab}	0.14 ^a	2.5 ^{ab,ab}	0.04 ^a
	Langhe	2.18 ^{ab}	0.18 ^{bc}	2.3 ^{ab}	0.04 ^{ab}
	Tortonese	2.18 ^{ab}	0.18 ^{ab,a}	2.1 ^{a,β}	0.05 ^b
	Monferrato	2.41 ^{b,β}	0.22 ^{c,β}	2.9 ^{b,β}	0.04 ^{ab}
	Sign (1)	*	***	**	*
1107 kg/m ³	Astigiano	2.44 ^{b,β}	0.20 ^{b,β}	2.4 ^b	0.04
	Collina Torinese	2.03 ^{a,β}	0.18 ^{ab}	2.7 ^{b,β}	0.04
	Langhe	2.19 ^{ab}	0.19 ^{ab}	2.5 ^b	0.04
	Tortonese	1.97 ^a	0.17 ^{a,a}	1.7 ^{a,a}	0.05
	Monferrato	2.10 ^{a,a}	0.18 ^{ab,a}	2.6 ^{b,ab}	0.04
	Sign (1)	**	*	**	ns
1115 kg/m ³	Astigiano	2.29 ^{c,a}	0.24 ^{c,γ}	2.6	0.04
	Collina Torinese	1.78 ^{a,a}	0.17 ^a	2.1 ^a	0.04
	Langhe	1.90 ^{ab}	0.21 ^{ab}	2.1	0.04
	Tortonese	1.97 ^{ab}	0.24 ^{bc,β}	2.0 ^{ab}	0.04
	Monferrato	2.03 ^{b,a}	0.21 ^{bc,β}	2.4 ^a	0.04
	Sign (1)	***	***	ns	ns
Sign (2)		** , * , ns , ns , *	*** , ns , ns , ** , **	ns , * , ns , * , *	ns , ns , ns , ns , ns

Values are expressed as average ($n = 3$). Different Latin letters within the same column indicate significant differences (1) among zones for the same berry density (Tukey HSD test; $p < 0.05$). Different Greek letters within the same column indicate significant differences (2) among the three density classes for the same zone (Tukey HSD test; $p < 0.05$). Sign: *, **, ***, and "ns" indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively.

Regarding seeds, although their weight was similar among the locations and the density classes, the variability of their number influenced the ratio between seeds and berry weight, the highest percentage value (5.4%) being in the grapes from Collina Torinese location belonging to the middle density class and the lowest value (3.6%) being in the grapes from Astigiano and Tortonese location belonging to the lower and the middle density class.

Grape berries of the same diameter and/or fresh weight have different total soluble solid concentrations as a consequence of the functional relationship among berry sugar accumulation, transpiration, and water accumulation (ŠUKLJE *et al.*, 2012). Indeed, they may belong to different density classes. This aspect could be related not only to the environmental conditions of the vineyard but also to the position of a specific berry in a bunch and the relative position of a bunch in the vine. All these factors are of great importance because they influence the relative skin and seed weight and therefore berry phenolic composition (ROBY *et al.*, 2004).

3.2. Berry skin mechanical properties

The berry skin mechanical parameters measured on “Freisa” grapes from the five different locations and sorted by density are available in Table 3. The data showed high values of skin break force ($F_{sk} \geq 0.824$ N) and skin break energy ($W_{sk} \geq 0.782$ mJ), in agreement with LETAIEF *et al.* (2008) who reported higher values of skin hardness parameters for “Freisa” grapes with relation to other six grapevine varieties, including the genetically related “Nebbiolo” grape variety. Although “Freisa” can be considered a grape variety with a ‘hard’ skin, when compared with other varieties growing in the same vineyard in a single vintage (i.e. same bioclimatic indexes), the skin hardness parameters (F_{sk} and W_{sk}) for “Freisa” berries did not show the highest values (ROLLE *et al.*, 2011a). In this sense, “Becuet” (variety grown in mountain environment) and some varieties of “Teinturier” (cultivar with coloured pulp) were characterized by harder skins.

Table 3. Berry skin mechanical properties of “Freisa” grapes harvested in five Piedmont growing locations and sorted according to density.

Density class	Location	Berry skin break force [F_{sk} , N]	Berry skin break energy [W_{sk} , mJ]	Berry skin Young's modulus [E_{sk} , N/mm]	Berry skin thickness [Sp_{sk} , μ m]
1100 kg/m ³	Astigiano	0.855±0.182	0.862±0.278	0.386±0.082 ^{ab}	190±35 ^a
	Collina Torinese	0.907±0.121	0.817±0.178	0.440±0.058 ^b	192±34 ^a
	Langhe	0.892±0.120	0.868±0.173	0.400±0.055 ^{b,β}	188±29 ^{a,α}
	Tortonese	0.869±0.141	0.884±0.256	0.386±0.057 ^{ab,β}	215±25 ^a
	Monferrato	0.879±0.201	0.893±0.272	0.342±0.061 ^a	244±35 ^b
	Sign (1)	ns	ns	***	***
1107 kg/m ³	Astigiano	0.875±0.175	0.896±0.268	0.380±0.074	199±29 ^a
	Collina Torinese	0.892±0.148	0.880±0.236	0.391±0.062	213±24 ^{ab}
	Langhe	0.897±0.137	0.906±0.256	0.380±0.050 ^{ab}	218±27 ^{ab,β}
	Tortonese	0.838±0.128	0.877±0.211	0.332±0.035 ^a	230±23 ^b
	Monferrato	0.835±0.229	0.822±0.280	0.354±0.099	225±34 ^b
	Sign (1)	ns	ns	ns	**
1115 kg/m ³	Astigiano	0.879±0.130	0.934±0.217	0.340±0.038 ^a	209±22
	Collina Torinese	0.870±0.162	0.782±0.217	0.417±0.090 ^b	211±28
	Langhe	0.857±0.156	0.860±0.223	0.356±0.064 ^{a,α}	224±31 ^β
	Tortonese	0.824±0.160	0.872±0.247	0.339±0.058 ^{a,α}	231±17
	Monferrato	0.945±0.137	0.953±0.219	0.387±0.066 ^{ab}	223±43
	Sign (1)	ns	ns	***	ns
Sign (2)		ns, ns, ns, ns, ns	ns, ns, ns, ns, ns	ns, ns, *, **, ns	ns, ns, ***, ns, ns

Values are expressed as average ± standard deviation ($n = 20$). Different Latin letters within the same column indicate significant differences (1) among zone for the same berry density (Tukey HSD test; $p < 0.05$). Different Greek letters within the same column indicate significant differences (2) among the three density classes for the same zone (Tukey HSD test; $p < 0.05$). Sign: *, **, ***, and "ns" indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively.

No significant differences in F_{sk} and W_{sk} were observed among density classes and growing areas. Although it is normal to observe high coefficients of variation for these parameters in berry skin analysis, this behaviour may highlight the assumption that the berry skin hardness traits (F_{sk} and W_{sk}) are firstly variety dependent and not markedly influenced by the ripeness degree and the growing area. In fact, ROLLE *et al.* (2008b) have observed similar values of F_{sk} and W_{sk} at the last weeks during ripening of “Nebbiolo” variety. These skin mechanical parameters give high resistance to “Freisa” grapes against fungal pathogens (ROSENQUIST and MORRISON, 1988) and to handling injury during harvest, transport, and postharvest treatments (KÖK and ÇELİK, 2004). From a technological point of view, a higher skin hardness is generally associated to a slower anthocyanin release into the must-wine during maceration-fermentation but, with a longer maceration, the anthocyanin extraction yield is higher (ROLLE *et al.*, 2008b). This aspect is particularly important and favorable for “Freisa” grapes and for all the wine grapes varieties rich in 3'-hydroxylated anthocyanins because these pigments are extracted preferentially during the initial phase of maceration and may be easily oxidized by the enzymes present in the juice (GONZÁLEZ-NEVES *et al.*, 2008).

Regarding other berry skin hardness-derived parameter, significant differences were found for Young's modulus (E_{sk}) that measures the rigidity or stiffness of tissues. Inside each density class, zone effects were observed. Particularly, the grapes of Collina Torinese location had the highest values of E_{sk} (> 0.390 N/mm). Furthermore, ripeness effects were evidenced in some growing areas (Langhe and Tortonese), with a tendency to decrease E_{sk} values when increasing the density class, as observed by TORCHIO *et al.* (2010) on “Barbera” grapes.

For skin thickness (Sp_{sk} , Table 3), the values obtained in the present study agreed with the reported by LETAIEF *et al.* (2008) for “Freisa” grapes. In this study, some significant differences were found among growing areas and density classes. Monferrato location showed a significantly higher value of skin thickness with respect to other locations when considering the grapes belonging to the lowest density class considered ($244 \mu\text{m}$). Furthermore, also in the other density groups this location evidenced high skin thickness values (225 and $223 \mu\text{m}$, respectively, in the middle and in the higher density class). On the other hand, Astigiano location was characterized by thinner skins (190 - $209 \mu\text{m}$). Previous studies have highlighted that precipitation indices, which could strongly influence berry water availability in the last ripening weeks, are responsible for differences in skin mechanical parameters among production areas (ROLLE *et al.*, 2011a). Moreover, the influence of rain on skin thickness has been already reported for Mondeuse grapes during on-vine withering (ROLLE *et al.*, 2009).

Significant differences in this skin mechanical parameter among the density groups inside each location were found only for Langhe samples ($p < 0.001$), with an increasing trend from 1100 kg/m^3 class to the higher density classes. A slight increasing tendency was found also for other locations and similarly observed by TORCHIO *et al.* (2010) on “Barbera” grapes, by RÍO SEGADÉ *et al.* (2011b) on Galician grapes, and by ROLLE *et al.* (2012) on “Nebbiolo”. Moreover, ROLLE *et al.* (2011b) showed on “Nebbiolo” grapes that stiffer and thicker berry skins allowed respectively the higher accumulation and extraction of proanthocyanidins, while harder skins provided higher concentration and extraction of oligomeric flavanols. VILLANGÓ *et al.* (2015) evidenced that thicker skins had the highest content of anthocyanins in “Syrah”, while RÍO SEGADÉ *et al.* (2011a) found on “Mencía” grapes that thinner skins were characterized by a higher release of anthocyanins.

Therefore, although the changes in the skin mechanical properties during ripening are generally limited, the preliminary knowledge of these parameters could help the planning

of the harvest time (i.e. selection among different vineyards) and the strategy of maceration process, in order to guarantee the rapid degradation of the grape skin and an improved extraction of its components (RÍO SEGADE *et al.*, 2014, RÍO SEGADE *et al.*, 2015).

3.3. Skin anthocyanin content and profile

The skin total anthocyanin content (TA_{sk}) and profile of “Freisa” samples are shown in Table 4. The first comparison shows that there were differences in total anthocyanin content among growing areas for all the density classes considered ($p < 0.05$ for density class of 1100 kg/m³, and $p < 0.001$ for 1107 and 1115 kg/m³). This behaviour highlighted a zone effect on grape total anthocyanin content because climate indices, sunlight and soil conditions are factors of great relevance on anthocyanin biosynthesis (SPAYD *et al.*, 2002). The lowest and highest contents of anthocyanins were found in the grapes from Astigiano and Monferrato locations, respectively, belonging to the three density classes, even though the differences between the two locations were more relevant for the grapes belonging to the highest density classes (156, 418, and 475 mg/kg for 1100, 1107, and 1115 kg/m³, respectively). Although probably there is not an only influential factor, the South-East exposure of the Monferrato vineyard could favour the biosynthesis of anthocyanins or reduce their degradation, with respect to the South orientation of the Astigiano vineyard, as a consequence of temperature and/or sunlight effects.

These same samples had also the lowest and highest values of Sp_{sk}, respectively, in accordance with VILLANGÓ *et al.* (2015) that observed on “Syrah” grapes a higher concentration of anthocyanins in the thicker skins. The second comparison showed that total extractable concentrations of anthocyanins almost always increased with increasing berry density, with significance at $p < 0.05$ in three cases (Collina Torinese, Langhe, and Tortonese locations) and at $p < 0.01$ in the case of Monferrato location. A density effect on anthocyanins concentration is evident and confirms the assumptions of TORCHIO *et al.* (2010), who previously observed this behaviour on “Barbera” grapes, and of ROLLE *et al.* (2011b) on “Nebbiolo” grapes.

In this study, “Freisa” grapes were characterized by a high average percentage of simple anthocyanin glucosides (95.3% of total forms on average), with a higher content of free di-substituted anthocyanins (66.0% on average) with respect to free tri-substituted free forms (29.3% on average), as already reported in literature (ROLLE and GUIDONI, 2007; FERRANDINO *et al.*, 2012). In the present study, peonidin-3-O-glucoside was the major anthocyanin compound found, with an average content of 48.1%, followed by malvidin-3-O-glucoside and cyanidin-3-O-glucoside with an average percentage of 19.2 and 17.8% of total forms, respectively. A similar anthocyanin profile was evidenced for “Nebbiolo” grapes and for other minor, ancient grape varieties diffused in North-West Italy close to the Alps such as “Avanà”, “Doux d’Henry”, “Grignolino”, “Neretto di Bairo”, “Pelaverga Piccolo”, and “Rastajola”, which showed a percentage of peonidin-3-O-glucoside of about 45-55% on total anthocyanin forms (GERBI *et al.*, 2005; FERRANDINO *et al.*, 2012).

Some differences in the anthocyanin profile among locations and density classes were observed, even if the variations are not easily attributable to environmental or ripeness factors, as already discussed by ORTEGA-REGULES *et al.* (2006). In the lower density class, the grapes from Collina Torinese location had the lowest content of di-substituted anthocyanins (cyanidin- and peonidin-3-O-glucoside, 56.1%) and the highest content of tri-substituted ones (delphinidin-, petunidin-, and malvidin-3-O-glucoside, 38.8%).

Table 4. Total extractable anthocyanins content and relative profile of “Freisa” grapes harvested in five Piedmont growing locations and sorted according to density.

Density class	Location	Total anthocyanins TA _{sk}	Delphinidin-3- O-glucoside [%]	Cyanidin-3-O- glucoside [%]	Petunidin-3-O- glucoside [%]	Peonidin-3-O- glucoside [%]	Malvidin-3-O- glucoside [%]	Acetyl-3-O- glucosides [%]	Cinnamoyl-3- O-glucosides [%]
1100 kg/m ³	Astigiano	834±53 ^{ab}	3.93±0.11 ^a	19.43±0.61 ^{b,a}	5.44±0.06 ^a	49.17±1.02 ^{b,a}	17.06±0.36 ^{a,b}	1.28±0.13 ^b	3.69±0.05 ^{ab,β}
	Collina Torinese	961±69 ^{ab,αβ}	5.23±0.30 ^{b,β}	14.02±0.93 ^a	7.09±0.29 ^{b,β}	42.06±2.27 ^{a,α}	26.52±2.18 ^{b,β}	0.31±0.05 ^b	3.77±0.39 ^b
	Langhe	924±76 ^{ab,α}	3.54±0.36 ^a	20.00±1.81 ^b	4.93±0.18 ^{a,α}	50.99±1.36 ^b	16.60±0.69 ^a	0.95±0.03 ^{a,α}	2.99±0.31 ^{a,α}
	Tortonese	777±16 ^{a,α}	3.24±0.65 ^{a,α}	17.57±0.66 ^{ab}	4.57±0.55 ^{a,α}	53.17±3.34 ^{b,β}	16.45±1.39 ^{a,α}	1.32±0.04 ^{b,α}	3.69±0.22 ^{ab}
	Monferrato	990±100 ^{b,α}	3.17±0.52 ^{a,α}	18.51±2.21 ^b	4.79±0.62 ^{a,α}	51.63±1.78 ^{b,β}	17.24±2.58 ^a	1.34±0.11 ^b	3.32±0.24 ^{ab}
	Sign (1)	*	***	**	***	***	***	***	*
1107 kg/m ³	Astigiano	773±75 ^a	3.53±0.96	21.35±0.60 ^{b,β}	5.05±0.95	50.47±5.09 ^{αβ}	14.96±2.70 ^{αβ}	1.28±0.13	3.36±0.69 ^{αβ}
	Collina Torinese	869±52 ^{a,α}	3.99±0.59 ^a	15.67±2.09 ^a	5.69±0.35 ^a	50.52±2.59 ^β	19.76±1.41 ^a	1.14±0.09	3.23±0.23
	Langhe	1122±44 ^{b,β}	4.67±0.42	18.89±2.07 ^{ab}	6.16±0.40 ^β	44.81±3.49	20.78±3.71	1.30±0.31 ^{αβ}	3.39±0.66 ^{αβ}
	Tortonese	847±45 ^{a,αβ}	4.22±0.38 ^{αβ}	17.65±1.09 ^{ab}	5.57±0.44 ^{αβ}	48.67±1.77 ^{αβ}	19.00±2.00 ^{αβ}	1.32±0.11 ^a	3.56±0.30
	Monferrato	1191±41 ^{b,β}	4.87±0.66 ^β	15.47±0.91 ^b	6.64±0.69 ^β	47.75±2.57 ^{αβ}	20.88±1.76	1.35±0.13	3.34±0.24
	Sign (1)	***	ns	**	ns	ns	ns	ns	ns
1115 kg/m ³	Astigiano	785±50 ^a	3.25±0.14 ^a	22.42±0.24 ^{b,β}	4.57±0.09 ^a	54.07±0.86 ^{b,β}	11.90±0.34 ^{a,α}	1.05±0.02 ^a	2.74±0.20 ^{a,α}
	Collina Torinese	1050±50 ^{b,β}	4.88±0.42 ^{b,αβ}	15.97±0.90 ^a	6.61±0.31 ^{b,β}	46.77±2.09 ^{ab,αβ}	21.23±0.68 ^{b,α}	1.11±0.15 ^a	3.43±0.31 ^b
	Langhe	1042±74 ^{b,αβ}	4.94±0.88 ^b	17.65±2.19 ^{ab}	6.51±0.90 ^{ab,β}	43.25±4.87 ^a	22.13±4.97 ^{ab}	1.52±0.16 ^{b,β}	4.01±0.14 ^{c,β}
	Tortonese	936±77 ^{ab,β}	5.40±0.76 ^{b,β}	16.31±0.18 ^a	6.87±0.70 ^{ab,β}	43.67±3.85 ^{a,α}	22.24±2.36 ^{b,β}	1.54±0.06 ^{b,β}	3.97±0.27 ^c
	Monferrato	1260±29 ^{c,β}	5.28±0.31 ^{b,β}	16.28±0.96 ^a	7.13±0.50 ^{b,β}	45.45±2.06 ^{a,α}	21.13±1.87 ^b	1.41±0.08 ^b	3.33±0.17 ^b
	Sign (1)	***	**	***	**	**	***	**	**
Sign (2)		ns,*,*,**	ns,*,ns,*,**	**,ns,ns,ns,ns	ns,**,*,**	**,*,ns,*,*	***,**,ns,*,ns	ns,ns,*,*,ns	*,ns,*,ns,ns

Values are expressed as average ± standard deviation ($n = 3$). Different Latin letters within the same column indicate significant differences (1) among zone for the same berry density (Tukey HSD test; $p < 0.05$). Different Greek letters within the same column indicate significant differences (2) among the three density classes for the same zone (Tukey HSD test; $p < 0.05$). Sign: *, **, ***, and "ns" indicate significance at $p < 0.05$, 0.01, 0.001, and not significantly respectively. TA_{sk} = total anthocyanins (mg malvidin-3-O-glucoside chloride/kg berries).

On the contrary, in the higher density class, the grapes from Astigiano location had the highest content of di-substituted anthocyanins (76.5%) and the lowest content of tri-substituted forms (19.7%). When the ripeness effect was evaluated for each location, in the grapes from Astigiano (and partly Collina Torinese) location there was a relative increase of di-substituted anthocyanins and a decrease of tri-substituted ones when increasing the density class considered, while in the grapes from Langhe, Tortonese, and Monferrato locations the opposite effect was observed. As previously commented for total anthocyanins, the South orientation of the Astigiano vineyard seems to influence negatively the percentage of the most stable forms, namely acylated- and malvidin-derivatives. It also can be hypothesized that higher rainfall and lower temperatures during the pre-veraison period could probably affect positively the F3'H activity, resulting in a higher di-substituted flavonoid accumulation, such as peonidin- and cyanidin-3-O-glucoside, and negatively the F3'5'H activity with a lower tri-substituted flavonoid accumulation, such as delphinidin-, petunidin- and malvidin-3-O-glucoside (FERRANDINO and GUIDONI, 2010).

3.4. Total flavonoids and flavanol composition of skins and seeds

The skin and seeds flavonoid content and flavanol composition of "Freisa" grapes harvested in the five different locations and sorted by flotation are shown in Table 5. Regarding grape skins, significantly higher contents of total flavonoids (TF_{sk}) were found in the grapes from Langhe and Monferrato locations when belonging to the middle and higher density classes ($p < 0.001$). However, an increase of TF_{sk} was observed with the increase of sugar level for all locations with exception of Astigiano, as also occurred for TA_{sk} content. Differently, few differences were found in skin proanthocyanidin and oligomeric flavanol contents (represented by PRO_{sk} and FVA_{sk} parameters, respectively) among locations for each density class and no differences were observed among the berries belonging to the different density classes for the same growing area. A similar behaviour of PRO_{sk} and FVA_{sk} was previously observed in "Nebbiolo" grapes with berry density (ROLLE *et al.*, 2012). Particularly, in this study, the highest contents of both larger and smaller molecular mass flavanols were found in the grapes skins from Astigiano, especially in the middle density class [PRO_{sk} , 2849 mg cyanidin chloride/kg berries; FVA_{sk} , 1122 mg (+)-catechin/kg berries]. This location had also the highest values of FVA_{sk}/PRO_{sk} ratio (0.36 and 0.39, respectively, in the lower and in the middle density class), thus evidencing an important contribution of the oligomeric flavanol fraction assessed by FVA_{sk} parameter.

Regarding seeds, a remarkable aspect of "Freisa" grapes is the high content of flavanols reactive to vanillin (FVA_s) with respect to proanthocyanidins (PRO_s) and, as a consequence, the high FVA_s/PRO_s ratio (> 0.57). ROLLE *et al.* (2008a) have also observed a similar relationship between oligomeric and polymeric flavanols in "Freisa" grapes and therefore a varietal behaviour is evident. This could represent an issue when seeds flavanol extraction into the must during the maceration is high, with the risk of producing unbalanced wines for bitterness and astringency. In fact, flavanols vanillin assay (FVA) quantifies monomeric and oligomeric flavanols, which are more bitter than the polymeric ones, while the astringency is positively correlated to mean degree of polymerization (mDP) and galloylation degree (VIDAL *et al.*, 2003, CHEYNIER *et al.*, 2006).

Table 5. Skin and seeds total extractable phenolic composition of “Freisa” grapes harvested in five Piedmont growing locations and sorted according to density.

Density class	Location	SKINS				SEEDS			
		Total flavonoids [TF _{sk}]	Proanthocyanidins [PRO _{sk}]	Vanillin assay [FVA _{sk}]	FVA _{sk} /PRO _{sk} ratio	Total flavonoids [TF _s]	Proanthocyanidins [PRO _s]	Vanillin assay [FVA _s]	FVA _s /PRO _s ratio
1100 kg/m ³	Astigiano	3770±154	2800±88	999±117 ^b	0.36±0.03 ^b	1310±115 ^{bc}	1476±5 ^b	847±69 ^a	0.57±0.03 ^a
	Collina Torinese	3802±29 ^a	2204±253	649±138 ^a	0.29±0.03 ^{ab}	860±49 ^a	961±22 ^a	621±70 ^a	0.65±0.06 ^a
	Langhe	3816±160 ^a	2305±161	750±18 ^{ab}	0.33±0.03 ^{ab}	1011±137 ^{ab}	981±126 ^a	758±125 ^a	0.77±0.03 ^b
	Tortonese	3482±70 ^a	2217±181	691±147 ^{ab}	0.31±0.04 ^{ab}	780±154 ^a	986±145 ^a	593±115 ^a	0.60±0.03 ^a
	Monferrato	3710±210 ^a	2501±380	648±122 ^a	0.26±0.01 ^a	1425±69 ^{c,β}	1684±87 ^{c,β}	1327±88 ^{b,β}	0.79±0.02 ^b
	Sign (1)	ns	ns	*	*	***	***	***	***
1107 kg/m ³	Astigiano	3481±104 ^a	2849±140 ^b	1122±206 ^b	0.39±0.06 ^b	1205±142 ^{cd}	1278±163 ^c	942±131 ^{cd}	0.74±0.05 ^b
	Collina Torinese	3736±149 ^{a,α}	2054±183 ^a	584±39 ^a	0.28±0.01 ^{ab}	1059±125 ^{bc}	1187±224 ^{bc}	771±96 ^{bc}	0.65±0.04 ^{ab}
	Langhe	4249±162 ^{b,β}	2787±73 ^b	878±242 ^{ab}	0.32±0.09 ^{ab}	898±25 ^b	903±49 ^{ab}	663±21 ^{ab}	0.74±0.06 ^b
	Tortonese	3813±24 ^{a,β}	2516±211 ^{ab}	722±135 ^{ab}	0.29±0.04 ^{ab}	642±111 ^a	800±89 ^a	470±89 ^a	0.58±0.04 ^a
	Monferrato	4241±212 ^{b,β}	2809±372 ^b	650±76 ^a	0.23±0.04 ^a	1384±49 ^{d,β}	1644±112 ^{d,β}	1137±116 ^{d,β}	0.69±0.08 ^{ab}
	Sign (1)	***	**	*	*	***	***	***	*
1115 kg/m ³	Astigiano	3541±210 ^a	2651±82	796±207	0.30±0.08	979±100 ^b	1075±134	693±78 ^{ab}	0.64±0.01
	Collina Torinese	4326±180 ^{bc,β}	2317±255	605±35	0.26±0.03	868±39 ^{ab}	1014±86	575±42 ^{ab}	0.57±0.01
	Langhe	4204±53 ^{bc,β}	2727±328	778±83	0.29±0.04	947±102 ^b	1042±126	698±53 ^{ab}	0.67±0.03
	Tortonese	3863±263 ^{ab,β}	2287±442	672±118	0.30±0.03	708±53 ^a	861±18	520±57 ^a	0.60±0.06
	Monferrato	4567±125 ^{c,β}	2885±141	819±53	0.28±0.03	949±111 ^{b,α}	1111±160 ^a	707±94 ^{b,α}	0.64±0.07
	Sign (1)	***	ns	ns	ns	*	ns	*	ns
Sign (2)		ns, **, *, **, *	ns, ns, ns, ns, ns	ns, ns, ns, ns, ns	ns, ns, ns, ns, ns	ns, ns, ns, ns, ***	ns, ns, ns, ns, **	ns, ns, ns, ns, ***	ns, ns, ns, ns, ns

Values are expressed as average ± standard deviation ($n = 3$). Different Latin letters within the same column indicate significant differences (1) among zone for the same berry density (Tukey HSD test, $p < 0,05$). Different Greek letters within the same column indicate significant differences (2) among the three density classes for the same zone (Tukey HSD test; $p < 0.05$). Sign: *, **, ***, and "ns" indicate significance at $p < 0.05$, 0.01, 0.001, and not significant, respectively. TF_{sk} = total flavonoids (mg (+) – catechin/kg berries); PRO_{sk} = proanthocyanidins (mg cyanidin chloride/kg berries); FVA_{sk} = flavanols vanillin assay (mg (+) – catechin/kg berries).

The content of flavanols in “Freisa” seeds showed differences mainly induced by the growing area, with the highest content of TF, FVA, and PRO, in the grapes from Monferrato location and belonging to the lower and middle density classes [PRO, 1684 and 1644 mg cyanidin chloride/kg berries; FVA, 1327 and 1137 mg (+)-catechin/kg berries, respectively]. Among density classes, a decreasing tendency of flavanols content from the lower to higher density class was observed, only being significantly relevant in the grapes from Monferrato location. These results agreed with the higher seed number found in the berries from the Monferrato vineyard, particularly in those belonging to the lower density class. Indeed, it is widely recognised that during the berry development the concentration of monomeric flavanols decreases rapidly (GONZÁLEZ-MANZANO *et al.*, 2004) and that the histological and histochemical modifications (i.e. solidification of the cells rich in tannins) occurring during grape ripeness influence negatively the aptitude for the extraction of these compounds (CADOT *et al.*, 2006).

3.5. “Freisa” experimental wines assessment

Monovarietal wines were produced for each single “Freisa” Designation of Origin considered in this study, and then analysed according to their general chemical composition, chromatic characteristics, and phenolic content (Table 6).

As expected, the alcohol content in all five “Freisa” wines were higher than the minimum limits imposed by disciplinary rules and always higher than 13% v/v. In the same way, also total acidity and dry net extract were satisfactory with values higher than 5.27 g/L as tartaric acid and 25.8 g/L, respectively. The wines produced by grapes from Astigiano and Collina Torinese locations had the highest values of total acidity (6.26 and 6.27 g/L as tartaric acid, respectively), with the latter presenting also the highest alcohol content (14.6% v/v). Wines produced by grapes from Tortonese location showed the highest value of dry net extract (28.1 g/L).

Regarding colour characteristics, the highest values of lightness (L^*), red/green colour coordinate (a^*), and yellow/blue colour coordinate (b^*), related to the lowest value of colour intensity, were found in the wine from Astigiano location that also had the lowest content of total anthocyanins (TA_{sk}). As opposed, the wine from Monferrato location showed the lowest values of L^* and of the coordinates a^* and b^* , related to the highest value of colour intensity, and a high value of colour hue (more red-orange nuances).

The anthocyanin content of “Freisa” wines ranged between 198 and 303 mg malvidin-3-O-glucoside chloride/L. It is well known that anthocyanins are strongly related to wine chromatic characteristics, but PAISSONI *et al.* (2018) demonstrated that they also can contribute to in-mouth properties in function of their content and acylation. These authors estimated the perception threshold of total anthocyanins at 255 mg/L, and evidenced that acetylated and cinnamoylated anthocyanins contribute to in-mouth sensory properties more than glucoside forms. Regarding “Freisa” wines analysed in the present study, only in two wines out of five (Langhe DOC Freisa and Monferrato DOC Freisa) the content of total anthocyanins was higher than the proposed threshold. Taking into account that “Freisa” grapes had a low amount of acylated forms (Table 4), it may be hypothesized that the wine contains limited acylated anthocyanins and, therefore, presents a very small contribution to in-mouth sensory traits. For these reasons, the contribution of anthocyanins to in-mouth sensory properties of “Freisa” wines can be excluded in most cases.

Table 6. Compositional characteristics of wines produced from “Freisa” grapes harvested in five Piedmont growing locations.

Wine parameter <i>Designation of Origin</i>	Location				
	Astigiano <i>Freisa d'Asti DOC</i>	Collina Torinese <i>Freisa di Chieri DOC</i>	Langhe <i>Langhe DOC Freisa</i>	Tortonese <i>Colli Tortonesi DOC Freisa</i>	Monferrato <i>Monferrato DOC Freisa</i>
<i>Minimum alcohol content [% v/v]</i>	11.0	11.0	11.0	11.0	11.0
<i>Minimum total acidity content [g/L as tartaric acid]</i>	5.50	4.50	4.50	4.50	5.00
<i>Minimum dry net extract [g/L]</i>	19.0	19.0	19.0	20.0	20.0
Alcohol content [% v/v]	13.7	14.6	14.1	14.1	14.1
Total acidity [g/L as tartaric acid]	6.26	6.27	5.85	5.27	5.48
Dry net extract [g/L]	25.8	26.7	27.0	28.1	27.3
L*	20.7	17.7	19.1	18.8	16.9
a*	55.1	52.4	54.0	52.7	51.1
b*	51.3	49.6	51.7	49.9	49.4
Color intensity [A.U., 10 mm optical path]	17.6	18.2	18.1	18.5	19.6
Color hue	0.87	0.79	0.78	0.92	0.90
TA _w [mg malvidin-3- <i>O</i> -glucoside chloride/L]	198	238	303	237	276
MA _w [mg malvidin-3- <i>O</i> -glucoside chloride/L]	64	83	135	85	107
MA _w /TA _w [%]	32	35	45	36	39
TF _w [mg (+)-catechin/L]	2217	2050	2048	2449	2155
FVA _w [mg (+)-catechin/L]	2136	1679	1580	2209	1665
PRO _w [mg cyanidin chloride/L]	3654	2875	3038	4044	3213
FVA _w /PRO _w	0.58	0.58	0.52	0.55	0.52

The values in the first three numerical rows are the levels to achieve in the wines for each appellation, in the latter three are the levels achieved in the produced wines. L* = lightness; a* = red/green color coordinate; b* = yellow/blue color coordinate; TA_w = total anthocyanins; MA_w = monomeric anthocyanins; TF_w = total flavonoids; PRO_w = proanthocyanidins; FVA_w = flavanols vanillin assay.

The highest percentage of monomeric anthocyanins on the total content (45%) was found in Langhe DOC Freisa wine, which had also the highest content of total anthocyanins (303 mg malvidin-3-*O*-glucoside chloride/L). On the contrary, the lowest monomeric/total anthocyanin ratio (32%) and total anthocyanin content (198 mg malvidin-3-*O*-glucoside chloride/L) were found in Freisa d’Asti DOC wine. In both wines the data confirmed the trends found in the grape anthocyanins (Table 1).

About wine flavanols, the two highest contents of smaller molecular mass tannins [FVA_w, 2209 and 2136 mg (+)-catechin/L] were found in the wines from Tortonese and Astigiano locations, respectively. These two wines were produced from the grapes with high values of seed maturity index (Mp%=82, Table 1). As previously mentioned, smaller molecular mass tannins (flavanol monomers and oligomers) are perceived as more bitter, and therefore high contents of FVA_w in wines could be involved in high bitter sensations. For this purpose, some production strategies could be useful: for instance, when both technological and phenolic maturity are not satisfactory, the grape

dehydration could aid to reach a better sugars/acids ratio and a higher level of seeds lignification with a lower flavanols release. Furthermore, when technological maturity is satisfactory, the removal of the seeds from the must 48-96 h after the beginning of the fermentation process could limit the extraction of flavanols during maceration-fermentation (ROLLE *et al.*, 2008a).

4. CONCLUSIONS

The physico-chemical characteristics of the Piedmont minor variety *Vitis vinifera* L. cv. "Freisa" were comprehensively studied, considering grapes from five different growing areas and at three ripeness levels defined by density sorting. Mechanical properties and phenolic composition of "Freisa" grapes were differently affected by these factors, and some parameters showed a strong varietal character. Particularly, berry skin hardness parameters (break force and break energy), which influence extraction kinetics of phenolic compounds, were slightly affected by environmental conditions and ripeness degree. Instead, the other two mechanical parameters, namely skin Young's modulus and thickness, varied among the locations and berry density classes considered. The first one decreased with increasing the ripeness degree while, on the contrary, the second one showed an increasing tendency that affects positively the skin extractable content of phenolic compounds as follows. Berry skin total anthocyanin content increased significantly with increasing the density class, in agreement with higher values of skin thickness, and also was significantly affected by the growing area. Nevertheless, berry skin flavanol contents (monomeric - oligomeric and polymeric) varied only among locations, while seed flavanol contents varied among the locations and the density classes with a tendency to decrease from the less ripe to the ripest grapes berries considered. Grapes from Monferrato growing area showed the thickest skins, and coherently the highest contents of total anthocyanins but also of seed flavanols.

The crucial point of "Freisa" grapes has been confirmed to be the high release of flavanols from the seeds during winemaking. This aspect highlights the importance of a careful management of the maceration process in cellar and the possibility of using process strategies such as partial grape dehydration, when both technological and phenolic maturity are not satisfactory to avoid an excessive alcoholic degree, or the seeds removal from the must to reduce an excessive extraction. This may be important especially when "Freisa" grapes have not achieved a satisfactory ripeness degree, and hence the risk to produce unbalanced wines for bitterness and astringency sensory traits is high.

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Paper Received March 19, 2019 Accepted June 27, 2019

LIKING AND SENSORY DESCRIPTION OF PROTEIN SUBSTITUTES IN PHENYLKETONURIA SUBJECTS: A CASE-STUDY IN NORTHERN AND SOUTHERN ITALY

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ABSTRACT

Nowadays, it is important to make effort to develop new formulations for subjects affected by rare diseases who need to follow a lifetime diet to maintain a good health.

The purpose of the study was to evaluate the acceptability and to obtain a sensory descriptive analysis of protein substitutes (glycomacropeptide GMP formulas *vs* L-amino acid formulas) involving subjects affected by phenylketonuria in Northern and Southern Italy. Results demonstrated in both groups of subjects a greater acceptability of GMP samples, characterized by sweet and mild taste, mild odor, and natural color, compared with amino acid formulations. These sensory attributes should be considered during product development as a key factor influencing subjects' satisfaction.

Keywords: acceptability, CATA, food development, food formulations, odor, taste

1. INTRODUCTION

Sensory perception and food preferences play a key role in determining food choice and, thus, directly influence the diet quality (COX *et al.*, 2015). The study of the influence of sensory and hedonic individual differences in products acceptability is extremely important in modern food product development, especially for the ones meant to satisfy the needs of specific target populations (e.g, diabetics, elderly, obese etc). In this context, it is also important to make effort to develop new formulations for subjects affected by rare diseases who need to follow a lifetime diet to maintain a good state of health.

Between rare diseases, phenylketonuria (PKU) is an inborn metabolism error, which, if untreated, could lead to severe brain damage (GIOVANNINI *et al.*, 2012). The main purpose of PKU patients' treatment is to control the blood phenylalanine (Phe) concentration to prevent severe health consequences (BLAU, 2016). The treatment for PKU is mainly a lifetime diet based on low-protein foods in combination with amino acid supplements. These supplements without Phe, are enriched with vitamins and minerals, and some products are also added with fat and carbohydrates to ensure normal growth and good health through adulthood (VAN SPRONSEN *et al.*, 2017).

Compliance with this strict diet becomes a challenge over time, especially in adolescence, and this is primarily due to the unpleasant taste of the amino acid supplements and the variety of available formulations (MACDONALD *et al.*, 2010, AGUIAR *et al.*, 2015). In order to achieve better compliance with diet through the lifespan, protein supplements should satisfy the need for better taste and easier management (VAN SPRONSEN *et al.*, 2008). The number of available protein substitutes for PKU subjects is increasing over time (FEILLET and AGOSTONI, 2010). In this context, beside the alternatives to traditional substitutes, the casein glycomacropeptide (GMP) is a 64-amino acid peptide from cheese whey which is rich in specific essential amino acids and is the only known natural protein source free from Phe (NEY *et al.*, 2009; SOLVERSON *et al.*, 2012).

The lack of empirical studies aimed at evaluating subjects' satisfaction for these substitutes reinforce the need to evaluate PKU subjects' perception regarding which sensory characteristics should have low-Phe formulations to be more appreciated. Only one recent study (PROSERPIO *et al.*, 2018) has been conducted in an ambulatory context involving PKU subjects leading to define the sensory drivers of liking of protein substitutes in Northern Italy. However, intracultural differences between the Northern and Southern regions of Italy are well recognized, not only referred to industrialization and economic prosperity, but also to cultural values and social structures (RUGGIERO *et al.*, 2000). These differences are also clearly reflected in different dietary patterns, which lead to a higher percentage of overweight and obese subjects in the Southern areas. In this context, some national data collected from the survey "Indagine Multiscopo dell'Istituto Nazionale di Statistica (ISTAT, 2015)" showed that, in the Southern regions, the consumption of food rich in carbohydrates prevails while healthy and low energy dense food consumption characterized the North West area.

Due of the above, the aim of the present study was to evaluate the acceptability of new GMP formulas compared with the more traditional amino-acid mixtures involving subjects affected by phenylketonuria in Northern and Southern Italy. A sensory descriptive analysis was also conducted in order to better understand which are the sensory characteristics that are related to the acceptance of these formulations that should be considered during the products development.

2. MATERIALS AND METHODS

2.1. Subjects

A total of sixty-six subjects (mean age: 25.6 ± 5.9 years, 34 women and 32 men) gave informed consent and completed the study. Thirty-three subjects who were admitted to Department of Pediatrics, San Paolo Hospital (Milan, Italy), and thirty-three subjects referred to the Department of Pediatrics, Federico II University Hospital (Naples, Italy) were recruited. All participants were following a low-phenylalanine diet (metabolic control Phe (uMol/L): Milan= 642.1 ± 309.2 ; Naples= 720.9 ± 295.2). The exclusion criteria were: pregnancy, food allergies to whey proteins, severe neurological and functional disorders. The present study was performed according to the principles established by the Declaration of Helsinki after the protocol was approved by the Institutional Ethics Committee (protocol approval n°210).

2.2. Samples

4 L-amino acid formulas (AA) and 4 Glycomacropeptide (GMP) formulas, flavored with neutral, chocolate, strawberry and tomato aromas were prepared as reported in PROSERPIO *et al.*, (2018).

The GMP flavored formulas (GMP_strawberry and GMP_tomato) were prepared by adding 2g of flavoring powder (aroMaxx erdbeere; aroMaxx tomate-basilikum, MetaX Istitut fur DiatetiK- MamoXi, Torino, Italy) to the neutral formulation (GMP_neutral) which consisted of 100 ml of Glytactin RTD™ (Cambrooke-Quaris, Roma, Italy). The GMP chocolate flavored sample (GMP_chocolate) consisted of 100 ml of Glytactin RTD™ Chocolate (Cambrooke-Quaris, Roma, Italy).

The AA neutral formula (AA_neutral) was prepared by mixing 16.5 g of powder high in L-amino acid (Xphe energy kid neutral, MetaX Istitut fur DiatetiK- MamoXi, Torino, Italy) and water to reach a final volume of 100 ml. The AA flavored formulas (AA_tomato; AA_chocolate) were prepared by adding 2g of flavoring powder (aroMaxx tomate-basilikum; aroMaxx schoko, MetaX Istitut fur DiatetiK- MamoXi, Torino, Italy). The L-amino acid strawberry flavored samples (AA_strawberry) were prepared using 16.5 g of Xphe energy kid erdbeere (MetaX Istitut fur DiatetiK- MamoXi, Torino, Italy) and water.

Each of these samples provides 5 g/100 ml protein equivalents. All the formulas were provided by MamoXi (Torino, Italy) and Cambrooke-Quaris (Roma, Italy).

30 ml of each sample were presented monadically, in a randomized and balanced order, to each participant in plastic cups labelled with three-digit codes. Water was available for rinsing the palate between the samples.

2.3. Experimental procedure

Sessions were conducted between 10:30-12:30 in quiet rooms under similar light conditions in both group of subjects (Milan and Naples). They were asked to refrain from consuming anything but water for 2 hours before the test (hungry state).

Subjects started by filling in the questionnaire on general appetite. Subsequently, they had to score their overall liking and, after a rest of 5 min, to made a sensory descriptive evaluation ('check-all-that-apply' questionnaire: CATA; VARELA and ARES, 2012) for each sample. All samples were prepared on the same day of the session and were presented at room temperature (20-22 °C).

2.4. General appetite

To ensure that both subjects from Milan and Naples were in a similar hunger state, they were asked to rate their appetite at the beginning of the session by filling out a questionnaire on general appetite (hunger, fullness, desire to eat, and thirst) all measured on 100mm Visual Analogue Scales (VAS, 'not at all': scored 0; 'very': scored 10).

2.5. Overall liking evaluation

Subjects were asked to taste the samples monadically and to express their liking scores on a 100mm VAS anchored by the extremes "extremely disliked" (rated 0) and "extremely liked" (rated 10). The experimenters provided instructions for the use of the scale prior to tasting (LAWLESS *et al.*, 2010).

2.6. Sensory descriptive evaluation

The sensory descriptive evaluation was performed using the 'check-all-that-apply' (CATA) questionnaire. A separate group of 12 untrained subjects (age range: 20-40 years) attended a pilot test to define the suitable terms to describe the samples using a free listing questionnaire (VARELA and ARES, 2012). The eight low protein samples were provided to the subjects and they were asked to evaluate the sensory characteristics and to write all attributes for describing their color, appearance, odor, taste, flavor and texture. The individual development of the attributes was followed by an open discussion. Subsequently, the experimenters finalized the list of terms, selecting the most mentioned and the most common words in order to avoid synonymous (JAEGER *et al.*, 2015).

Finally, the questionnaire consisted of a list of 27 sensory attributes: 10 for the appearance (light brown, dark brown, light yellow, dark yellow, light pink, dark pink, natural color, artificial color, brightness and opaque), 6 for the odor (natural odor, artificial odor, mild odor, strong odor, milk odor and vanilla odor), 8 for the taste/flavor (sweet, bitter, salty, sour, mild taste, strong taste, milk flavor and vanilla flavor) and 3 for the texture (thin, thick and floury).

Subjects who took part to the experimental sessions were asked to check from the list all the terms that they considered appropriate to describe each sample. The position of attributes was randomized.

2.7. Data analysis

A paired samples t-test was performed to compare the general appetite ratings (100mm VAS: hunger, fullness, satiety, desire to eat, and thirst) in the Northern and Southern groups before the samples evaluation.

A linear mixed model procedure was carried out on overall liking scores considering 'samples', 'gender' (women and men), 'city' (Northern and Southern) and their two-way interactions as fixed factors. Subjects were added as random effect while age as covariate. When a significant difference ($p < 0.05$) was found, least significant difference (LSD) *post hoc* test was used. These statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk NY).

For the sensory descriptive evaluation, a data set was generated as 0/1 matrix, that is, "1" if the term was selected by the subjects and "0" if term was not selected. A frequency table was made from the total count of each term for each sample. Cochran's Q test was carried

out for each of the 27 terms to detect differences in participants' perception of the evaluated samples.

Correspondence analysis (CA) was used to obtain a bi-dimensional representation of the samples and to show relationship between samples and terms from the CATA questionnaire. Since the results provided by Northern and Southern subjects were similar to each other, the CA results are reported showing all the 66 subjects.

Penalty-lift analysis (PLAEHN, 2012; MEYNER *et al.*, 2013) was also performed in order to study which CATA terms were positively or negatively related with liking scores. These statistical analyses were performed using XLSTAT-Sensory® software for Windows, Version 2015.6.01 (Addinsoft™, France). A p-value of <0.05 was considered significant.

RESULTS

3.1. General appetite

The baseline general appetite ratings (Table 1) confirmed that feelings of hunger, fullness, desire to eat, and thirst were not significantly different in the two groups of subjects (Northern and Southern).

Table 1. General appetite ratings (means±SEM), as measured on 100 mm VAS, provided by Northern and Southern subjects.

General appetite	Northern	Southern	t	p
Hunger	4.47±0.45	4.53±0.46	0.08	0.94
Fullness	3.43±0.43	3.53±0.49	1.69	0.09
Desire to eat	4.99±0.48	4.45±0.45	0.82	0.41
Thirsty	4.77±0.44	5.86±0.37	1.90	0.07

3.2. Overall liking evaluation

The main factor 'samples' was found to have a significant effect on liking scores ($F_{(7;441)} = 58.75$; $p < 0.001$). Generally, GMP samples obtained significant higher liking scores ($M = 4.3 \pm 0.1$) compared with the AA formulas ($M = 2.7 \pm 0.2$).

A significant effect of the interaction 'samples' * 'city' on liking scores was also found ($F_{(7;441)} = 2.95$, $p < 0.01$). The mean liking scores by samples in subjects from Milan and Naples are provided in Table 2.

Looking at the results gave by Northern subjects, the highest liking scores were obtained by the GMP_chocolate and GMP_strawberry, which were comparable to each other. The AA_strawberry samples obtained the highest score between the AA formulas, even if all AA samples were not acceptable (score <5). Similarly, Southern subjects preferred the GMP and AA samples flavored with strawberry aroma. Contrariwise, the tomato flavored samples, both GMP and AA formulas, obtained the lowest hedonic ratings in both subjects from Milan and Naples.

Table 2. Liking scores (means±SEM) for each samples provided by Northern and Southern subjects. Different letters (in column) indicate significant differences according to *post hoc* test within each group of subjects.

Samples		Liking scores		
		Northern	Southern	p
GMP	tomato	1.10 ^a ±0.38	1.23 ^a ±0.39	0.78 ^{n.s}
	neutral	4.48 ^c ±0.37	4.45 ^{cd} ±0.39	0.89 ^{n.s}
	chocolate	5.64 ^d ±0.37	4.84 ^d ±0.38	0.13 ^{n.s}
	strawberry	6.45 ^d ±0.38	6.13 ^e ±0.40	0.57 ^{n.s}
AA	tomato	1.17 ^a ±0.38	0.94 ^a ±0.38	0.72 ^{n.s}
	neutral	1.23 ^a ±0.37	3.10 ^b ±0.39	0.00 ***
	chocolate	3.00 ^b ±0.38	3.33 ^{bc} ±0.39	0.55 ^{n.s}
	strawberry	4.05 ^c ±0.37	5.17 ^{de} ±0.38	0.03*

*p<0.05; *** p<0.001.

Comparing the results obtained in the two groups of subjects no significant differences have been highlighted between the GMP samples, while significant differences have been found in AA samples. In particular, significant higher scores were provided to AA_neutral and AA_strawberry by Southern subjects.

The main factor 'gender' and the interactions 'sample' * 'gender'; 'city' * 'gender' were not significant ($F_{(1,64)} = 0.015$, $p=0.90$; $F_{(7,441)} = 0.70$, $p=0.80$; $F_{(1,64)} = 0.004$, $p=0.95$, respectively).

3.3. Sensory descriptive evaluation

The contingency table below (Table 3) shows the frequency of terms checked by Northern and Southern subjects to describe the eight samples.

Significant differences ($p<0.001$) were found in the frequency for all the 27 terms within the five sensory attributes categories evaluated, suggesting that the PKU subjects involved perceived differences between samples in terms of their sensory characteristics.

The Correspondence Analysis, used to obtain a bi-dimensional representation of the samples and the relationship between samples and terms from the CATA questionnaire, resulted in two dimensions accounting for 60.62% of variance in the data.

As shown from Fig. 1, samples were clearly discriminated by subjects according to their aroma. Indeed, samples flavored with strawberry aroma (AA_strawberry and GMP_strawberry) are positioned in the upper left side of the map while the samples added with tomato aroma (AA_tomato and GMP_tomato) are situated in the upper right side of the map. In the lower part of the map, GMP samples without aroma (GMP_neutral) and the chocolate one (GMP_chocolate) are well distinguished from the L-amino acid formulas with the same aromas (AA_neutral and AA_chocolate).

Table 3. Contingency table for the sensory descriptive analysis evaluation.

Sensory attributes	AA samples				GMP samples			
	Chocolate	Strawberry	Neutral	Tomato	Chocolate	Strawberry	Neutral	Tomato
<i>Appearance</i>								
artificial color	9	28	12	20	10	24	14	26
natural color	17	7	24	6	19	7	25	7
light yellow	0	0	5	1	12	0	19	1
dark yellow	0	0	0	3	12	0	1	7
brightness	2	18	15	4	11	5	12	5
light brown	5	0	0	45	25	0	3	35
dark brown	60	0	0	7	4	0	0	4
opaque	19	3	6	18	13	13	16	23
light pink	0	58	0	0	0	15	0	4
dark pink	0	2	0	6	0	47	0	9
<i>Odor</i>								
artificial odor	21	25	32	34	12	23	10	35
mild odor	15	38	19	2	26	24	27	6
milk odor	4	4	8	0	14	6	32	2
vanilla odor	1	7	13	1	13	4	11	2
strong odor	21	5	11	43	10	19	7	40
natural odor	20	14	8	3	20	9	18	3
<i>Taste</i>								
sweet	14	39	6	1	41	51	33	5
sour	10	12	17	26	2	3	6	23
salty	12	6	15	38	3	0	5	31
bitter	27	9	28	24	8	1	6	18
mild taste	11	27	18	2	31	27	32	5
strong taste	35	20	28	51	7	23	9	45
<i>Flavor</i>								
milk flavor	6	9	2	0	24	10	35	1
vanilla flavor	1	4	8	0	16	3	8	2
<i>Texture</i>								
thin	18	44	39	29	24	25	32	25
thick	32	0	17	16	18	21	20	25
floury	21	2	14	12	11	8	6	8

3.4. Relating sensory profiling with liking

A penalty-lift analysis was carried out to understand which sensory attributes were mainly associated to the overall liking of the samples. The analysis showed to which extent liking increased or decreased when the subjects related a certain CATA terms to the samples.

As inferred from Fig. 2 liking scores were significantly positively associated with the sensory attributes: 'sweet' ($p < 0.0001$), 'mild taste' ($p < 0.0001$), 'mild odor' ($p < 0.0001$) and

'natural color' ($p < 0.0001$). Contrariwise, the terms that significantly decreased the samples' acceptability were: 'salty' ($p < 0.0001$); 'strong taste' ($p < 0.0001$); 'bitter' ($p < 0.0001$); 'strong odor' ($p < 0.0001$); 'light brown' ($p < 0.0001$); 'artificial odor' ($p < 0.0001$), 'opaque' ($p < 0.001$) and 'thick' ($p < 0.05$).

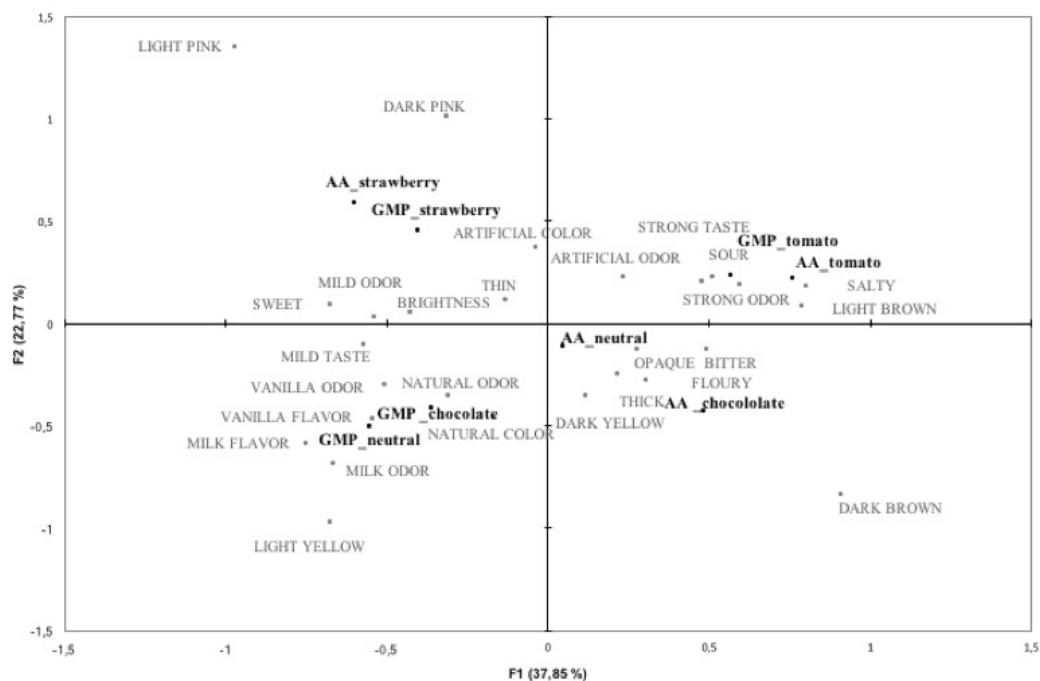


Figure 1. Attributes and products plot obtained from CATA total frequency counts.

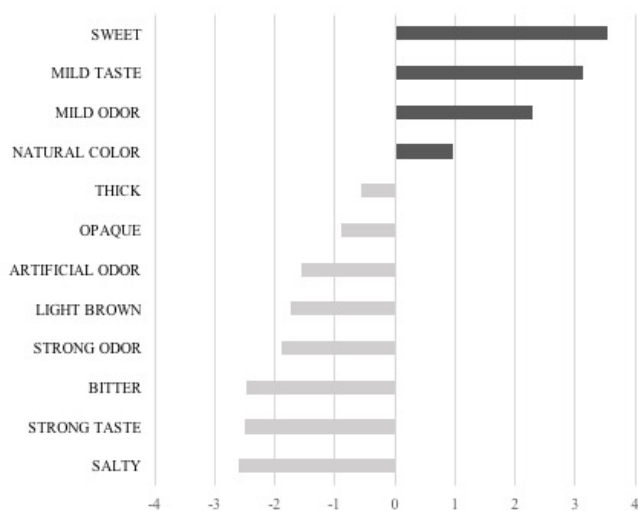


Figure 2. Penalty-lift analysis of sensory attributes across all samples.

4. DISCUSSION

The purpose of the present study was to deepen the evaluation of the acceptability of protein substitutes (GMP formulas *vs* L-amino acid formulas) involving subjects affected by phenylketonuria in Northern and Southern Italy. A sensory descriptive evaluation was performed to define which sensory characteristics are mainly related to the acceptance or to the refusal of these products that should be taken into account in developing new formulations for specific target populations.

Overall, the present results demonstrated in both subjects from Milan and Naples a greater acceptability of GMP samples compared with the more traditional amino acid formulations, besides the reported differences in dietary pattern among the regions of Italy (RUGGIERO *et al.*, 2000; ISTAT, 2015). In particular, GMP samples flavored with chocolate and strawberry, were the most appreciated by the Northern group, while the sample flavored with strawberry was the most appreciated by the Southern group. These results confirmed our previous findings with a group of Northern subjects (PROSERPIO *et al.*, 2018) in which a greater acceptability was depicted for GMP beverages flavored with chocolate and strawberry compared with the L-amino acid formulations flavored with the same aromas. According to the present results Lim and collaborators (2007) showed that a GMP chocolate beverage was significantly more liked compared with the same flavored amino acid beverage.

Both groups of subjects from Milan and Naples gave to the tomato flavored samples the lowest liking scores. Even if the GMP samples received generally average higher liking scores ($M=4.3\pm0.1$) compared with the AA formulas ($M=2.7\pm0.2$), it is important to consider that the type of added aroma was a key driver of the acceptance. Indeed, the GMP sample, as well as AA formulation, flavored with the tomato aroma were evaluated as not acceptable by both group of subjects. These samples were not appreciated since they were perceived as salty, sour and characterized by strong odor and taste. The tomato flavored formulations maybe were perceived as unpleasant since tomato aroma, signaling savory product, is not usually used as flavoring in low-phenylalanine products, especially as beverages. Thus, subjects maybe have perceived tomato flavored samples as really far from their usual food habits. Indeed, it is well known that food habits and also consumers' expectations could influence the sensory perception, the liking, and consequently the actual food consumption (KÖSTER, 2009). Confirming this hypothesis, two different studies demonstrated that cracker samples with GMP, a product expected to be characterized by salty taste, obtained higher liking scores compared with the low-protein crackers (LIM *et al.*, 2007; VAN CALCAR *et al.*, 2012).

Considering the sensory attributes mainly associated to the overall liking of the samples, the present study demonstrated that the acceptability increased when the samples were characterized by the attributes: 'sweet', 'mild taste', 'mild odor' and 'natural color'. The information achieved by the present results could be useful to understand which sensory characteristic should have a low-phenylalanine product to be more accepted by the subjects. Consequently, more appreciated products could facilitate dietary compliance that is a challenge over time, especially in adolescence (MACDONALD *et al.*, 2010, AGUIAR *et al.*, 2015). Moreover, it has also been demonstrated that, besides the higher acceptance of GMP formulations compared the more traditional ones, GMP could also be considered as a more physiological source of dietary protein and promote higher satiety compared with synthetic amino acids (VAN CALCAR *et al.*, 2012).

As future perspective, it should be useful to evaluate other GMP base products using validated sensory approaches, as it has been performed in the present study, besides considering mainly the nutritional composition of these products.

In conclusion, the present results suggest that, in order to improve the protein substitutes' sensory quality, these formulations should be characterized by a sweet and mild taste, a mild odor, and a natural color. Indeed, it is important to consider the PKU patients' satisfaction as a key factor during the product development in order to improve the diet quality through the lifespan.

ACKNOWLEDGMENTS

We would like to express our thanks to MamoXi (Torino, Italy) and Cambrooke-Quaris (Roma, Italy) for supplying the samples.

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Paper Received February 5, 2019 Accepted June 20, 2019

VARIATIONS IN NUTRITIVE COMPOSITION OF THREE SHELLFISH SPECIES

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ABSTRACT

Nutritive composition, fatty acid profile and health-related lipid indices of natural-born European flat oyster (*Ostrea edulis*), variegated scallop (*Chlamys varia*) and smooth scallop (*Flexopecten glaber*) in 108 samples originating from the Adriatic sea, recovered on a monthly basis were investigated. Out of three shellfish species, the lowest share of saturated fatty acids, the most favourable ratio of polyunsaturated over saturated fatty acids, the most favourable atherogenic and thrombogenic index, and the most favourable ratio of hypocholesterolaemic over hypercholesterolaemic fatty acids were seen in oysters, sampled during springtime. No statistically significant inter-seasonal differences between basic chemical parameters and fat quality indices were established.

Keywords: European flat oyster, lipid quality indices, seasonal variations, smooth scallop, variegated scallop

1. INTRODUCTION

Aquaculture is the fastest-growing food production sector worldwide, shellfish thereby being an important component of global aquatic food supply. The production of marine organisms mainly takes place in sheltered areas or coastal embayments (POGODA, *et al.*, 2011), oyster cultivation thereby being a particularly good example of an extensive production of high value-added products (GIBBS, 2004). Due to the high nutritional and gastronomic value of these products, consumer demand for cultivated, but also wild shellfish continuously increases. In general, shellfish is a highly nutritious foodstuff, since it contains appreciable quantities of digestible proteins, essential amino acids, bioactive peptides, long-chain polyunsaturated fatty acids, astaxanthin and other carotenoids, vitamin B12 and other vitamins, minerals including copper, zinc, inorganic phosphate, sodium, potassium, selenium, iodine and also other nutrients, which offer a variety of health benefits to consumers (VENUGOPAL and GOPAKUMAR, 2017). In comparison to other shellfish, European flat oyster (*Ostrea edulis*) represents a product with a higher nutritional value and is hence much higher priced than other shellfish (FAO, 2011).

Literature data have shown that seasonal metabolic activities of shellfish molluscs result from complex interactions between food availability, environmental and growth factors, type of shellfish, but also other parameters (GABBOTT, 1983). For example, lipid changes seen throughout an annual cycle may be related to the increase in energy and nutritional requirements during gonad development (LUZZANA *et al.*, 1996), temperature changes (VARLJEN *et al.*, 2004) or diet (HENDERSON *et al.*, 1996). GULLIAN and AGUIRE-MACEDO (2009) pointed out that, although oysters are tolerant to a broad range of natural variables, this shellfish is susceptible to various forms of physical and chemical disturbances, which cause extreme changes in their metabolism, physiology and defence related-functions, seasonal variations thereby also changing their physiology. Variations that occur in different varieties of European shellfish over a 12-month period have not been fully explored yet.

Additionally, studies of shellfish nutritive composition have been performed only on some species or during certain seasons, mainly on the Atlantic oysters (*Crassostrea gigas*) as cultivated shellfish of great economic importance (PAZOS *et al.*, 1996; SOUDANT *et al.*, 1999; DAGORN *et al.*, 2016). On top of that, data on natural-born oysters inhabiting the Adriatic Sea and other types of shellfish is scarce. Investigations into the shellfish composition could provide producers with a useful background information that could also well serve the needs of consumers keen to evaluate health benefits of their shellfish consumption. In view of the above, the aim of this study was to investigate into, and compare, the nutritional properties of European flat oyster (*Ostrea edulis*), variegated scallop (*Chlamys varia*) and smooth scallop (*Flexopecten glaber*) originating from natural beds in the Adriatic Sea. To the best of our knowledge, this is the first study of basic nutritional composition, fatty acid profile and lipid quality of three natural-born shellfish species populating the Adriatic Sea and seasonal variations in the above parameters witnessed throughout a year.

2. MATERIALS AND METHODS

2.1. Sampling and sample preparation

Samples of European flat oyster (*Ostrea edulis*), variegated scallop (*Chlamys varia*) and smooth scallop (*Flexopecten glaber*) were retrieved during 2016 and 2017 from the western coast of the Istrian Peninsula (Fig. 1). This area is extending from the Savudrija Cape on the north to the border of the territorial sea of the Republic of Croatia, and from the Barbariga Cape on the south to the border of the territorial sea of the Republic of Croatia. This geographic area is highly influenced by strong currents and the vicinity of the Mirna River mouth. It hosts natural beds of different species of bivalves like European flat oyster (*Ostrea edulis*), smooth scallop (*Flexopecten glaber*), variegated scallop (*Chlamys varia*), clam (*Venus verrucosa*) and Noah's Ark (*Arca noae*). Fishermen are collecting mollusks by trawling. Variegated scallop samples were collected at the B1 point (45°31'30"N; 13°27'18"E), those of smooth scallop at the B2 point (45°13'15,3"N; 13°30'00"E) and those of European flat oyster at the B3 point (45°01'14,7"N; 13°41'46,8"E) of the area detailed above.

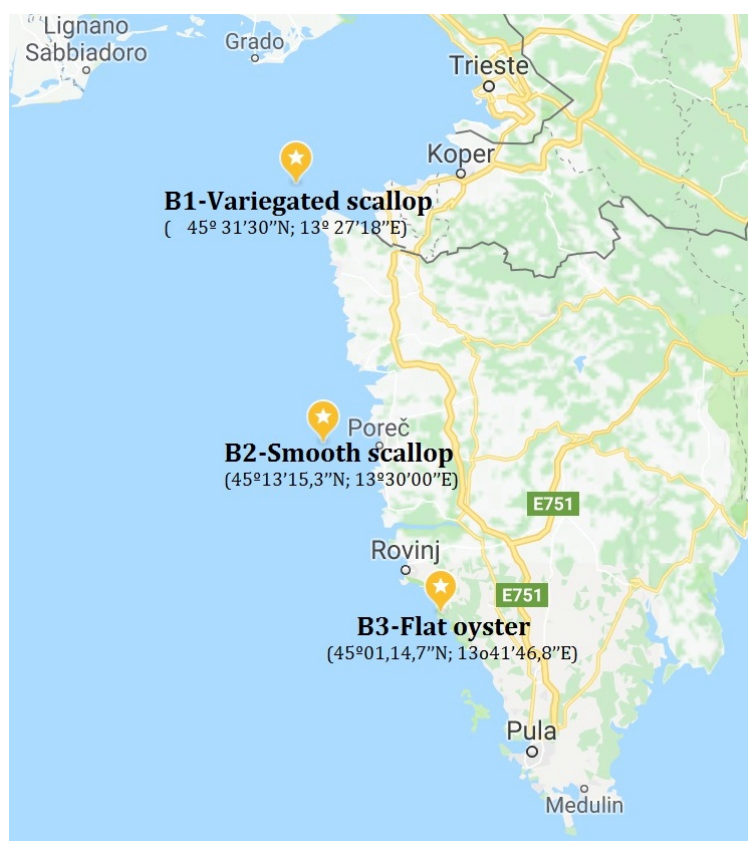


Figure 1. Sampling points at the west coast of the Istrian Peninsula.

Shellfish samples were grouped based on the recovery season, that is to say, into the group of samples retrieved during springtime (March, April & May 2016), those retrieved during summertime (June, July & August 2016), those retrieved during autumn time (September, October & November 2016) and those retrieved during wintertime (December 2016 and January & February 2017). Each month, samples containing 3 kg of each shellfish under study were sampled from the locations of their growth. In total, 108 shellfish samples (36 oyster, 36 variegated scallop and 36 smooth scallop samples) were analysed within 48 hours after sampling. From a 3 kg-shellfish sample, 300 to 400 g of muscle tissue were obtained and further homogenized using a Grindomix GM200 knife mill (Retch, Germany), so as to obtain a homogeneous sample allowing for the determination of basic chemical composition and fatty acid profile.

2.2. Determination of basic chemical composition

The moisture content was determined using gravimetric analysis. The samples were dried at 103 ± 2 °C (ISO 1442:1997) in an UF75 Plus Memmert oven (Schwabach, Germany). The total protein content was determined by virtue of the Kjeldahl method (HRN ISO 937:1999) using an 8 - Basic Digestion Unit (Foss, Höganäs, Sweden) for sample digestion and an automated device for distillation and titration (Vapodest 50s, Gerhardt, Germany). The total fat content was determined using the Soxhlet method (HRN ISO 1443:1999) that implies the digestion of samples by virtue of acid hydrolysis, followed by the extraction of fats using petroleum ether and a Soxtherm 2000 automated device (Gerhardt, Munich, Germany). The ash content was determined according to the ISO 936:1998 and made use of a LV9/11/P320 Nobertherm furnace (Lilienthal, Germany). All chemicals used for the analyses were of an analytical grade. Carbohydrate content was determined by calculation, based on the determination of water, ash, total protein and fat content. The mean of data obtained from two parallel runs in form of weight percentage (%) and with the accuracy of 0.01% was considered as a result descriptive of a single sample.

2.3. Fatty acid profile

Sample preparation method for the analysis of fatty acid methyl esters was described earlier by PLEADIN *et al.* (2015). Methyl esters of fatty acids were analysed using gas chromatography (GC) according to the EN ISO 12966-2:2011 and EN ISO 12966-4:2015. To the above effect, a 7890BA gas chromatographer equipped with flame ionization detector (FID), a 60-m DB-23 capillary column having an internal capillary diameter of 0.25 mm and the stationary phase thickness of 0.25 µm (Agilent Technologies, Santa Clara, USA) was used. The components were detected by FID at the temperature of 280 °C, hydrogen flow of 40 mL/min, air flow of 450 mL/min and nitrogen flow of 25 mL/min. The initial column temperature was 130 °C; after a minute, it was increased by 6.5 °C/min until the temperature of 170 °C was reached. The temperature was further increased by 2.75 °C/min until the temperature of 215 °C was attained. The latter temperature was maintained for 12 min and then further increased rate by 40 °C/min until the final column temperature of 230 °C was reached, the latter being maintained for 3 min. One mL of a sample was injected into a split-splitless injector at the temperature of 270 °C and with the partition coefficient of 1:50. The carrier gas was helium (99.9999%), flowing at the constant rate of 43 cm/sec. Fatty acid methyl esters were identified by comparing their retention times with those of fatty acid methyl esters contained by the standard mixture, as

described earlier by PLEADIN *et al.* (2015). The results are expressed as a percentage (%) of a particular fatty acid in total fatty acids, the accuracy thereby being 0.01%.

2.4. Nutritional quality of lipids

Data on fatty acid composition were used for the calculation of the following lipid quality indices: the atherogenic index (AI), the thrombogenic index (TI) and the hypocholesterolaemic/hypercholesterolaemic ratio (HH). The atherogenic index (AI) indicates the relationship between the sum of the main saturates and the sum of the main non-saturates. This parameter was calculated as: $AI = [(C12:0 + (4 \times C14:0) + C16:0)] / [\sum MUFA + PUFA\ n-6 + PUFA\ n-3]$ (ULBRITCTH and SOUTHGATE, 1991). The thrombogenic index (TI) is defined as the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic FAs (MUFA, PUFA n-6 & PUFA n-3). The index was calculated as: $TI = (C14:0 + C16:0 + C18:0) / [0.5 \times \sum MUFA + 0.5 \times PUFA\ n-6 + 3 \times PUFA\ n-3] + (PUFA\ n-3 / PUFA\ n-6)$. The ratio of hypocholesterolaemic over hypercholesterolaemic fatty acids (HH) takes into account well-known effects of certain fatty acids on cholesterol metabolism (SANTOS-SILVA *et al.*, 2002). It was calculated as: $HH = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3) / (C14:0 + C16:0)$ (ULBRITCTH and SOUTHGATE, 1991).

2.5. Statistical analysis

Statistical analysis was performed using the SPSS Statistics Software 22.0 (SPSS Statistics, NY IBM, 2013). In order to determine the differences between the sample groups (season-based, shellfish type-based), one-way ANOVA and the robust Brown-Forsythe test were used. The decisions on statistical significance were made at the significance level of $p < 0.001$ and $p < 0.05$.

3. RESULTS AND DISCUSSION

This study provides data on nutritional composition of three natural-born shellfish species originating from the Adriatic Sea, with a special emphasis on fatty acids and health-related lipid indices determined over four seasons of a one-year period. Literature data have revealed that habitats, season, feed, species, but also gametogenesis and spawning cycle, can influence the proximate shellfish composition (GABBOTT, 1983).

Basic chemical composition of the investigated shellfish determined in this study is shown in Table 1. As regards the moisture content, oysters had a significantly lower average value (around 82 g/100 g) as compared to variegated (around 84 g/100 g, $p = 0.0028$) and smooth scallops (around 87 g/100 g, $p < 0.001$), representing the shellfish richer in nutrients in comparison to the other two. However, oysters were the only one out of the three shellfish species in which significant seasonal moisture content variations were found (higher moisture content in autumn as compared to spring and summer). Moisture contents similar to those we found, with values of 82.1 g/100 g during summer and 81.4 g/100 g during winter, were reported by MARTINO and CRUZ (2004) for oysters of the *Crassostrea rhizophorae* species.

Oysters also had significantly higher carbohydrate content (average value around 4.3 g/100 g) as compared to variegated (around 3.2 g/100 g) and smooth scallops (1.4 g/100

g). However, the significant seasonal carbohydrate content variations weren't found for any shellfish ($p > 0.05$).

Table 1. Basic chemical composition of the analysed shellfish types during a one-year period.

Shellfish	Season	Mean $\pm$ SD (g/100 g)				
		Moisture	Protein	Ash	Fat	Carbohydrate
European flat oyster (n=36)	Spring	80.30 $\pm$ 0.53 ^d	10.99 $\pm$ 0.27 ^d	2.04 $\pm$ 0.16	2.37 $\pm$ 0.32 ^{a,d}	4.29 $\pm$ 0.63
	Summer	81.43 $\pm$ 0.58 ^d	10.00 $\pm$ 0.27	2.14 $\pm$ 0.43	2.03 $\pm$ 0.25 ^d	4.40 $\pm$ 0.07
	Autumn	83.87 $\pm$ 0.61 ^{b,c}	8.13 $\pm$ 1.13 ^b	2.21 $\pm$ 0.04	1.20 $\pm$ 0.10 ^{b,c}	4.57 $\pm$ 0.77
	Winter	81.87 $\pm$ 1.22	10.27 $\pm$ 1.47	2.33 $\pm$ 0.20	1.67 $\pm$ 0.23 ^b	3.88 $\pm$ 0.75
	Average	81.87 $\pm$ 1.50 ^{B,C}	9.85 $\pm$ 1.36	2.18 $\pm$ 0.24	1.82 $\pm$ 0.50 ^{B,C}	4.29 $\pm$ 0.30 ^{B,C}
Variegated scallop (n=36)	Spring	80.43 $\pm$ 7.51	8.88 $\pm$ 0.48	3.40 $\pm$ 2.42	0.90 $\pm$ 0.20	6.39 $\pm$ 7.94
	Summer	85.27 $\pm$ 0.40	9.43 $\pm$ 0.24	2.56 $\pm$ 0.57	0.97 $\pm$ 0.23	1.77 $\pm$ 0.57
	Autumn	85.23 $\pm$ 1.31	9.90 $\pm$ 1.52	1.94 $\pm$ 0.20	0.93 $\pm$ 0.12	1.99 $\pm$ 0.44
	Winter	85.27 $\pm$ 1.25	9.02 $\pm$ 2.03	2.06 $\pm$ 0.35	0.77 $\pm$ 0.15	2.87 $\pm$ 1.81
	Average	84.05 $\pm$ 3.95 ^{A,C}	9.31 $\pm$ 1.18	2.49 $\pm$ 1.23	0.89 $\pm$ 0.17 ^{A,C}	3.24 $\pm$ 1.83 ^A
Smooth scallop (n=36)	Spring	86.57 $\pm$ 0.35	9.32 $\pm$ 1.26	1.70 $\pm$ 0.17	1.07 $\pm$ 0.31 ^d	1.34 $\pm$ 0.29
	Summer	86.70 $\pm$ 0.79	8.71 $\pm$ 0.39	2.55 $\pm$ 0.61	0.77 $\pm$ 0.15	1.28 $\pm$ 0.33
	Autumn	87.63 $\pm$ 0.95	8.65 $\pm$ 0.88	1.91 $\pm$ 0.16	0.30 $\pm$ 0.10 ^b	1.51 $\pm$ 0.46
	Winter	87.03 $\pm$ 1.42	8.68 $\pm$ 0.96	2.27 $\pm$ 0.71	0.53 $\pm$ 0.23	1.49 $\pm$ 0.53
	Average	86.98 $\pm$ 0.92 ^{A,B}	8.84 $\pm$ 0.32	2.11 $\pm$ 0.53	0.67 $\pm$ 0.35 ^{A,B}	1.40 $\pm$ 0.11 ^A

Results are expressed as the mean value (mean $\pm$ SD) of six results (3 months per season; each month, one sample was taken and analysed in duplicate).

Statistically significant difference ($p < 0.05$) within the same column for every shellfish type separately:

^avs. winter; ^bvs. spring; ^cvs. summer; ^dvs. autumn; ^Avs. European flat oyster; ^Bvs. variegated scallop; ^Cvs. smooth scallop.

The average protein content was almost equal in all three studied shellfish species, ranging from 8.84 g/100 g in smooth scallops to 9.85 g/100 g in oysters. The proportion of proteins significantly differed among the shellfish species only in summer. Oysters and smooth scallops contained the highest protein levels in spring (10.99 g/100 g and 9.32 g/100 g, respectively) while variegated scallops presented with the highest protein levels in autumn (9.90 g/100 g), although the only statistically significant difference ($p = 0.014$) was that in the protein content of oysters, which was higher in those collected in spring as compared to those collected in autumn. Three shellfish species had quite similar average ash contents, ranging from 2.11 g/100 g in smooth scallop to 2.49 g/100 g in variegated scallop and showing no statistically significant differences, neither across seasons nor across species.

Based on linear correlation coefficient and slope values from correlation equations related to moisture and fat ($y = -0.1818x + 16.453$; $R^2 = 0.6275$), moisture and protein ($y = -0.1153x + 19.232$; $R^2 = 0.0885$) as well as moisture and ash ($y = -0.0679x + 7.986$; $R^2 = 0.1681$), it is clear that fat content shows the strongest inversely proportional relationship with the moisture content found in the three shellfish. Therefore, a decrease in proportion of water is primarily reflected in an increase of fat content, especially in case of oysters. Oysters had

a significantly higher average fat content (1.82 g/100 g) as compared to variegated (0.89 g/100 g) and smooth scallops (0.67 g/100 g) ($p < 0.001$), as well as the highest share of fat in winter ($p = 0.002$), spring ($p = 0.001$), summer ($p = 0.001$) and autumn ($p < 0.001$) in comparison to other shellfish types (*data not shown*). As regards seasonal influence, both oysters and smooth scallops showed a significantly higher fat content in spring than in autumn ($p = 0.005$ and $p < 0.05$, respectively), while in variegated scallops no significant variations were found. Since fats have been shown to be involved in spawning-related biochemistry of marine species (REN *et al.*, 2003), the observed variability in fat levels in different sampling times was to be expected.

In comparison to the results of LIRA *et al.* (2013) that revealed these species to have a higher fat content in winter than in summer, our study failed to confirm such a pattern. However, in the study quoted above, the composition of oysters was analysed using the Brazilian cultivated *Crassostrea rhizophorae* oysters sampled in only two seasons - winter and summer. Nevertheless, our spring sampling could be compared to their winter sampling, confirming the same variability pattern. Also, it should be emphasized that the majority of studies were conducted on the Pacific oysters (*Crassostrea gigas*) in particular months or seasons, either rendering the inter-comparison impossible or limiting its extent (PAZOS *et al.*, 1996; SOUDANT *et al.*, 1999; DAGORN *et al.*, 2016).

In oysters, 27 fatty acids were identified, in all four investigated seasons mostly in the following order of representation: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n-9c) and docosahexaenoic acid (DHA; C22:6n-3) (Table 2). The fatty acid composition and the prevalence of certain fatty acids could be compared to the results of some earlier studies performed on different oyster types (LINEHAN *et al.*, 1999; EZGETA-BALIĆ *et al.*, 2012; HURTADO *et al.*, 2012; LIRA *et al.*, 2013; POGODA *et al.*, 2013; DAGORN *et al.*, 2016). The highest SFA content was determined in summer and autumn, whereas the highest PUFA content was determined in spring. It could be assumed that in our samples fatty acid composition of the muscle tissue indicates the differences in selective incorporation of dietary PUFAs. The study of bivalve food sources populating the Adriatic Sea (EZGETA-BALIĆ *et al.*, 2012) confirmed that bivalves feed on mixed food, the quality of which strongly depends on seasonal changes in food composition. During the period of high phytoplankton presence (spring/summer), bivalve species mainly ingest phytoplankton, but also zooplankton and detritus. During the period of low phytoplankton presence (autumn/winter), bivalves rely on zooplankton and detritus.

In line with the findings of EZGETA-BALIĆ *et al.* (2012), we confirmed that oysters accumulate a significant amount of PUFAs during springtime. As oppose to the results of LIRA *et al.* (2013) (although obtained on *Crassostrea rhizophorae*, not *Ostrea edulis*), who determined the DHA (C22:6n-3) content to be twice higher in winter than in summer, in our study the highest DHA oyster content was observed in spring, with moderately high levels in winter and autumn and the lowest level in summer. The ratios in favour of DHA over EPA throughout the year confirm the presence of animal component in oyster diets (EZGETA-BALIĆ *et al.*, 2012).

Generally, variegated and smooth scallops were shown to harbour a significantly lower number of fatty acids in comparison to oysters, which could be explained by the fact that food selection is an active process and that different species have different affinities when it comes to food, i.e. various preferences for microalgae (GONZÁLEZ-ARAYA *et al.*, 2012).

Table 2. Fatty acid composition (% of total fatty acids) of European flat oyster (*Ostrea edulis*).

Fatty acids	Season			
	Spring	Summer	Autumn	Winter
C8:0	0.21±0.18	< LOD	< LOD	0.08±0.14
C10:0	0.26±0.23	< LOD	< LOD	< LOD
C12:0	0.06±0.11	< LOD	< LOD	0.60±1.04
C14:0	7.32±0.73	6.51±1.74	5.30±1.28	5.01±1.48
C15:0	1.45±0.00	1.54±0.37	1.43±0.31	1.24±0.45
C16:0	31.57±2.41	39.56±7.40	36.54±3.68	34.90±3.75
C17:0	3.15±0.22	4.42±0.87	4.20±0.67	3.33±1.03
C18:0	6.72±5.88	14.75±1.92	20.27±2.86	14.09±7.13
C20:0	< LOD	< LOD	< LOD	0.11±0.19
C23:0	0.28±0.48	< LOD	< LOD	0.56±0.49
C14:1	< LOD	0.43±0.74	< LOD	< LOD
C16:1n-7t	0.50±0.07	< LOD	0.47±0.81	0.48±0.48
C16:1n-7c	4.26±0.67	3.14±0.71	2.96±0.50	3.14±0.83
C17:1	0.21±0.18	< LOD	< LOD	< LOD
C18:1n-9c	8.03±0.20	10.18±0.75	9.08±4.91	14.82±9.69
C18:1n-7	2.96±0.24	3.13±0.39	3.12±0.26	2.13±1.85
C20:1n-9	0.60±0.02	1.63±2.12	< LOD	0.12±0.20
C24:1n-9	< LOD	< LOD	< LOD	0.25±0.43
C18:2n-6c	2.72±0.26	2.26±0.44	1.74±1.60	4.31±3.53
C18:3n-6	0.40±0.69	< LOD	< LOD	< LOD
C20:4n-6	0.89±0.19	0.41±0.70	0.37±0.64	0.54±0.66
C18:3n-3	4.35±1.40	1.71±1.72	1.78±1.58	1.11±1.42
C18:4n-3	5.22±0.91	1.69±1.62	1.47±1.41	1.62±2.39
C20:4n-3	0.49±0.43	< LOD	< LOD	0.22±0.38
C20:5n-3	7.31±1.58	3.40±2.96	5.03±1.23	5.15±3.85
C22:5n-3	0.60±0.52	< LOD	< LOD	< LOD
C22:6n-3	10.44±2.05	5.21±4.62	6.25±2.29	6.18±5.28
SFA	51.02±7.19	66.79±11.40	67.74±8.74	59.93±7.99
MUFA	16.55±1.12	18.52±2.88	15.63±3.92	20.95±7.68
n-6	4.01±0.88	2.67±1.14	2.11±2.12	4.85±3.41
n-3	28.42±5.67	12.02±10.78	14.52±6.01	14.28±13.01
PUFA	32.43±6.46	14.69±11.63	16.64±7.38	19.13±11.66

Results are expressed as the mean value (%), mean ± SD) of six results obtained for total fatty acids (3 months per season; each month, one sample was taken and analysed in duplicate);

LOD (limit of detection) = 0.05%.

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

RABY *et al.* (1997) found that different species ingest microalgae of different sizes, which is an indicator of their active food selection, the size of microalgae thereby being the major factor influencing the ingestion of food particles. Although the three shellfish types were collected from different locations in the Adriatic Sea (along the coast of the Istrian

Peninsula), the influence of water temperature, salinity and other environmental factors on fatty acid composition should be negligible, due to the small distances between the sampling locations (the same area of the Adriatic Sea). So, the differences in nutritive composition of three shellfish investigated in this study are probably mainly coming as a result of their different diet preferences.

Table 3 presents the fatty acid composition of variegated scallop determined in various seasons. Same as with oysters, the most dominant fatty acids in variegated scallop were C16:0, C18:0 and C18:1n-9c, whereas DHA was not detected. In this shellfish, no statistically significant inter-seasonal differences ($p > 0.05$) in individual fatty acid, SFA, MUFA and PUFA contents as were found. However, PUFA contents were highly variable within the same annual period, as can be seen from high intra-seasonal standard deviations. Higher PUFA content was observed in winter in comparison to summertime.

Table 3. Fatty acid composition (% of total fatty acids) of variegated scallop (*Chlamys varia*).

Fatty acids	Season			
	Spring	Summer	Autumn	Winter
C8:0	0.52±0.90	< LOD	< LOD	0.40±0.69
C10:0	0.35±0.61	< LOD	< LOD	< LOD
C12:0	< LOD	< LOD	< LOD	0.50±0.87
C14:0	8.13±4.77	10.02±1.33	7.18±3.36	3.72±0.96
C16:0	41.20±6.34	44.34±1.10	44.60±1.81	44.56±4.67
C17:0	2.52±4.36	< LOD	< LOD	< LOD
C18:0	31.68±12.47	24.20±4.35	38.73±10.02	35.70±9.55
C16:1n-7	3.67±3.29	3.31±2.95	2.95±2.60	0.70±1.21
C18:1n-9c	7.86±3.61	15.27±2.63	4.48±3.93	12.27±6.89
C18:1n-7	< LOD	1.96±1.75	0.78±1.35	< LOD
C18:2n-6c	1.69±2.93	0.90±1.56	< LOD	2.15±3.73
C18:3n-3	2.38±4.13	< LOD	1.28±2.21	< LOD
SFA	84.40±8.64	78.56±4.91	90.51±8.46	84.87±11.83
MUFA	11.53±6.32	20.54±5.07	8.21±7.11	12.98±8.10
n-6	1.69±2.63	0.90±1.56	< LOD	2.15±3.73
n-3	2.38±4.13	< LOD	1.28±2.21	< LOD
PUFA	4.07±7.05	0.90±1.56	1.28±2.21	2.15±3.73

Results are expressed as mean value (%; mean ± SD) of six results obtained for total fatty acids (3 months per season; each month, one sample was taken and analysed in duplicate);

LOD (limit of detection) = 0.05%.

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

Fatty acid composition of smooth scallop seen in various seasons is presented in Table 4. Same as with variegated scallop, the most dominant fatty acids in smooth scallop were C16:0, C18:0 and C18:1n-9c, while DHA presence was not detected. A statistically significant inter-seasonal difference was determined only for C18:0 found in summer (lower value) as compared to that in autumn (higher value) ($p = 0.001$), which can also be achieved thanks to low intra-seasonal variability witnessed in these two seasons. As for

the content of other individual fatty acids, SFA, MUFA and PUFA, no statistically significant inter-seasonal differences were found ($p > 0.05$). PUFAs were not quantified, while a higher SFA content was observed in winter in comparison to summertime.

Table 4. Fatty acid composition (% of total fatty acids) of smooth scallop (*Flexopecten glaber*).

Fatty acids	Season			
	Spring	Summer	Autumn	Winter
C14:0	8.63±3.10	5.91±1.81	1.10±1.90	5.80±3.08
C16:0	44.67±0.89	46.01±3.45	45.29±0.43	44.30±2.45
C17:0	1.88±2.66	< LOD	< LOD	< LOD
C18:0	29.12±11.47	33.47±1.33*	47.22±1.14*	39.49±5.57
C14:1	< LOD	1.17±2.02	< LOD	< LOD
C16:1n-7	4.78±1.60	1.19±2.06	< LOD	2.00±3.46
C18:1n-9c	8.99±0.49	12.25±2.44	6.39±1.31	8.41±3.13
C18:1n-7	1.93±2.73	< LOD	< LOD	< LOD
SFA	84.29±4.82	85.39±5.78	93.61±1.31	89.59±2.11
MUFA	15.71±4.82	14.61±5.78	6.39±1.31	10.41±2.11
PUFA	< LOD	< LOD	< LOD	< LOD

Results are expressed as mean value (% , mean ± SD) of six results obtained for total fatty acids (3 months per season; each month, one sample was taken and analysed in duplicate);

LOD (limit of detection) = 0.05%.

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids.

* Statistically significant difference ($p < 0.05$).

The representation of all fatty acid groups (SFA, MUFA and PUFA) significantly differed ($p < 0.05$) between the analyzed shell species. Oysters contained the smallest proportion of SFAs ($p < 0.001$) and the highest ($p < 0.001$) share of PUFAs as compared to other shellfish types. The share of MUFA was significantly higher in oysters than in smooth scallop ($p = 0.031$).

It is known that shellfish fatty acid composition usually reflects a fatty acid composition of their diet (phytoplankton or zooplankton), although shellfish have shown a certain ability to elongate (e.g. C16:1 to C18:1, C18:1 to C20:1, C20:5 to C22:5, C20:4 to C22:4) or desaturate (e.g. C20:3 to C20:4) fatty acids (ALBENTOSA *et al.*, 1996; DELAPORTE *et al.*, 2005). Given that basic shellfish nutrient composition and fatty acid profile are influenced by many parameters, both a correct interpretation of the obtained results and a plausible comparison with the results of other studies require the knowledge on ecological characteristics of areas in which shellfish are cultivated or natural-born.

The results pertaining to the nutritional fat quality indices (n-6/n-3, PUFA/SFA, AI, TI and HH) determined for each shellfish type in each season and in total, the latter being expressed as the mean value descriptive of the entire one-year study period, are presented in Table 5. Ratios n-6/n-3 and PUFA/SFA are the parameters most commonly used for the assessment of nutritional fat quality. Literature has shown that in case of lower n-6/n-3 ratios, the body is more able to make use of n-3 fats (WOOD *et al.*, 2008). Ratio n-6/n-3 has been suggested to be a good tool for comparing relative nutritional values of different species, but this index is of a limited value should the share of individual fatty acids be

unknown. Due to the fact that fatty acids containing C20 and C22 are more valuable from the nutritional standpoint as compared to fatty acids containing C18, and taking into account their predominance over other n-3 fatty acids, EPA and DHA are largely responsible for the changes in n-6/n-3 ratio, the latter otherwise being considered as a reliable indicator that enables comparison of relative nutritive lipid values (PLEADIN *et al.*, 2017). As recently reviewed by WEYLAND *et al.* (2015), beneficial effects of these fatty acids have been reported for a number of disorders, including cardiovascular, neuropsychiatric and inflammatory diseases, as well as some cancers (mainly colorectal, mammary and prostatic cancer).

Table 5. Nutritional fat quality indices established for the analysed shellfish types during a one-year period.

Shellfish type	Season	Fat quality indices (target values)				
		n-6/n-3	PUFA/SFA	AI	TI	HH
European flat oyster (n=36)	Spring	0.14±0.01	0.65±0.21	1.27±0.26	0.46±0.16	0.89±0.17
	Summer	0.11±0.10	0.24±0.20	2.32±1.53	2.92±3.72	0.54±0.28
	Autumn	0.13±0.14	0.26±0.13	1.95±0.91	1.30±0.79	0.60±0.28
	Winter	0.77±1.02	0.34±0.24	1.43±0.32	1.17±0.62	0.81±0.17
	Average	0.29±0.53	0.37±0.24	1.74±0.89 ^{B,C}	1.46±1.90 ^{B,C}	0.71±0.25 ^{B,C}
Variegated scallop (n=36)	Spring	0.24±0.41	0.05±0.09	6.12±3.82	14.29±15.52	0.25±0.16
	Summer	n.d.	0.01±0.02	4.08±0.96	7.68±2.33	0.30±0.03
	Autumn	n.d.	0.02±0.03	3.80±3.36	6.36±7.32	0.11±0.10
	Winter	n.d.	0.03±0.05	5.68±3.40	16.33±10.03	0.32±0.26
	Average	0.24±0.41	0.03±0.05	4.92±2.84 ^A	11.17±9.61 ^A	0.23±0.15 ^A
Smooth scallop (n=36)	Spring	n.d.	n.d.	5.15±0.74	11.17±4.38 ^d	0.17±0.00
	Summer	n.d.	n.d.	5.48±2.86	13.19±5.81 ^d	0.24±0.07
	Autumn	n.d.	n.d.	8.08±2.45	30.12±5.95 ^{b,c}	0.14±0.03
	Winter	n.d.	n.d.	6.65±1.74	17.82±4.54	0.17±0.08
	Average	n.d.	n.d.	6.45±2.22 ^A	18.70±8.93 ^A	0.18±0.06 ^A

Results are expressed as mean value (%), mean ± SD) of six results obtained for total fatty acids (3 months per season; each month, one sample was taken and analysed in duplicate);

SFA saturated fatty acids; PUFA polyunsaturated fatty acids;

HH hypo-/hyper-cholesterolaemic fatty acids ratio = $(C18:1n-9+C18:2n-6+C20:4n-6+C18:3n-3+C20:5n-3+C22:5n-3+C22:6n-3)/(C14:0+C16:0)$

AI atherogenic index = $[(C12:0+(4 \times C14:0)+C16:0)]/(\sum MUFA+PUFA\ n-6+PUFA\ n-3)$

TI thrombogenic index = $(C14:0+C16:0+C18:0)/[(0.5 \times \sum MUFA+0.5 \times PUFA\ n-6+3 \times PUFA\ n-3)+(PUFA\ n-3/PUFA\ n-6)]$

Statistically significant difference ($p < 0.05$) within the same column for every shellfish type separately:

^avs. winter; ^bvs. spring; ^cvs. summer; ^dvs. autumn; ^avs. European flat oyster, ^bvs. variegated scallop, ^cvs. smooth scallop

n.d. (not detected) - fatty acids needed for calculation were not detected (< LOD).

According to health recommendations, n-6/n-3 ratio should be lower than 4, thereby reducing the incidence of chronic food-related illnesses (CORDAIN *et al.*, 2005; SIMOPOULOS, 2002). In an annual form, this index was calculable only for oysters, while for variegated scallop it could be provided only for the samples recovered during springtime. In both cases, the determined n-6/n-3 ratios fell within the recommended

boundaries, although high intra-seasonal variations were evident. In an annual form, n-6/n-3 ratio was not calculable for smooth scallop because of the absence of PUFAs (values below the LOD).

PUFA/SFA ratio is recommended to be higher than 0.4, so as to reduce the risk of cardiovascular, autoimmune and other chronic diseases (SIMOPOULOS, 2002). Generally, for two shellfish types in which PUFAs were present in values above the LOD (oyster and variegated scallop), the determined PUFA/SFA ratios were significantly lower than the recommended minimum, except in oysters during springtime (0.65 ± 0.21). Same as with n-6/n-3 ratio, high intra-seasonal variations were noticeable. Some authors are of the opinion that an index such as PUFA/SFA may prove inadequate for the evaluation of nutritional value of fats, because some SFAs do not increase plasma cholesterol. Therefore, MENSINK and KATAN (1992), and DALEY *et al.* (2010), suggested that C12:0 and C14:0 have a more pronounced total cholesterol raising effect than C16:0, whereas C18:0 is neutral when it comes to the concentration of total serum cholesterol, with no apparent impact on either LDL or HDL. Myristic acid (C14:0) has a 4-6 times higher potential to increase cholesterol concentrations as compared to C16:0 (ULBRITTH and SOUTHGATE, 1991; BRESSAN *et al.*, 2011). On top of that, PUFA/SFA index ignores the effects of MUFAs, which may have more profound health benefits in terms of coronary disease prevention (ORELLANA *et al.*, 2009).

Therefore, two additional indices, which take into account different effects that a single fatty acid might have on the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation, i.e. the atherogenic (AI) and the thrombogenic index (TI), were calculated, too. The atherogenic index takes into account the fact that some saturates are considered to be pro-atherogenic (since they facilitate the adhesion of lipids onto the cells the immune and the circulatory system are composed of), while non-saturates are considered to be anti-atherogenic (since that inhibit the formation of plaques and diminish the levels of esterified fatty acids, cholesterol, and phospholipids, therefore preventing micro- and macro-coronary disease) (ULBRITTH and SOUTHGATE, 1991). The thrombogenic index (TI) shows the tendency towards blood clotting. It is assumed that AIs and TIs below 1 are beneficial to human health (PLEADIN *et al.*, 2017). According to the data reported in Table 5, only oysters approach the recommended values, while other two shellfish types exceed the maximum limits by far.

In order to gain insight into the effect of fatty acids on blood cholesterol, an additional indicator of nutritional quality, i.e. the ratio between hypocholesterolaemic and hypercholesterolaemic fatty acids (HH), was calculated. It is preferable for that index to be higher (SANTOS-SILVA *et al.*, 2002). The obtained HH values ranged from 0.18 ± 0.06 in smooth scallop and from 0.23 ± 0.15 in variegated scallop, whereas the highest HH index was determined in oysters during springtime (0.89 ± 0.17).

In case of oysters and variegated scallop, no statistically significant seasonal differences ($p > 0.05$) in any of the fat quality indices were determined. In smooth scallop, a significant seasonal difference was determined only for TI ($p < 0.05$; $p = 0.016$), with a significantly higher TI in autumn in comparison to spring and summer. With the exception of n-6/n-3 ratio, which was actually determined only in oysters, a significant difference in TI, HH and PUFA/SFA indices was determined across the studied shellfish types, while a statistically significant difference in the AI value among the analysed shellfish over different seasons failed to be seen. In spring, oysters had significantly higher PUFA/SFA and HH ratios than other shellfish types. In winter and autumn, oysters also showed a significantly higher HH ratio as compared to variegated and smooth scallop. The TI established for smooth scallop in autumn was significantly higher as compared to other

shellfish; in winter, the latter index determined for smooth scallop was also higher than that found for oysters. As in the study by LIRA *et al.* (2013), fatty acids-related nutritional quality indices were more favourable in winter in comparison to summer period.

4. CONCLUSION

The proportion of fat found in all shellfish types under study was low, with the highest average representation in oysters. The proportion of proteins and total minerals in meat of all three shellfish was found to be similar. The representation of saturated fatty acids was generally found to be high, with an unfavourable PUFA/SFA ratio that might increase the risk of chronic diseases. Out of the three shellfish species under study, the lowest SFA content, the most favourable PUFA/SFA ratio and the most favourable AI, TI and HH indices were established in oysters. Although oysters harvested in springtime contained the highest proportion of fats and proteins, and therefore presented with the most favourable PUFA/SFA, AI, TI and HH indices in that particular season, intra-seasonal variations were huge, so that statistically significant inter-seasonal differences in these parameters in oysters harvested in different times of the year failed to be found. During springtime, smooth scallop also showed the highest representation of fats and proteins, and hence also the most favourable AI and TI indices, but inter-seasonal variations were proven to be either statistically insignificant or significant only in comparison to one out of the three remaining seasons. As for variegated scallop, none of the seasons could be considered as the most favourable when it comes either to fat and protein content or to fat nutritional quality indices. In summary, no statistically significant inter-seasonal differences in basic chemical parameters and fat quality indices descriptive of an edible part of the three shellfish were determined.

ACKNOWLEDGEMENTS

This study was carried out as a part of the FAIMMAC project (*Fishery and aquaculture integrated management model along the Adriatic coasts*) funded from the European Maritime and Fishery Fund EASME/EMFF/2015/1.2.1.7/02/SI2.735915.

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ANTIOXIDANT CAPACITY AND HEAT DAMAGE OF POWDER PRODUCTS FROM SOUTH AMERICAN PLANTS WITH FUNCTIONAL PROPERTIES

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ABSTRACT

Aim of the study was to evaluate color, total polyphenol content (TPC), antioxidant capacity (ABTS, FRAP, DPPH), reducing sugars and heat damage (furosine, hydroxymethylfurfural, glucosylisomaltol) of 21 commercial powder products obtained from South-American fruits (mesquite, lucuma, camu camu), seeds (amaranth, purple maize), roots and tubers (yacon, maca, mashua, tocosh), bark (cat's claw) and leaves (graviola). TPC and antioxidant capacity were maximum in camu camu and cat's claw powders, and minimum in tocosh, amaranth, lucuma and maca; graviola, mashua, purple maize and mesquite also showed good antioxidant properties. Yacon, mashua and lucuma powders had high reducing sugars content (40.9, 34.4 and 21.2 g/100 g DM, respectively) and heat damage (HMF 146.6 mg/kg, furosine 2399.8 and 2228.4 mg/100 g protein, respectively). Overall, camu camu powders and cat's claw were the most interesting products, having high levels of total polyphenols and antioxidant capacity together with very low heat damage.

Keywords: camu camu, cat's claw, maca, mashua, mesquite, yacon

1. INTRODUCTION

A major threat to human wellbeing is the oxidative stress, an “imbalance between oxidants and antioxidants in favour of the oxidants” (SIES, 1997), which can lead to cellular damage and facilitate the insurgence of cardiovascular and neurodegenerative diseases, diabetes mellitus, cancer and inflammatory illness (UTTARA *et al.*, 2009). An effective approach to prevent oxidative stress is to include in the daily diet products rich in antioxidants, which can quench the oxygen free radicals, preventing the oxidation of the cell membrane.

Plants and plant-derived ingredients have been used as medical remedies from prehistoric ages, and still are a major source of health-promoting elements. In recent years, the interest in the identification and utilization of plants rich in antioxidant compounds to limit the oxidative stress (ALMEIDA *et al.*, 2011; KRISHNAIAH *et al.*, 2011) has been steadily growing, because they may behave as preventive medicine. Several authors have reviewed the beneficial uses of underexploited and little-known plant species used in food production but also in traditional medicine (e.g. BIEL *et al.*, 2017; CAMPOS *et al.*, 2013; CHIRINOS *et al.*, 2013; CONTRERAS-CALDERÓN *et al.*, 2011; KRISHNAIAH *et al.*, 2011). Peru, thanks to its widely diversified climatic zones, is home to a broad array of endemic plants, which show huge differences in the content and type of nutrients and that are potential sources of valuable bioactive compounds (CAMPOS *et al.*, 2018).

The antioxidant capacities of plant-derived products vary depending on their content in polyphenols, vitamin C, tocopherols and carotenoids, (SAURA-CALIXTO and GOÑI, 2006), as well as on the different processing conditions. While in some cases the plant products are consumed fresh, most often they undergo some type of transformation and/or drying to improve shelf life, to lower transport costs and to reach far off consumers (CINAR, 2018). Accordingly, powders from South-American plants with known health-promoting features (Supplementary Table 1) are manufactured by several industries to find new market niches and to foster the consumption of health-promoting natural products. These innovative powder products, obtained from fruits (mesquite, lucuma and camu camu), seeds (amaranth and purple maize), roots and tubers (yacon, maca, mashua and tocosha), bark (cat's claw) and leaves (graviola), are currently used for the preparation or enrichment of infusions, juices, shakes/smoothies, yogurts, desserts, as well as ingredients in cosmetic and pharmaceutical recipes.

The aim of our study was to evaluate some characteristics of these powder products for their possible utilization as enhancing ingredients in wheat-based oven products. To achieve this goal, 21 commercial powder samples of the above-mentioned species were assessed for color, total polyphenol content, antioxidant capacity, reducing sugars and heat damage.

2. MATERIALS AND METHODS

2.1. Samples

The powders analyzed were acquired in 2016 at an industrial fair dedicated to Peruvian export products (Expoalimentaria, Lima, Peru; www.expoalimentariaperu.com) except amaranth, obtained from the Peruvian market, and two maca samples, bought from the Italian market. Several samples (3-5) of each powder product were collected. A detailed list of the products tested is presented in Table 1.

2.2. Physical and chemical analyses

2.2.1 Color

The color coordinates L^* (luminosity), a^* (red-green) and b^* (yellow-blue) of the samples were scored with a tristimulus colorimeter (Chroma meter CR-300, Minolta Italia S.p.A., Italy) using the standard-white reflector plate and illuminant C. Four measurements for each sample were performed.

Table 1. Samples analyzed: species, brands, codes, average dry matter and protein contents (g/100 g).

Product	Species	Brand code	Sample code	Dry matter	Protein
<i>Bark</i>					
Cat's claw	<i>Uncaria tomentosa</i> L.	A	Cat's claw 1	91.8	0.3
Cat's claw bio	<i>Uncaria tomentosa</i> L.	B	Cat's claw 2	92.8	2.7
Cat's claw tea	<i>Uncaria tomentosa</i> L.	B	Cat's claw tea	92.5	3.0
<i>Seeds</i>					
Amaranth flour	<i>Amaranthus caudatus</i> L.	C	Amaranth FR	90.4	11.5
Amaranth flakes	<i>Amaranthus caudatus</i> L.	D	Amaranth FS	90.4	8.8
Purple maize	<i>Zea mays</i> L.	B	Purple maize	90.3	7.0
<i>Roots</i>					
Yacon	<i>Smallanthus sonchifolius</i> (Poepp. & Endl.) H. Robinson	B	Yacon	87.8	1.8
Maca gluten free	<i>Lepidium meyenii</i> Chacon	E	Maca 1	85.9	10.6
Maca bio	<i>Lepidium meyenii</i> Chacon	B	Maca 2	87.3	9.0
Maca HP	<i>Lepidium meyenii</i> Chacon	B	Maca 3	90.3	8.5
Maca extract	<i>Lepidium meyenii</i> Chacon	A	Maca 4	85.4	9.3
Maca	<i>Lepidium meyenii</i> Chacon	A	Maca 5	86.6	7.7
Maca root	<i>Lepidium meyenii</i> Chacon	F	Maca 6	92.9	12.0
Maca energia	<i>Lepidium meyenii</i> Chacon	G	Maca 7	90.4	7.0
<i>Tubers</i>					
Tocosh	<i>Solanum</i> spp.	H	Tocosh	83.4	2.2
Mashua	<i>Tropaeolum tuberosum</i> Ruiz & Pav.	E	Mashua	84.7	9.0
<i>Leaves</i>					
Graviola bio	<i>Annona muricata</i> L.	B	Graviola	91.9	10.8
Graviola tea	<i>Annona muricata</i> L.	B	Graviola tea	91.0	11.0
<i>Fruits</i>					
Mesquite	<i>Prosopis</i> spp.	B	Mesquite	89.3	8.7
Lucuma	<i>Pouteria lucuma</i> Ruiz & Pav.	B	Lucuma	88.9	3.4
Camu camu	<i>Myrciaria dubia</i> (Kunth) McVaugh	B	Camu Camu	87.4	5.4

2.2.2 Dry matter and protein content

Dry matter was determined following the gravimetric method, drying 2 g of product at 130 °C for 90 min; protein content was assessed by Kjeldahl ($N \times 6.25$). These and all the following analyses were performed in triple.

2.2.3 Samples preparation for total polyphenols content and antioxidant capacity analysis

All the reagents, of analytical grade, were purchased from Sigma-Aldrich Co. (Milan, Italy). Two different solvents were tested for the extraction of total polyphenols and the evaluation of the antioxidant capacity, i.e. ethanol:H₂O (EtOH:H₂O; 80:20) and methanol:H₂O:acetic acid (MeOH:H₂O:acet; 50:42:8).

Exactly 0.15 g of powdered product were weighed in 2 mL tubes and subjected to three extractions, adding 1 mL of an EtOH:H₂O solution each time. In the first extraction, the samples were stirred with a Vortex (Reax 2000, Meindolph Heidolph, Schwabach, Germany) for 1 min and sonicated (F5200b, Decon, UK) twice for 20 min; in the second extraction the samples were stirred with a Vortex for 1 min, an orbital shaker (Multi-Rotator GRANT-BIO, Cambridge, UK) for 20 min and sonicated for another 20 min; in the third extraction the samples were stirred with a Vortex for 1 min and sonicated for 5 min. After each extraction, the samples were centrifuged with a 4224 centrifuge (ALC Apparecchi per Laboratori Chimici Srl, Milan, Italy) for 5 min at 8048 g and all the supernatants were mixed in a single tube. The extractions were performed at 10 °C and away from light as far as possible.

Following the same procedures, 0.3 g of powdered product underwent three extraction cycles, adding respectively 1.5, 1.5 and 1.0 mL of a MeOH:H₂O:acet solution.

2.2.3.1 Total polyphenol content

Total polyphenol content (TPC) in samples extracted with EtOH:H₂O and MeOH:H₂O:acet was assessed with the Folin-Ciocalteu method as described by BRANDOLINI *et al.* (2013) using a Du-62 Beckman spectrophotometer (Beckman Coulter, Nyon, VD, Switzerland). The TPC, in mg gallic acid equivalent (GAE)/kg DM, was computed from a reference curve obtained from six gallic acid concentrations (range: 0-150 mg/L).

2.2.3.2 Assessment of antioxidant capacity using the ABTS method

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging capacity was analysed as described by YILMAZ *et al.* (2015). A stable stock solution of the ABTS radical cation was prepared by reacting 10 mL of an aqueous solution of 2-2'-azinobis-3-etilenbenzotiazoline 7 mM and 176 μ L potassium persulfate 140 mM in the dark at room temperature for 12-16 h. The EtOH:H₂O or MeOH:H₂O:acet extracts (150 mL) were reacted with 5 mL of a diluted ABTS radical solution in ethanol (absorbance: 0.70 ± 0.02 AU at 734 nm); the absorbance was measured at 734 nm, after 6 min at 30 °C, with a V650 spectrophotometer (Jasco, Japan), using ethanol as blank. The antioxidant capacity was evaluated as percentage of absorbance decrease (inhibition percentage). A reference curve was built with 11 concentrations (from 0.05 to 0.72 mM) of Trolox. The results are expressed as mmol Trolox equivalents (TE)/kg DM.

2.2.3.3 Assessment of the reduction power using the FRAP method

The ferric reducing antioxidant power (FRAP) was determined as described by YILMAZ *et al.* (2015), following the method proposed by Benzie and Strain (1996). Briefly, 200 mL of EtOH:H₂O or MeOH:H₂O:acet extracts were mixed with 4.5 mL FRAP reagent. Absorption was measured with a V650 spectrophotometer (Jasco, Japan) at a wavelength of 593 nm after 60 min incubation at 37 °C; acetate buffer 0.3 M pH 3.6 was used as blank. The FRAP reagent, prepared daily, consisted of 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃ (10:1:1 v/v/v). FRAP values were obtained by comparing the results to a calibration curve built with 18 concentrations (0.06 - 0.90 mM) of Trolox. The antioxidant capacity was expressed as mmol TE/kg DM.

2.2.3.4 Assessment of antioxidant capacity using the DPPH method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical cation scavenging capacity of EtOH:H₂O and MeOH:H₂O:acet extracts was evaluated according to BRANDOLINI *et al.* (2013) using a DU-62 spectrophotometer (Beckman, USA). For each extract five different dilutions were analysed. A dose-response line was computed for each sample and the powder quantity needed to scavenge 50% of the radical (I₅₀) was determined. A reference regression line was computed for the antioxidant Trolox, with concentrations between 3 and 50 µM. The antioxidant capacity was expressed as ratio between I₅₀ of Trolox and I₅₀ of the sample, i.e. mmol TE/kg DM.

2.2.4 Sugars content

Fructose, glucose, maltose and sucrose were assessed by HPLC, following HIDALGO and BRANDOLINI (2011). For peak quantification, sugars calibration curves were constructed using 15 different concentrations (between 0 and 155 mg/L) of fructose, 19 different concentrations (between 0 and 428 mg/L) of glucose, 19 different concentrations (between 0 and 385 mg/L) of maltose, and 15 different concentrations (between 0 and 153 mg/L) of sucrose standards (Sigma, St. Louis, MO, USA). The calibration curves, after log transformation, were linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration ranges considered. The results are reported as g/100 g DM.

2.2.5 Heat damage indices

Furosine was determined by HPLC as described by HIDALGO and BRANDOLINI (2011). A calibration curve was built using nine different concentrations (between 0.33 and 5.13 mmol/L of furosine dihydrochloride (NeoMPS, PolyPeptide Laboratories, Strasbourg, France) in 3 N HCl. The calibration curve was linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration ranges considered. The results are expressed as milligrams of furosine/100 g of protein.

Hydroxymethylfurfural (HMF) and glucosylisomaltol (GLI) were determined following the HPLC method of RUFÍAN-HENARES *et al.* (2008) as described by Hidalgo and Brandolini (2011). For peak quantification, a calibration curve was constructed using 13 different concentrations (between 0 and 6.25 mg/L) of HMF (Safc, St. Louis, MO, USA). The calibration curve was linear ($r^2 = 1.00$; $p \leq 0.001$) in the concentration range considered. GLI quantification was computed considering the response factor of HMF at 280 nm. The results are expressed as mg/kg DM.

2.3. Statistical analysis

The data were processed by one-way analysis of variance (ANOVA) considering the samples as factors. The distribution of the data was checked and, for normalization purposes, L^* and b^* values were squared, while the other parameters were $\log_{10}$ -transformed; however, for easier comprehension, in Tables and Figures the original data are reported.

When significant differences were found ($p \leq 0.05$), Fisher's lowest significant difference (LSD) was computed at a 95% significance level. To compare the results of the two solvents used for the preparation of the extracts, the t-test was applied ($p \leq 0.05$). ANOVA, LSD test and t-test were conducted using the statistical program STATGRAPHICS® Centurion. Mean, standard error and coefficient of variation were computed using the program Excel (Microsoft® Office Excel 2007). Principal Components Analysis (PCA), performed considering the mean values of the 21 samples and all the parameters, was carried out with the software The Unscrambler X 10.2 (CAMO software AS, Norway).

3. RESULTS AND DISCUSSION

3.1. Powders color

Supplementary Table 2 shows the average values and the results of the LSD test for the color coordinates L^* , a^* , b^* of the twenty-one samples. The results obtained grouping the samples by species are reported in Fig. 1.

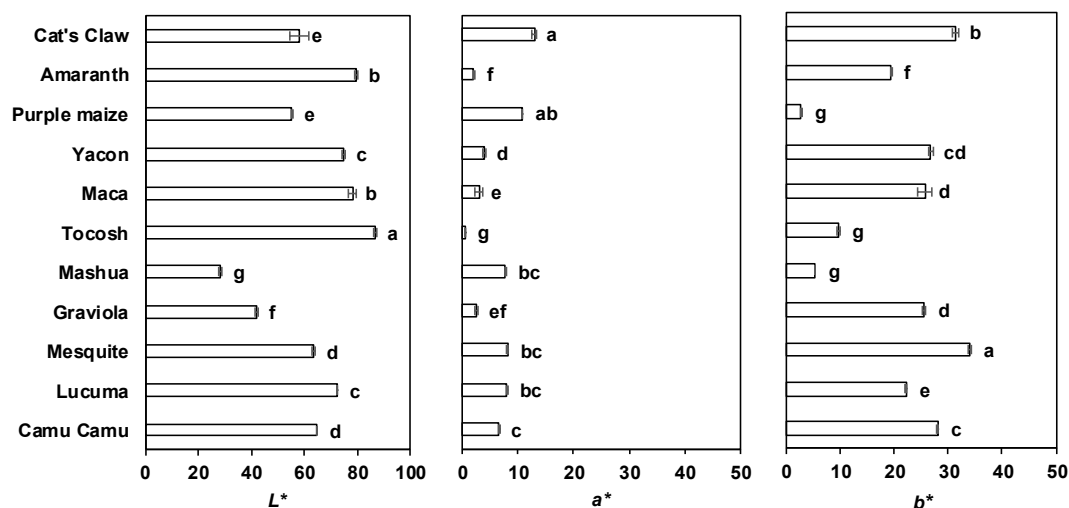


Figure 1. Colour coordinates (L^* , a^* , b^*) of powdered products from 11 species. Different letters indicate significant differences (LSD, $p \leq 0.05$) among species.

The broad heterogeneity of the samples led to an ANOVA (not presented) showing significant differences for all the parameters. In fact, a preliminary visual control gave the following color characterization: mashua and purple corn were purple; maca, yellow-

orange; cat's claw, mesquite and yacon, orange; graviola, green-brown; camu camu, yellow-brown; tocosh, white; amaranth, cream-white; lucuma, ocher.

The tocosh powder was the brightest (L^* : 86.6), followed by most maca samples (76.2-83.9) and amaranth (78.5-80.3). One maca (maca 4) had an L^* of 72.0, lower than the other maca samples. Camu camu had a L^* like the lyophilized samples (60.45 ± 2.78) and higher than the spouted bed dried samples (36.6-40.8) described by FUJITA *et al.* (2013). Overall, mashua presented the lowest brightness (28.3), followed by graviola (43.3-41.0). Cat's claw and purple corn scored the highest a^* red component values (12.1-13.5 and 10.8, respectively), while tocosh presented the lowest (0.5). The variation among the different maca samples was quite limited, ranging from 1.2 (Maca 7) to 5.1 (Maca 4). Mesquite presented the highest b^* yellow component (34.1), followed by cat's claw (on average 31.6), five maca samples (22.2-26.2) and graviola (on average, 25.6); maca 1 and maca 4 had values different from the other maca (28.3-30.2). Purple corn presented the lowest b^* value, hence the major blue component (2.8), followed by mashua (5.3) and tocosh (9.7). The differences observed between maca samples may be due either to the different treatments utilized for their preparation (ONWUDE *et al.*, 2017) or to cultivars with different chromatic characteristics. No information or comparisons for the color components are available in literature.

3.2. Total polyphenol content

Supplementary Table 3 reports the results of TPC, performed on the EtOH:H₂O and MeOH:H₂O:acet extracts, as well as the results of the LSD test comparing the products. The great heterogeneity of the samples led to ANOVAs (not shown) always with significant differences. The average values, obtained by grouping the samples according to the species, are depicted in Fig. 2.

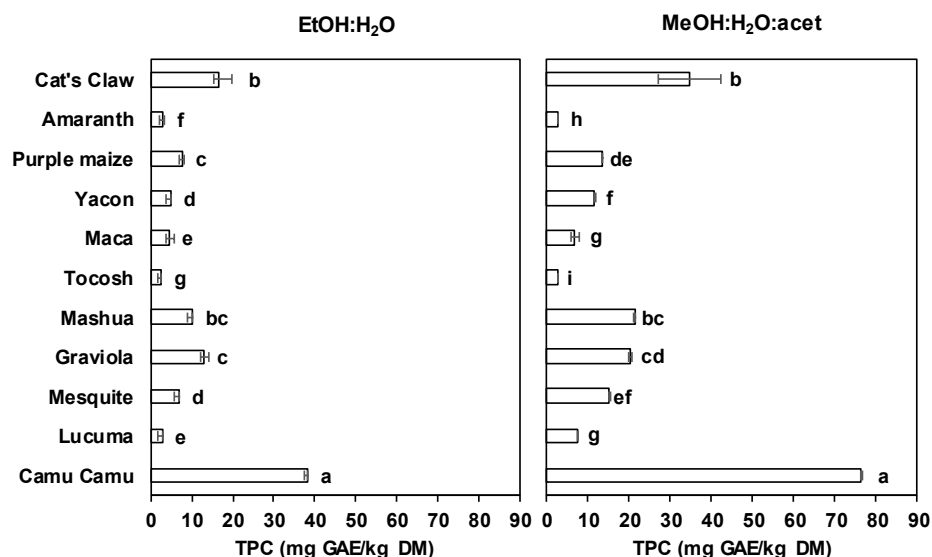


Figure 2. Total polyphenol content (TPC) of the ethanol 80% (EtOH:H₂O) and methanol:H₂O:acetic acid (MeOH:H₂O:acet) extracts of powdered products from 11 species. Different letters indicate significant differences ($p \leq 0.05$) among species.

EtOH:H₂O showed a lower TPC extraction capacity than MeOH:H₂O:acet, but the information provided was similar, as demonstrated by their very high linear coefficient of correlation ($r=0.98$). TPC was maximum for camu camu (38.3 and 76.4 g GAE/kg DM, respectively), followed by cat's claw 2 (24.2 and 53.6 g GAE/kg DM, respectively); the lowest TPCs were recorded in tocosh (2.5 and 2.8 g GAE/kg DM), amaranth (on average, 3.1 and 2.8 g GAE/kg DM), lucuma (2.8 and 7.5 g GAE/kg DM) and six maca samples (on average, 3.6 and 6.7 g GAE/kg DM). The values generally fell within the range of variation reported in the literature for camu camu (FUJITA *et al.*, 2013), cat's claw (BERLOWSKI *et al.*, 2013; GALVEZ RANILLA *et al.*, 2010), amaranth (REPO-CARRASCO-VALENCIA *et al.*, 2010), lucuma (FUENTEALBA *et al.*, 2016), mesquite (CARDOZO *et al.*, 2010), maca (GALVEZ RANILLA *et al.*, 2010; CAMPOS *et al.*, 2013), mashua (CHIRINOS *et al.*, 2007; CHIRINOS *et al.*, 2013), yacon (CAMPOS *et al.*, 2013), but were lower than those described for graviola frozen pulp (ZIELINSKI *et al.*, 2014). For Peruvian purple maize the information available on TPC is reported in chlorogenic acid equivalent and is not directly comparable to our results, while for tocosh no similar information was found in literature.

The Folin-Ciocalteu method sometimes overstates total phenolics content because other compounds, including reducing sugars (e.g. glucose and fructose), may interfere with the results; however, in this research the powders with the highest sugars content (yacon, mashua, lucuma and maca), generally have low TPC; conversely, the two highest TPC values were from camu camu and cat's claw, which showed very low sugars content.

3.3. Antioxidant capacity

The antioxidant capacity of the samples, assessed by the ABTS, FRAP and DPPH tests carried out on the EtOH:H₂O and MeOH:H₂O:acet extracts are shown in Supplementary Table 3, along with the results of the LSD test. The great heterogeneity among samples led to ANOVAs (not presented) always indicating significant differences, as previously remarked for color and total polyphenols content. The average antioxidant capacities obtained by grouping the samples according to the type of product are presented in Fig. 3. The ABTS, FRAP and DPPH tests give similar and highly correlated results (r between 0.98 and 1.00 for both EtOH:H₂O and MeOH:H₂O:acet extracts). A higher antioxidant capacity was observed in the MeOH extracts, the exceptions being amaranth (for all three methods), maca and tocosh (ABTS), graviola tea and maca 5 (DPPH). Camu camu, which had the highest TPC concentration (Fig. 2) but also an outstanding vitamin C content (FUJITA *et al.*, 2013), showed the highest antioxidant capacity (Fig. 3), followed by cat's claw, graviola, mashua, purple maize and mesquite. On the other hand, tocosh, amaranth, yacon, maca and lucuma had low antioxidant activities, like that of wheat (YILMAZ *et al.*, 2015). Comparable results were reported for camu camu (DPPH: 153-185 mmol TE/g FW; CHIRINOS *et al.*, 2010), cat's claw (ABTS: 513 mmol TE/kg DM; BERLOWSKI *et al.*, 2013), graviola (ABTS: about 200 mmol TE/kg DM; BERLOWSKI *et al.*, 2013), mashua (ABTS: 24.3-247.7 mmol TE/g DM; DPPH: 23.2-157.1 mmol TE/g DM; CHIRINOS *et al.*, 2013), MESQUITE (ABTS: 57.0-61.6 μ mol TE/g DM; CARDOZO *et al.*, 2010), yacon (ABTS 23-136 mmol TE/g DM; CAMPOS *et al.*, 2012), purple maize (DPPH: 23.1 mmol TE/g DM; CEVALLOS-CASALS and CISNEROS-CEVALLOS, 2003), lucuma (ABTS: 5.6-304.6 mmol TE/g DM; DPPH: 0.7-132.9 mmol TE/g DM; FUENTEALBA *et al.*, 2016) and amaranth (ABTS: 3.7 mmol TE/g DM; DPPH: 1.2 mmol TE/g DM; CHIRINOS *et al.*, 2013). On the other hand, those of maca were slightly lower than the levels (ABTS: 67 mmol TE/g DM) observed by FUENTEALBA *et al.* (2016).

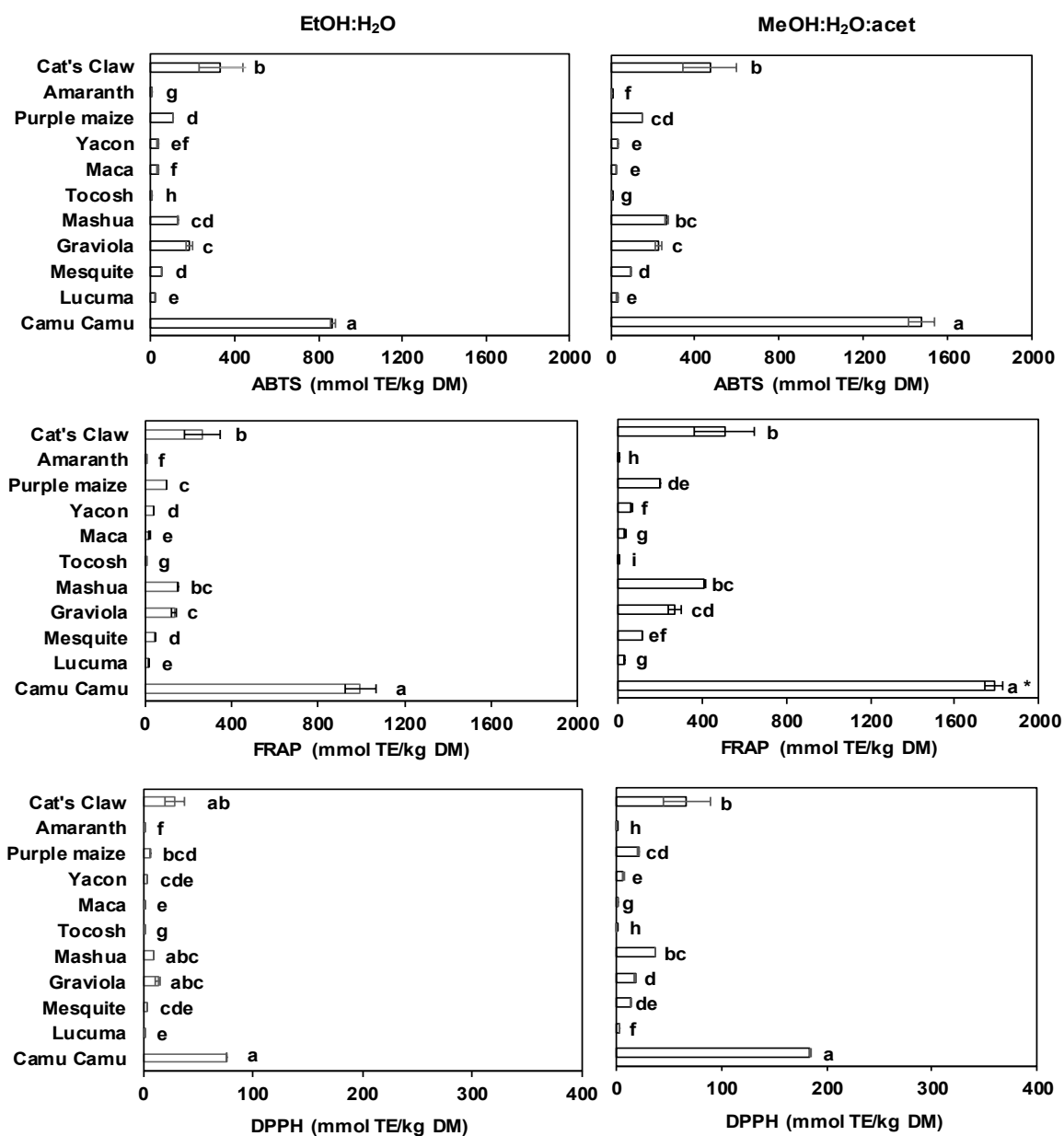


Figure 3. Antioxidant capacity (ABTS, FRAP and DPPH tests,)of the ethanol 80% (EtOH:H₂O) and methanol:H₂O:acetic acid (MeOH:H₂O:acet) extracts of powdered products from 11 species. Different letters indicate significant differences ($p \leq 0.05$) among species.

3.4. Sugars content

The ANOVA (not presented) showed the existence of significant differences for sugars content among samples. The average values and the results of the LSD test for the different sugars are reported in Supplementary Table 2. The reducing sugars results obtained grouping the samples by species are presented in Fig. 4.

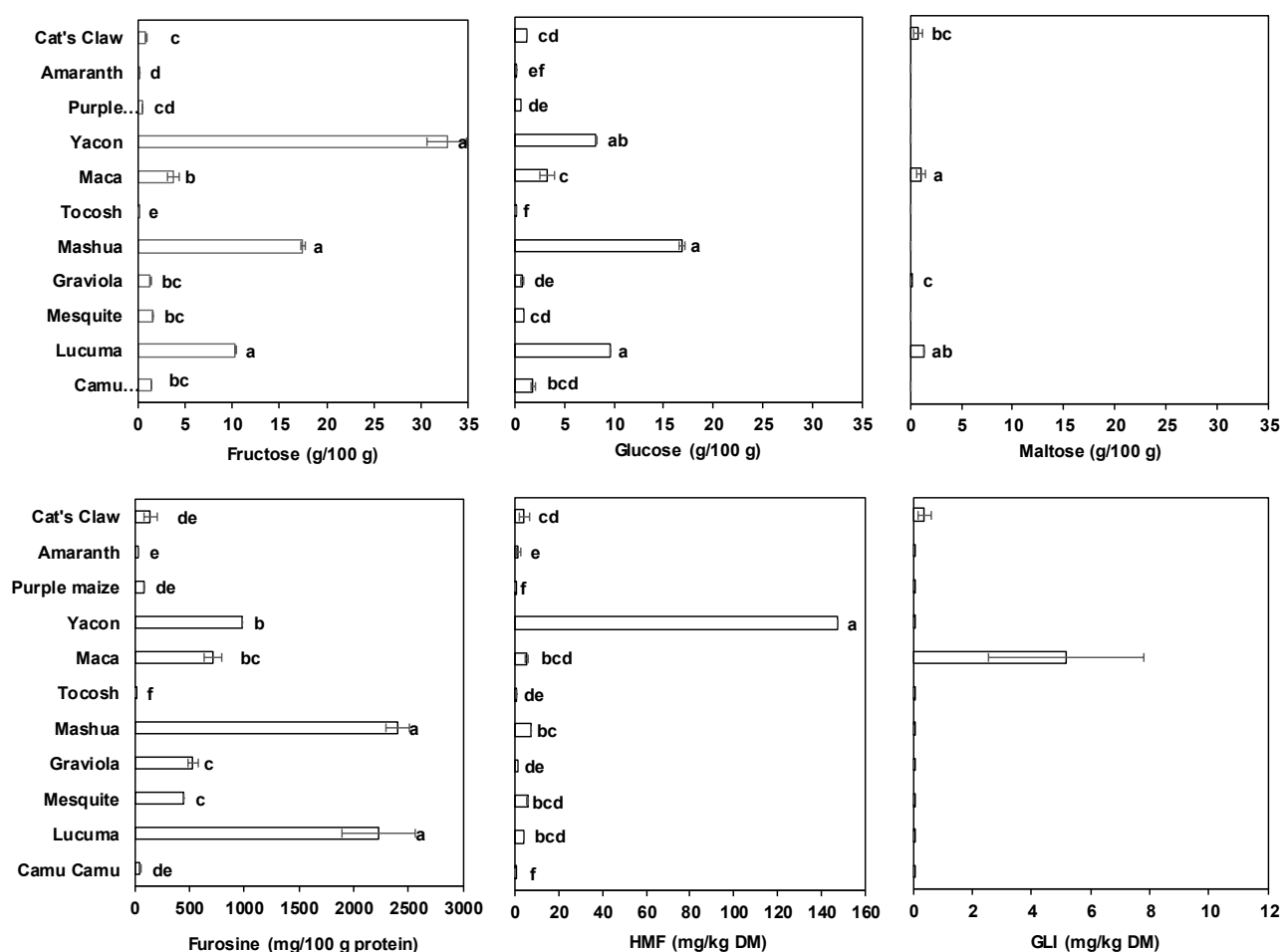


Figure 4. Reducing sugars (fructose, glucose and maltose) and heat damage indices (furosine; hydroxymethylfurfural, HMF; glycosylisomaltol, GLI) of powder products from 11 species. Different letters indicate significant differences ($p \leq 0.05$) among species.

Fructose and glucose were detected in all samples, and were particularly abundant in yacon, mashua and lucuma; maltose was detected only in cat's claw, most maca samples, lucuma and graviola. Overall, yacon (40.9 g/100 g DM), mashua (34.4 g/100 g DM) and lucuma (21.2 g/100 g DM) showed the highest content of reducing sugars, which, on the other hand, were almost absent in tocosh (0.11 g/100 g DM) and amaranth (0.30 g/100 g DM). Sucrose (a non-reducing sugar, but a possible source of monosaccharides) was present in moderate quantities in mesquite, maca, mashua, lucuma and yacon (42.5, 25.9, 16.4, 8.30, 8.20 g/100 g DM, respectively) and was very scarce in all the other products. The presence of reducing sugars is important, because they are one of the basic reactants involved in the formation of Amadori products during the Maillard reaction, when exposed to high temperatures (e.g. oven drying, cooking, baking): therefore, higher reducing sugars concentrations forebode higher heat damage during products manufacturing. Among the plants tested, yacon is a well-known source of fructo-oligosaccharides (CAMPOS *et al.*, 2012) and our results are confirmed by the observations (49.2 g/100 g) of SCHER *et al.* (2009). The reducing sugars content found in mashua is

analogous to the quantity (6.4-45.3 g/100 g DM, average 28.4 g/100 g DM) reported by GUEVARA-FREIRE *et al.* (2018), while those of maca are slightly lower than the value (13.10±0.17 g/100 g DM) described by RONDÁN-SANABRIA and FINARDI-FILHO (2009), and that of mesquite is slightly inferior to the data (3.17-3.74 g/100 g DM) reported by CARDOZO *et al.* (2010) for different *Prosopis* spp. For fructose and glucose content our lucuma results are within the broad range of variation (1.28-12.71 and 2.48-17.37 g/100 g DM) reported by FUENTEALBA *et al.* (2016), and the amaranth ones are very similar to those (0.12 and 0.34 g/100 g DM) presented by GAMEL *et al.* (2006).

3.5. Heat damage

The ANOVA (not presented) showed the existence of significant differences for heat damage among the samples. The average values and the results of the LSD test for heat damage indices, i.e. furosine, GLI and HMF, are reported in Supplementary Table 2. The results obtained grouping the samples by species are reported in Fig. 4.

Non-enzymatic browning in dried products may be influenced by water activity, drying temperature, pH and chemical composition of foods (SAGAR and SURESH KUMAR, 2010). Furosine is an index of the first steps of Maillard reaction, while GLI and HMF are markers of intermediate phases; GLI is formed by the heating of maltose and aminoacids (especially glutamine), while HMF is created not only by degradation of Amadori compounds but also of sugars.

Furosine content was very high in mashua and lucuma (>2000 mg/100 g protein), high in yacon and most maca samples and low in camu camu, amaranth, purple maize as well as in two cat's claw samples. Maca 6 and maca 7 had significantly lower furosine content than the other maca samples. HMF was high only in yacon, but was detected, at lower levels, in several other samples, while GLI was found only in maca 3, maca 6 and cat's claw 1. No Maillard reactions developed in tocosh (a characteristic food, obtained by natural bacterial fermentation of straw-wrapped potatoes kept in running water for several months) as furosine and GLI were lower than the detection limit and HMF was very low, while camu camu, purple maize, amarant, and cat's claw tea had limited heat damage (low furosine levels and generally below-detection GLI and HMF). Furfural, an indicator of more advanced Maillard reaction stages mainly produced by pentose degradation or thermal degradation of HMF during caramelization, was absent in all samples, even if the method used for GLI and HMF analysis is able to determine its presence.

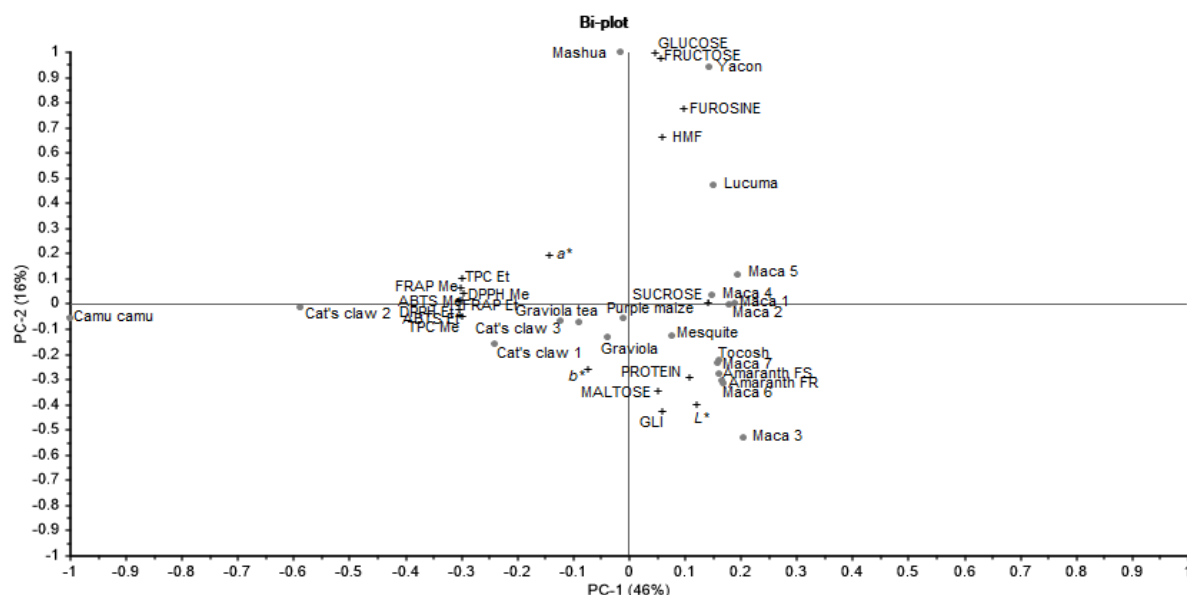
Since water activity values for all the samples were very similar, ranging between 0.492 and 0.585, and no correlation between heat damage indices and protein content (Table 1) exists, the development of the Maillard reaction seems completely attributable to processing conditions and reducing sugar concentration. The lofty heat damage of yacon (981.3 mg/100 g protein of furosine and 146.6 mg/kg DM of HMF) is justified by its high fructose and glucose content and by the strong thermal treatment needed to inactivate its highly thermostable polyphenol oxidase enzyme (NEVES DA SILVA, 2007) for a luminous color (Fig. 1). These conditions lead to the degradation of the Amadori compounds and to the formation of intermediate compounds (i.e. HMF). The furosine levels of the other samples correlate well to the concentration of reducing sugars ($r = 0.93$). Thus, mashua, lucuma and most of maca powders with relevant content of reducing sugars have high furosine levels while samples with low reducing sugars content present limited furosine. However, furosine alone is not suitable to completely describe the heat damage of all powder products. The presence of HMF (0.9-11.8 mg/kg DM) in most samples points to

an intermediate development of Maillard reaction. The samples with detectable GLI (maca 3, maca 6 and cat's claw 1) showed the highest maltose levels but relatively low reducing sugar concentrations (4.0-7.7 g/100 g DM). The simultaneous formation of HMF suggests higher-than-the-average processing temperatures for these samples. A similar hypothesis can be made for some samples with very low reducing sugar concentrations (0.2-2.5 g/100 g DM) and significant HMF content (1.3-6.0 mg/kg DM).

To the best of our knowledge, no information on heat damage in this type of powder products is available. With reference to other vegetables, furosine contents of 14-262 mg/100 g protein (RUFÍAN-HENARES *et al.*, 2013) and of 457-1172 mg/100 g protein (BIGNARDI *et al.*, 2016) are reported in sweet pepper and in dried red chili pepper, respectively; similarly, RÍOS-RÍOS *et al.* (2018) describe furosine concentrations of 46.6-110.1 mg/100 g protein in black garlic powder, while HMF levels of 1.3-9.5 mg/kg DM are recorded by SORIA *et al.* (2009) in carrots dried under different conditions.

3.6. Principal components analysis

Fig. 5 depicts the biplots of scores and loadings obtained by the principal components analysis (PCA) performed considering all samples and parameters. PC1 and PC2 describes 46% and 16% of variation (Fig. 5A), while PC3 and PC4 10% and 9% (Fig. 5B); therefore, the initial four PC explain 81% of total variation. The PCA unmistakably separates the different species. PC1, characterized by antioxidant properties, differentiates camu camu and cat's claw along the left side, while PC2, mainly related to heat damage, positions mashua, yacon and lucuma in the upper side (high furosine, HMF, glucose and fructose contents), and maca 3, maca 6, amaranth and tocosh in the bottom side (high L^* , GLI, maltose and protein). PC3 further splits purple maize, graviola, amaranth and tocosh, from mesquite and maca samples, while PC4 divides mashua (top of the plot, characterized by high protein and furosine contents) from yacon (bottom, high HMF and L^*); maca 3 sits alone in a spot defined by high GLI content.



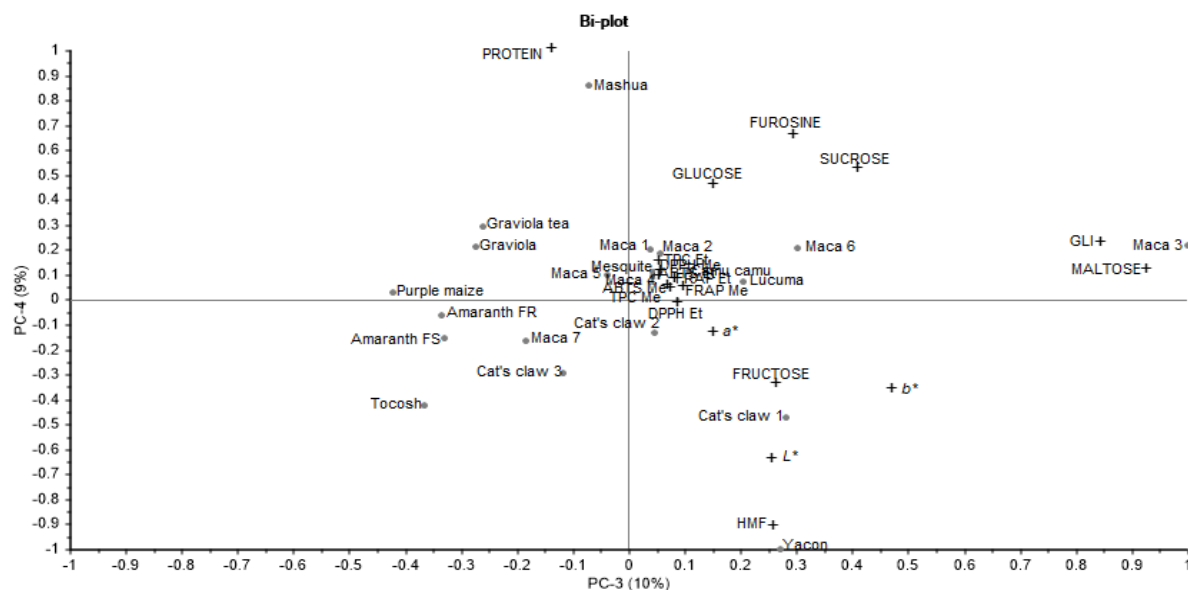


Figure 5. Bi-plot of scores and loads for the first four principal components (PC1 vs. PC2, A; PC3 vs. PC4, B) of the principal component analysis carried out on colour coordinates (L^* , a^* , b^*), protein content, total polyphenol content (TPC) and antioxidant capacity (ABTS, FRAP and DPPH tests) of the ethanol:H₂O (Et) and methanol:H₂O:acetic acid (Me) extracts, reducing sugars, furosine, hydroxymethylfurfural (HMF), and glycosylisomaltol (GLI) of 21 powder products.

4. CONCLUSIONS

Our results show that, for the traits analysed, camu camu and cat's claw powders are excellent products, because they have high levels of total polyphenols and antioxidant capacity together with low heat damage. Other interesting products are the powders of graviola, purple maize and mesquite, while the high antioxidant properties of mashua are coupled to severe heat damage.

For an effective use in the food industry it will be necessary to evaluate the stability of the antioxidant capacity of the powders during the manufacturing process and the digestion of innovative high-nutritional-value foods, as well as to assess the sensorial quality of the end products.

Supplementary Table 1. Beneficial properties of the species analyzed, and bibliographic references.

Species	Putative properties	References
Cat's claw	immune-modulatory, antioxidant, antiviral, antibacterial and anti-inflammatory	Steinberg, 1995; Heitzman <i>et al.</i> , 2005
Amaranth	balanced aminoacid composition	Repo-Carrasco <i>et al.</i> , 2010; Bressani <i>et al.</i> , 1993
Purple maize	anti-inflammatory, prevention of obesity, diabetes, hyperglycemia and hypertension	Tsuda <i>et al.</i> , 2003; Finkel <i>et al.</i> , 2013
Yacon	low glycemic index, resistance to infections and allergic reactions, prevention of colon cancer	Delgado <i>et al.</i> , 2013 ; de Moura <i>et al.</i> , 2012
Maca	sexual dysfunctions, menopausal symptoms, osteoporosis, benign prostatic hyperplasia, lipid and glucose metabolism, skin protection from UV	Gonzales <i>et al.</i> , 2005; Gonzales-Castañeda and Gonzales, 2008; Rubio <i>et al.</i> , 2011; Vecera <i>et al.</i> , 2007 ; Zhang <i>et al.</i> , 2006

Tocosh	antimicrobial	Lopez Campos, 2017
Mashua	anticancer	Noratto <i>et al.</i> , 2004
Graviola	anti-inflammatory and anti-malarial, prevention of some cancers	Foong and Hamid, 2012; <i>Mishra et al.</i> , 2013; Yang <i>et al.</i> , 2015
Mesquite	antidiabetic, anti-inflammatory, anticancer and antimicrobial	Henciya <i>et al.</i> , 2017
Lucuma	antioxidant, antihyperglycemic	Fuentealba <i>et al.</i> , 2016
Camu camu	antioxidant, anti-inflammatory, antimicrobial and anti-diabetic	Akter <i>et al.</i> , 2011; Fujita <i>et al.</i> , 2015

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Supplementary Table 2. Mean±standard error and LDS results for colour coordinates (L^* , a^* , b^*), sugars content (fructose, glucose, maltose and sucrose, g/100 g DM) and heat damage indices (furosine, mg/100 g protein; hydroxymethylfurfural, HMF, mg/kg DM; and glycosylisomaltol, GLI, mg/kg DM) of 21 commercial powder samples.

	L^*	a^*	b^*	Fructose	Glucose	Maltose	Saccharose	Furosine	HMF	GLI
Cat's claw 1	66.3 ^l ±0.4	13.2 ^{ab} ±0.2	31.1 ^c ±0.3	0.7 ^l ±0.003	1.1 ^{il} ±0.02	2.2 ^b ±0.08	0.9 ⁿ ±0.01	nd ^p	11.8 ^b ±0.7	1.2 ^c ±0.03
Cat's claw 2	57.7 ^o ±0.4	12.1 ^b ±0.1	30.3 ^d ±0.1	1.0 ^h ±0.01	1.2 ⁱ ±0.07	0.1 ^g ±0.01	1.0 ^l ±0.03	324.5 ⁱ ±20.4	nd ^l	nd ^d
Cat's claw tea	50.2 ^q ±0.5	13.5 ^{a±} 0.2	32.8 ^b ±0.2	0.8 ⁱ ±0.03	1.1 ^{il} ±0.09	0.1 ^h ±0.01	0.9 ⁿ ±0.01	88.6 ^l ±0.6	0.9 ^j ±0.02	nd ^d
Amaranth FR	80.3 ^c ±0.3	2.2 ^l ±0.0	19.6 ^m ±0.1	0.1 ^q ±0.002	0.1 ^r ±0.002	nd ⁱ	1.8 ^h ±0.03	34.9 ⁿ ±1.1	3.1 ^h ±0.2	nd ^d
Amaranth FS	78.5 ^{ef} ±0.2	1.9 ^m ±0.0	19.3 ^m ±0.2	0.2 ^p ±0.01	0.2 ^q ±0.005	nd ⁱ	1.9 ^h ±0.02	29.0 ^o ±0.2	nd ^k	nd ^d
Purple maize	55.3 ^{p±} 0.3	10.8 ^c ±0.1	2.8 ^o ±0.1	0.5 ⁿ ±0.01	0.6 ^o ±0.01	nd ⁱ	1.5 ⁱ ±0.02	79.5 ^l ±2.0	nd ^k	nd ^d
Yacon	74.9 ^h ±0.5	3.9 ^g ±0.2	26.8 ^f ±0.5	32.7 ^a ±2.09	8.2 ^c ±0.04	nd ⁱ	8.2 ^g ±0.13	981.3 ^{cd} ±1.9	146.6 ^a ±0.1	nd ^d
Maca 1	77.8 ^f ±0.2	2.0 ^m ±0.1	28.3 ^e ±0.1	5.4 ^d ±0.01	5.8 ^e ±0.2	0.5 ^e ±0.01	28.8 ^c ±0.35	825.3 ^e ±6.8	3.3 ^h ±0.1	nd ^d
Maca 2	76.2 ^g ±0.3	3.8 ^g ±0.1	24.6 ^h ±0.2	4.2 ^e ±0.19	3.6 ^g ±0.15	0.5 ^{ef} ±0.03	35.4 ^b ±0.43	971.9 ^{cd} ±8.2	5.5 ^{de} ±0.8	nd ^d
Maca 3	78.4 ^{ef±} 0.2	4.0 ^g ±0.1	26.2 ^{fg} ±0.3	0.2 ^o ±0.01	0.7 ⁿ ±0.01	4.2 ^a ±0.04	21.2 ^e ±0.25	728.5 ^f ±17.5	4.4 ^{fg} ±0.2	27.3 ^a ±0.3
Maca 4	72.0 ⁱ ±0.2	5.1 ^f ±0.1	30.2 ^d ±0.3	5.3 ^d ±0.06	4.6 ^f ±0.15	0.4 ^f ±0.05	24.4 ^d ±0.96	877.7 ^{de} ±20.8	10.4 ^b ±0.3	nd ^d
Maca 5	83.9 ^b ±0.5	1.9 ^m ±0.0	22.2 ^l ±0.2	5.6 ^d ±0.04	7.0 ^d ±0.20	nd ⁱ	26.4 ^{cd} ±0.47	1008.6 ^c ±0.0	4.3 ^{fg} ±0.03	nd ^d
Maca 6	79.4 ^d ±0.3	2.8 ⁱ ±0.1	23.5 ^l ±0.2	5.4 ^d ±0.002	0.5 ^p ±0.02	1.8 ^c ±0.01	27.8 ^c ±0.16	378.0 ⁱ ±6.0	3.0 ^h ±0.2	8.9 ^b ±0.4
Maca 7	78.7 ^{de} ±0.1	1.2 ⁿ ±0.0	24.8 ^h ±0.1	0.6 ^m ±0.005	0.5 ^o ±0.004	nd ⁱ	17.1 ^f ±0.14	167.7 ^k ±3.7	5.0 ^{ef} ±0.07	nd ^d
Tocosh	86.6 ^a ±0.7	0.5 ^o ±0.1	9.7 ⁿ ±0.2	0.01 ^r ±0.002	0.1 ^s ±0.004	nd ⁱ	0.1 ^o ±0.003	nd ^p	nd ^k	nd ^d
Mashua	28.3 ^t ±0.4	7.7 ^d ±0.1	5.3 ^o ±0.0	17.5 ^b ±0.27	16.9 ^a ±0.25	nd ⁱ	16.4 ^f ±0.05	2399.8 ^a ±107.6	7.5 ^c ±0.1	nd ^d
Graviola	43.3 ^r ±0.3	3.1 ^h ±0.0	26.1 ^g ±0.1	nd ^s	0.6 ^o ±0.03	0.1 ^g ±0.001	0.9 ⁿ ±0.03	445.6 ^h ±23.4	1.3 ⁱ ±0.02	nd ^d
Graviola tea	41.0 ^s ±0.3	2.0 ^{lm} ±0.0	25.1 ^h ±0.1	1.3 ^g ±0.08	1.0 ^l ±0.05	0.1 ^h ±0.01	1.0 ^m ±0.07	608.2 ^g ±6.0	1.3 ⁱ ±0.02	nd ^d
Mesquite	63.4 ⁿ ±0.4	8.1 ^d ±0.2	34.1 ^a ±0.2	1.6 ^f ±0.04	0.9 ^m ±0.02	nd ⁱ	42.5 ^a ±0.1	450.5 ^h ±0.1	6.0 ^d ±0.6	nd ^d
Lucuma	72.3 ⁱ ±0.2	8.0 ^d ±0.1	22.2 ^l ±0.2	10.3 ^c ±0.1	9.6 ^b ±0.02	1.3 ^d ±0.03	8.3 ^g ±0.02	2228.4 ^b ±334.5	4.1 ^g ±0.2	nd ^d
Camu camu	64.6 ^m ±0.3	6.6 ^{e±} 0.1	28.0 ^e ±0.1	1.4 ^g ±0.02	1.8 ^h ±0.19	nd ⁱ	0.9 ^{mn} ±0.04	48.2 ^m ±0.7	nd ^l	nd ^d

For each parameter, diverse letters indicate significant differences (LSD, $p \leq 0.05$) among samples; nd, not detectable.

Supplementary Table 3. Mean and LDS results for total polyphenols content (TPC; mg GAE/kg DM) and ABTS, FRAP and DPPH antioxidant capacity (mmol TE/kg DM) of 21 powder samples extracted with ethanol 80% (EtOH:H₂O) and methanol:H₂O:acetic acid (MeOH:H₂O:acet).

	TPC		ABTS		FRAP		DPPH	
	EtOH:H ₂ O	MeOH:H ₂ O:acet	EtOH:H ₂ O	MeOH:H ₂ O:acet	EtOH:H ₂ O	MeOH:H ₂ O:acet	EtOH:H ₂ O	MeOH:H ₂ O:acet
Cat's claw 1	16.1 ^c ±0.5	30.7 ^c ±0.0	267.4 ^c ±0.0	331.5 ^c ±6.1	229.9 ^c ±4.2	391.9 ^c ±10.0	26.2 ^c ±0.1	43.4 ^c ±0.04
Cat's claw 2	24.2 ^b ±0.4	53.6 ^b ±0.8	590.9 ^b ±21.0	802.6 ^b ±10.6	460.7 ^b ±52.8	872.8 ^b ±2.3	49.6 ^b ±0.2	125.9 ^b ±1.1
Cat's claw tea	9.2 ^f ±0.1	20.1 ^e ±0.6	135.6 ^f ±0.2	280.7 ^d ±14.1	110.0 ^{ef} ±4.0	255.1 ^e ±2.6	9.9 ^e ±0.02	32.3 ^e ±0.3
Amaranth FR	3.5 ^m ±0.0	2.9 ^q ±0.0	5.7 ^o ±0.0	4.9 ^o ±0.0	5.1 ⁿ ±0.1	4.6 ^s ±0.0	0.2 ^q ±0.0	nd
Amaranth FS	2.6 ^q ±0.0	2.6 ^r ±0.0	5.5 ^o ±0.0	4.6 ^o ±0.0	5.2 ⁿ ±0.1	3.3 ^t ±0.0	nd	nd
Purple maize	7.8 ^g ±0.3	13.7 ^g ±0.1	103.1 ^g ±0.2	148.8 ^g ±0.3	97.3 ^f ±0.8	200.1 ^g ±3.1	5.8 ^h ±0.1	21.1 ^f ±0.3
Yacon	4.8 ⁱ ±0.1	11.7 ^h ±0.1	31.6 ^l ±0.3	34.5 ⁱ ±1.7	42.2 ^g ±1.2	65.2 ^j ±0.7	3.8 ⁱ ±0.01	7.1 ^j ±0.1
Maca 1	3.5 ^m ±0.3	7.8 ^{mn} ±0.0	31.4 ^l ±0.4	26.0 ^m ±0.7	13.9 ^m ±0.3	24.3 ^r ±0.7	0.8 ^o ±0.002	0.8 ⁿ ±0.001
Maca 2	3.4 ^m ±0.1	9.2 ⁱ ±0.1	32.8 ^l ±0.0	31.7 ^{il} ±0.7	20.5 ^l ±0.3	30.1 ^p ±0.1	0.9 ⁿ ±0.002	1.5 ^m ±0.001
Maca 3	3.4 ^m ±0.1	5.9 ^o ±0.1	31.6 ^l ±0.7	26.1 ^m ±0.7	23.6 ⁱ ±0.4	35.6 ⁿ ±1.1	1.5 ^k ±0.003	1.5 ^m ±0.004
Maca 4	4.0 ^l ±0.2	7.8 ^{lm} ±0.1	48.0 ⁱ ±1.5	33.4 ^{il} ±0.1	29.3 ^h ±0.7	49.3 ^l ±1.4	1.6 ^j ±0.01	1.5 ^m ±0.004
Maca 5	3.0 ⁿ ±0.1	6.0 ^o ±0.2	32.2 ^l ±0.5	31.2 ^l ±2.0	14.3 ^m ±0.2	26.1 ^q ±0.1	1.0 ^m ±0.002	0.9 ⁿ ±0.001
Maca 6	10.4 ^{de} ±0.2	8.1 ^l ±0.0	28.7 ^m ±0.5	25.8 ^m ±0.8	24.6 ⁱ ±0.2	38.4 ^m ±0.3	1.1 ^l ±0.02	1.6 ^l ±0.0
Maca 7	4.2 ^l ±0.1	3.9 ^p ±0.1	20.2 ⁿ ±0.6	13.3 ⁿ ±0.1	18.2 ^l ±0.4	32.7 ^o ±0.8	0.4 ^p ±0.0	0.8 ⁿ ±0.0
Tocosh	2.5 ^o ±0.0	2.8 ^q ±0.0	2.1 ^p ±0.0	1.0 ^p ±0.0	nd	nd	nd	nd
Mashua	10.0 ^{ef} ±0.2	21.4 ^d ±0.5	129.9 ^f ±1.2	264.2 ^{de} ±10.1	152.3 ^d ±2.1	410.6 ^c ±3.9	8.7 ^g ±0.02	36.6 ^d ±0.01
Graviola	11.2 ^d ±0.5	19.7 ^e ±0.5	155.3 ^e ±3.5	198.1 ^f ±1.0	116.3 ^e ±10.2	215.3 ^f ±3.5	9.0 ^f ±0.1	19.9 ^g ±0.1
Graviola tea	14.8 ^c ±0.7	21.0 ^d ±0.1	209.9 ^d ±1.1	253.8 ^e ±1.0	155.2 ^d ±5.1	327.6 ^d ±1.4	17.6 ^d ±0.02	16.6 ^h ±0.03
Mesquite	6.8 ^h ±0.1	15.3 ^f ±0.2	51.0 ^h ±0.7	95.3 ^h ±0.1	47.7 ^g ±0.3	115.0 ^h ±0.2	3.8 ⁱ ±0.04	13.4 ⁱ ±0.02
Lucuma	2.8 ^{no} ±0.0	7.5 ⁿ ±0.0	19.5 ⁿ ±0.4	31.5 ^{il} ±1.5	18.2 ^l ±0.2	33.5 ^o ±0.1	0.9 ⁿ ±0.001	3.5 ^k ±0.03
Camu camu	38.3 ^a ±0.1	76.4 ^a ±0.5	868.6 ^a ±16.8	1473.5 ^a ±90.3	996.0 ^a ±97.8	1786.5 ^a ±60.9	75.7 ^a ±0.4	184.33 ^a ±1.1

For each parameter, diverse letters indicate significant differences (LSD, $p \leq 0.05$) among samples; nd, not detectable.

ACKNOWLEDGEMENTS

We thank Giulia Malizia and Mattia Magistrelli for their assistance in the lab analyses.

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Paper Received February 8, 2019 Accepted May 9, 2019

IMMOBILIZATION AND CHARACTERIZATION OF β -GLUCOSIDASE FROM GEMLIK OLIVE (*OLEA EUROPEA* L.) RESPONSIBLE FOR HYDROLIZATION OF OLEUROPEIN

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ABSTRACT

The β -Glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) enzyme was purified from Gemlik variety olive (*Olea europea* L.). The purified enzyme was immobilised onto Supermagnetic Nanoparticles in order to stabilise the enzymatic efficiency and increase usage in the food industry. The purified and immobilised enzyme was characterised by molecular weight, kinetic parameters and optimum pH and temperature values comparatively. The enzyme is a monomer with a mass of approximately 40 kDa. The Kinetic values of the immobilised and purified enzymes were 1.34 mM and 384.61 U/mg; 0.37 mM as K_m and 370.37 U/mg as V_{max} respectively.

Keywords: β -glucosidase, *Olea europea* L., pNPG, purification, SPMN

1. INTRODUCTION

Olive is a fruit which consists a lot of biotransformal compounds. There is a wide variety of phenolic compounds in *Olea europaea* L. which are important for sensorial properties. They also have substantial effects on human health such as nutritional, physiological and pharmaceutical effects. Unripe olive fruit has important phenolic secoiridoids causing bitter taste. These polyphenolic substances called oleuropeins have many aldehydic or dialdehydic forms such as hydroxytyrosol and tyrosol, transformed by β -glucosidase as a part of the defence mechanism in the plant tissue (GARCIA-RODRIGUEZ *et al.*, 2011; DE LEONARDIS *et al.*, 2015). As a result of this system, many oleuropein-related compounds are also available from olives, rearranged via aglycon by the elenolic acid ring. The quality and quantity of these substances change by variety, tissue by tissue (leave, fruit, etc.) and in terms of ripening stage (Bianchi, 2003). Olives gradually get rid of their bitterness at the ripening stage. This occurs by β -glucosidase gradual hydrolyzation of oleuropein and leads to changes in taste (GUIRIMAND *et al.*, 2010).

β -glucosidases are biologically active enzymes that hydrolyse 1.4 β -glycoside bonds between carbohydrate molecules (ÜNAL and SENER, 2017). Olive β -glucosidases (DE LEONARDIS *et al.*, 2015) (β -D-glucoside glucohydrolases, EC 3.2.1.21) show high substrate specificity (MAZZUCA *et al.*, 2006) as in other plant species (SAVAS *et al.*, 2018). They hydrolyse the β -glycosidic bonds in oligosaccharides or other glucose moieties and ester bonds in oleuropein, which is responsible for bitterness (VELÁZQUEZ-PALMERO *et al.*, 2017).

After the reaction, β -glucosidases lose their catalytic activities like other enzymes. Some techniques may be used in food processing systems (debittering, flavour enrichment *etc.*) to stabilise enzyme usage. Enzyme immobilisation by covalent bonding is used for stability and reuse of enzymes. For this purpose, different bulk or magnetic materials could be used as a matrix.

Superparamagnetic iron oxide nanoparticles (SPMN) that are used for high colloidal stability, magnetism and be biocompatible materials are preferred as immobilisation matrices (MA *et al.*, 2009). These are small synthetic γ -Fe₂O₃ or Fe₃O₄ particles with a core size of < 10 nm and well dispersed in a liquid for biomedical applications. They may be removed easily with simple magnetism from the reaction medium.

Gemlik variety olives are from the north-west Turkey (UYLAŞER and ŞAHİN, 2004). This variety, which are processed olives for direct consumption has high oil content.

Considering the rare reports on the topic indicates the catalytic activity of olive β -glucosidases. The objective of this study is to show that Gemlik variety olive fruits could be used as a one β -glucosidase source for biotechnological application in the food industry. In this context, purified and immobilised forms of β -glucosidases (β -D-glucoside glucohydrolase) were investigated as a naturally occurring enzyme involved in the biotransformation of oleuropein.

2. MATERIAL AND METHODS

2.1. Materials

Gemlik variety olives that were used in our study were obtained from Balıkesir at their black maturity stage. They were brought to the laboratory in cold storage conditions

(+4°C). After the washing and sorting steps, they were used for obtaining acetone powder as the enzyme source.

All chemicals that were used in our study were supplied from Sigma-Aldrich (St. Louis, MO, USA), and protein molecular weight markers were supplied from Thermo Scientific (Waltham, MA, USA). They were of the highest grade available.

2.2. Preparation of acetone powder

Acetone powder was used as the enzyme source in this study (SAVAS *et al.*, 2018). 100 g of olive pulp was homogenised for 2 min in 750 mL of cold acetone (-20°C) using a homogenizer for preparation. The homogenate was filtered by Whatmann No 1 filter paper, and retentate was extracted three times with 500 mL of acetone (-20°C) to remove oil residues. Reddish purple residues on the filter were air-dried at room conditions on blotting papers and held at -20°C for enzyme assays. 2 g of the acetone powder was homogenised in 100 mL of a cold extraction buffer (4°C) (pH 9.5) using an Ultra Torrax homogenizer. The mixtures were centrifuged at 15,000 rpm for 30 min at 4°C, and crude extracts were obtained from the supernatant.

2.3. Chromatographic Study

The further step of enzyme purification was carried out based on the method by KARA *et al.* (2011) by Hydrophobic Interaction Chromatography. As defined in the method, the solid ammonium sulphate in concentrations from 0 to 50% was added to the crude extract at +4°C for ammonium sulphate precipitation. The reaction mixture was centrifuged at 15000 rpm for 30 min (+4°C), the sediment was dissolved in 50 mM of the sodium phosphate buffer (pH 6.8), and the final saline concentration of the mixture was set to 1M ammonium sulphate.

The hydrophobic column was synthesized using 10% CNBr in a 1:1 solution of Sepharose 4B and distilled water for the second step of the purification process. The pH of the mixture was stabilised at 11 for 8–10 min. The gel obtained was filtrated and washed with a cold 0.1M NaHCO₃ buffer (pH 10). After the reaction mixture was combined with the saturated L-tyrosine, the solution was stirred for 90 min. After the gel, washing and diazotization of 1-naphthylamine in this complex was fixtured to the sepharose-4B-l-tyrosine. The further steps were carried out as described in the method by 3 mL of enzyme solution loaded onto the hydrophobic column. 1 mL fractions were gathered at a flow rate of 30 mL/h in a linear gradient. The fractions were collected with the highest protein content and used in next studies as the purified enzyme.

2.4. Immobilisation

Superparamagnetic nanoparticles (SPMN) were synthesized specifically for the use of enzyme immobilization (KOCKAR *et al.*, 2010). 20-100 mg of Fe^{+2/+3} superparamagnetic nanoparticles (SPMN) was placed into 2 ml of a 0.003 M phosphate buffer (pH 6) with 0.1 M of NaCl, and 0.5 ml of carbodiimide solution (0.025 g/mL in buffer) was placed into the reaction medium. The reaction medium was sonicated for 10 minutes. 2 ml of purified β-glucosidase enzyme was added and sonicated for 30 minutes.

2.5. Characterisation assays and protein determination

In all steps for olive β -glucosidase extraction, purification and further studies, activity of the enzyme was measured at 410 nm against para-nitrophenyl- β -D-glucopyranosides (p-NPG) as substrate (LOWRY *et al.*, 1951). 70 μ L of enzyme solution in 50 mM sodium acetate (pH 5.5) and 70 μ L of substrate were added in a 96 well plate. Incubation of the well content was facilitated at 37°C for 30 min in triplicates. 70 μ L of 0.5M Na₂CO₃ was added into the medium for stopping the reaction, and the absorbance values were determined by spectrophotometry. Enzyme activity was expressed as μ mol p-nitrophenol composed per minute in the reaction medium under these terms. Molecular mass values of protein were estimated using a commonly used standard (bovine serum albumin - BSA).

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to estimate the molecular weight of olive β -glucosidase according to the method reported by LI *et al.* (1997) using a Minigel system (Bio-Rad Laboratories, USA). After gel colorization with Coomassie brilliant blue R-250 and decolorization with 7.5% acetic acid in 5% methanol to detect protein bands, the gel was photographed with UV Light.

25 mM sodium acetate (2.0–10.0) buffers were used for the pH optima assays (KARA *et al.*, 2011). Activity measurements were achieved by using 5 mM of substrate (pH 5.5) at the temperature range of 25 to 65°C for 30 min to determine the optimum temperature. The thermal stability was determined by incubating at 70 for 30 min and then cooling down to 4 °C.

p-NPG (concentration range from 0.12-2.38 mM) and oleuropein were studied for determining K_m and V_{max} values. Glucose, citric acid, lactic acid, sodium hydroxide and sodium chloride were studied as potential inhibitors. Activity measurements of the samples were performed, and 1.75 mM of pNPG and 1 mM of Ni⁺¹, Zn⁺¹, Cr⁺¹, Mn⁺², Mg⁺² and Co⁺¹ were used in the reaction medium. The effect of various concentrations (0.0029-0.0297 mM) of Deltamethrin, Chlorpyrifos and Alphacypermethrin as widely used pesticides against olive insects on olive β -glucosidase were also studied using 1.25 mM of pNPG as the substrate. Results are given as relative activity, which the enzyme activity in the non-inhibitor medium is considered 100. The inhibitor concentration, which reduces the enzymatic activity by 50% (IC_{50} values), was determined by the relative plots.

A Fourier Transform-Infrared Spectroscopy (FT-IR) analysis was performed to prove the correctness of the enzyme immobilisation after the purification step. IR spectra of Fe₃O₄ superparamagnetic nanoparticles, β -glucosidase and immobilised β -glucosidase on the superparamagnetic nanoparticles were obtained by using the KBr pellets preparation technique in ATR cells (600-4000cm⁻¹) with a Perkin Elmer-1600 Series device.

3. RESULTS AND DISCUSSION

3.1. Enzyme Extraction and Purification

Protein assays were achieved by using acetone powder as previously reported (KOUDOUNAS *et al.*, 2015). Although it is known that the use of acetone leads to mutual effects, thanks to usage of acetone, it is possible to obtain concentrated proteins without pigments derived from fresh fruits (ROMERO-SEGURA *et al.*, 2009). Furthermore, acetone powder usage also makes it possible to use as stock enzyme source in the absence of the olive fruit.

Hydrophobic interaction chromatography (SAVAS *et al.*, 2018) was used by precipitation with ammonium sulphate to separate β -glucosidase from the olive acetone powder. After precipitation of the β -glucosidase active fractions with ammonium sulphate, 97% of the activity was measured (Table 1). In this part of the process, great proteins except β -glucosidase were removed, and the quantity of the protein was reduced from 297 to 23 mg.

The elution pattern of enzyme activity and total protein concentrations for all fractions that were collected on the hydrophobic column are shown in Fig. 1. The fractions that had the highest enzyme activity were pooled. The enzyme was purified 163-fold from the remaining particles with clear homogeneity with an overall enzyme yield of 9.90% and a specific activity of 6291.7777 U/mg (Table 1).

The purification yield values were higher than those previously reported for olive (LI *et al.*, 2005; MAZZUCA *et al.*, 2006) and several sources (CAMERON *et al.*, 2001; LI *et al.*, 2005; VERMA *et al.*, 2011). Minimal sequential steps, matrix and ligand characteristics (hydrophobic structure of 1-naphthylamine, sepharose-4B gel matrix and l-tyrosine arm) led to increased purification factors. More purification steps could result in better purification rates. However, more steps cause a dramatic decrease in enzyme activity and protein amounts.

Table 1. Purification of β -glucosidase from olive (*Olea europaea* cv. Gemlik).

Purification steps	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	40	297.27	11430.88	38.4528	100
Ammonium sulphate	10	23.19	11123.31	479.6597	97.30
Hydrophobic chromatography	2	0.18	1132.52	6291.7777	9.90

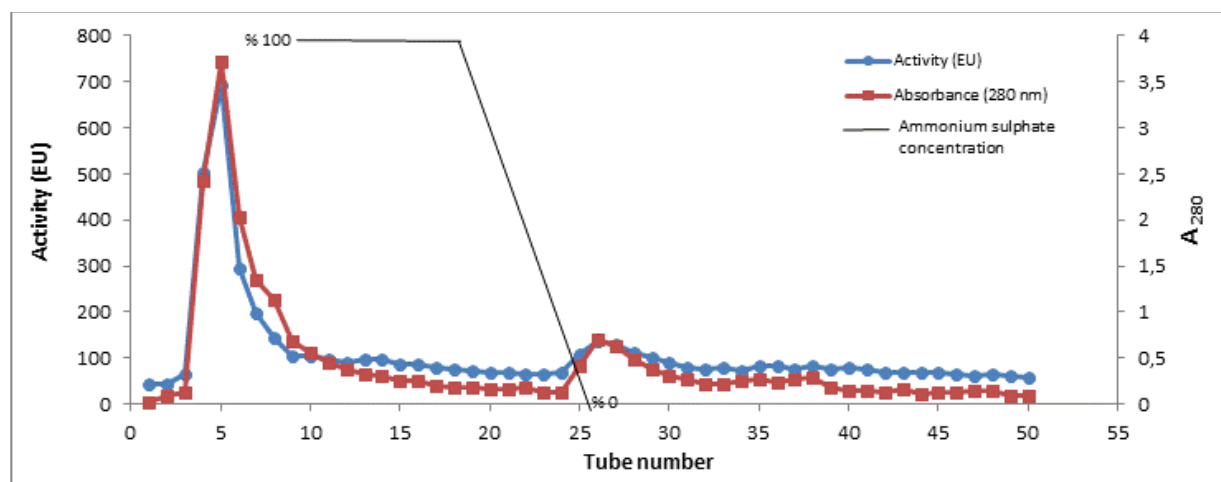


Figure 1. Purification of olive fruit β -glucosidase by hydrophobic interaction chromatography. The enzyme activity and total protein concentrations were determined from all fractions that were collected, as described in Section 2. The enzyme activity was expressed as μ mol of p-/o-nitrophenol liberated per minute in the reaction.

A single band with an apparent molecular mass of ca. 42 kDa was seen by standard methods of SDS-PAGE electrophoresis (Fig. 2). The β -glucosidase of the olive is a

monomer like other plant sources e.g. tea, citrus. There are many β -glucosidase results reported as monomer and oligomer from different plant sources e.g. 68 kDa from *Rauvolfia serpentina*, 92 kDa (VERMA *et al.*, 2011), 37 kDa from tea leaves (LI *et al.*, 2005), 65 kDa from almond (HE and WITHERS, 1997), from sweet cherry fruit (*Prunus avium* L.) (GERARDI *et al.*, 2001) and 55 kDa from *Citrus sinensis* Valencia (KAYA, 2014). Estimated molecular mass of β -glucosidase from olive was reported previously as 55-65.5 kDa (ROMERO-SEGURA *et al.*, 2009; KARA *et al.*, 2011; KAYA, 2014). These different results indicate the molecular mass of β -glucosidases from olives depending on variety.

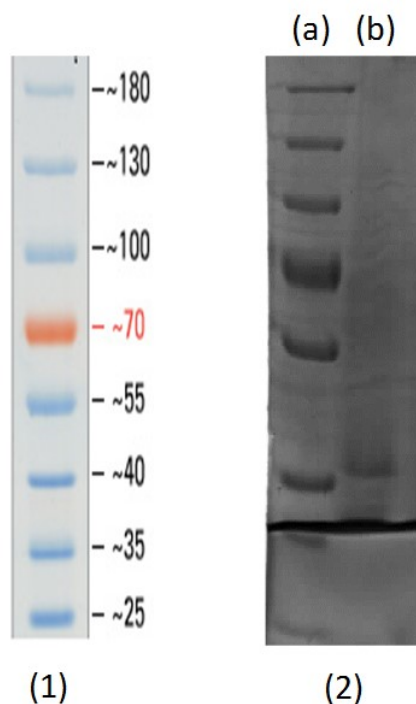


Figure 2. SDS-PAGE analysis of the β -glucosidase purified from olive (*Olea europaea* cv Gemlik) fruit. The enzyme was electrophoresed at pH 8.3 on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1: molecular weight standards (β -galactosidase, 116kDa; bovine serum albumin, 66.2kDa; egg albumin, 45kDa; lactate dehydrogenase, 35kDa; Rease Bsp981 (*E. coli*), 25kDa; β -lactoglobulin, 18.4kDa; lysozyme, 14.4kDa); Lane 2: purified β -glucosidase.

3.2. Characterisation of enzyme sources

The activities of purified and immobilised olive β -glucosidase in different pH are shown in Fig. 3. The optimal pH was found at 5.5 for both enzymes. The purified enzyme showed higher activity relatively in the range of pH 4.5-6 with any activity at pH 2 and 8.

The optimal pH values of β -glucosidases that were determined in previous studies from olive fruit (ROMERO-SEGURA *et al.*, 2009; KARA *et al.*, 2011; KOUDOUNAS *et al.*, 2015), from citrus (CAMERON *et al.*, 2001), from wheat (SUE *et al.*, 2000a) and rye (SUE *et al.*, 2000b) were similar and higher than β -glucosidases from rice (pH 4.5) (AKIYAMA *et al.*, 1998), soybean (pH 4.5) (MASARU *et al.*, 1995), barley (pH 5.0) (LEAH *et al.*, 1995) and lower than vanilla bean (pH 6.5) (ODOUX *et al.*, 2003) and maize (pH 5.8) (CUEVAS *et al.*, 1992).

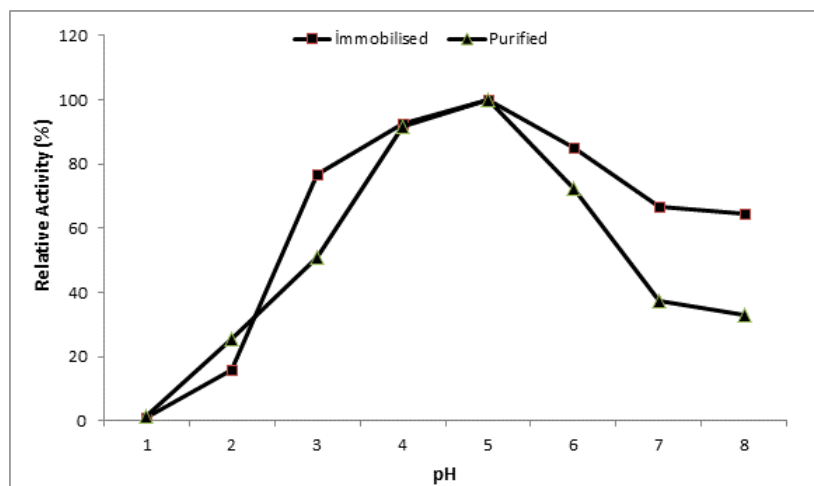


Figure 3. Effect of pH on activity of purified olive (*Olea europaea* cv Gemlik) fruit β -glucosidase. pH optima of β -glucosidase purified from olive (*Olea europea* L.) and immobilised form.

The optimum temperature was determined in the purified and immobilised enzyme sources respectively as 50 and 55°C by using p-NPG as a substrate (Fig. 4A). Temperature optima of the immobilised enzyme were higher than the free enzyme by 5°C like other immobilised enzymes (SINGH *et al.*, 2011). The enzyme became more stable after the immobilization process by means of the vineyard structures. The enzyme purified from olive fruit showed maximum activity at 50°C and 35°C with p-NPG and oleuropein as substrates, respectively (Fig. 4B). The purified enzyme lost its catalytic activity at the end of the 30th min at 70°C with p-NPG as a substrate in the 50 mM acetate buffer (Fig. 4C).

It was reported that plant β -glucosidase showed maximal hydrolytic activity towards p-NPG at 40–45°C in *Citrus sinensis* var. Valencia fruit (CAMERON *et al.*, 2001), 25–30°C in rye (SUE *et al.*, 2000b), 45°C in soybean (MASARU *et al.*, 2005), 50°C in rice (AKIYAMA *et al.*, 1998), 60°C in barley (LEAH *et al.*, 1995) and 40°C in vanilla bean (ODOUX *et al.*, 2003). The temperature optima of our enzyme were similar to that for β -glucosidases taken from tea (LI *et al.*, 2005), rice (AKIYAMA *et al.*, 1998) and maize (CUEVAS *et al.*, 1992) using p-NPG as a substrate. The enzyme was still active by 33% at 4°C and 11% of the initial level at 25°C after 8 weeks (data not shown). This temperature-sensitive enzyme was more stable at cold conditions.

3.3. F-TIR analysis

The F-TIR charts indicated patterns of purified and immobilised olive β -glucosidase on to SPIONs, Fe₃O₄ Superparamagnetic Nanoparticles (SPMN) and processed SPIONs (Fig. 5). The peak of the enzyme at 1400cm⁻¹ was lost on the SPMN activated by carbodiimide (without enzyme) and enzyme-bond SPIONs, while the new peak occurred in the range of 1000-1100 cm⁻¹. It was concluded according to the IR spectrum that, the new bond indicated SPMN-carbodiimide activation. In the enzyme bond SPMN pattern, the characteristic peaks revealed that bonding of the enzyme onto nanoparticles took place.

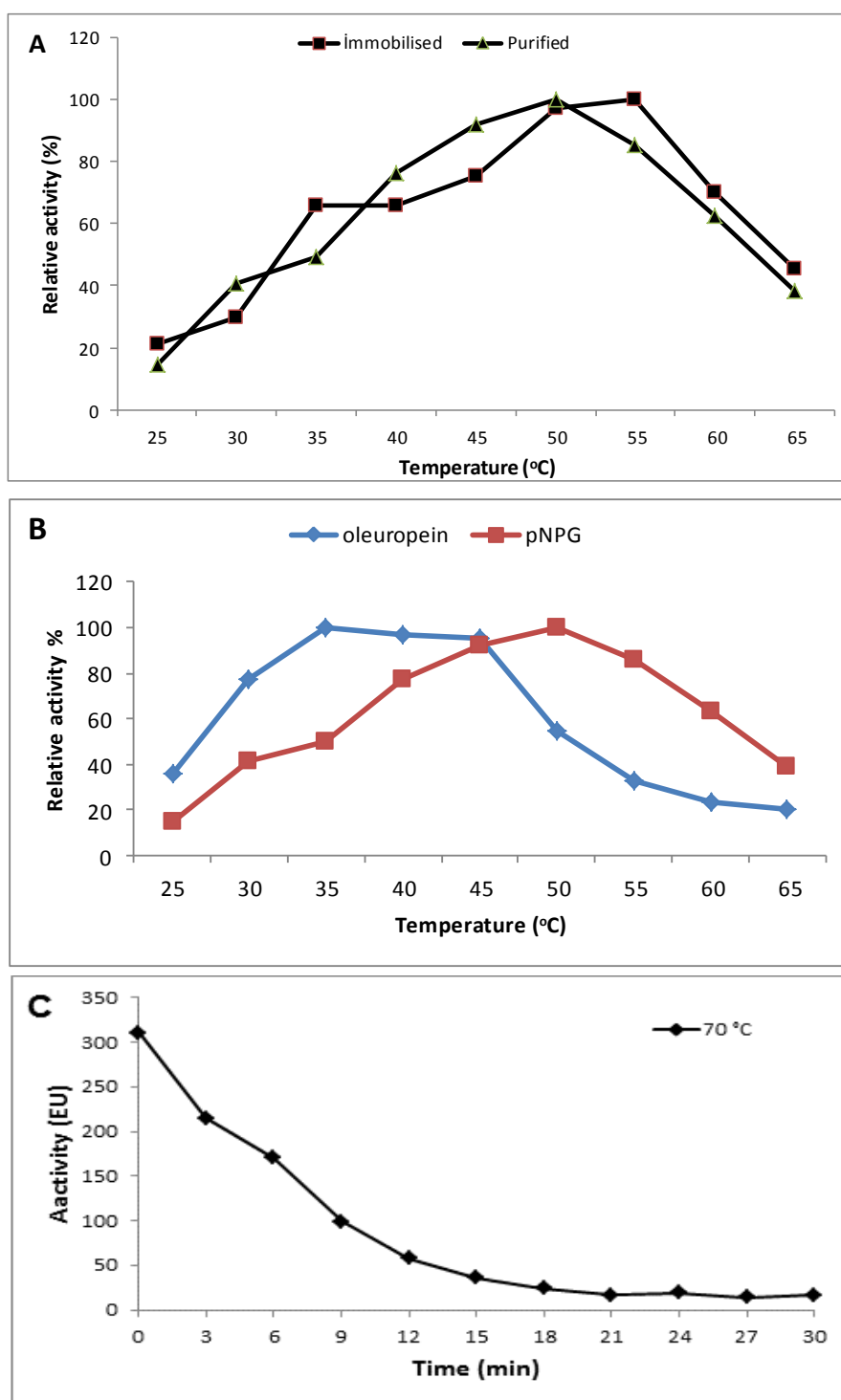


Figure 4. Effect of temperature on olive β -glucosidase activity. Temperature optima of A) β -glucosidase purified from olive (*Olea europaea* L.) and immobilised form using p-NPG as the substrate, and B) purified enzyme using different substrates. C) Thermal stability of purified olive β -glucosidase by using p-NPG as the substrate.

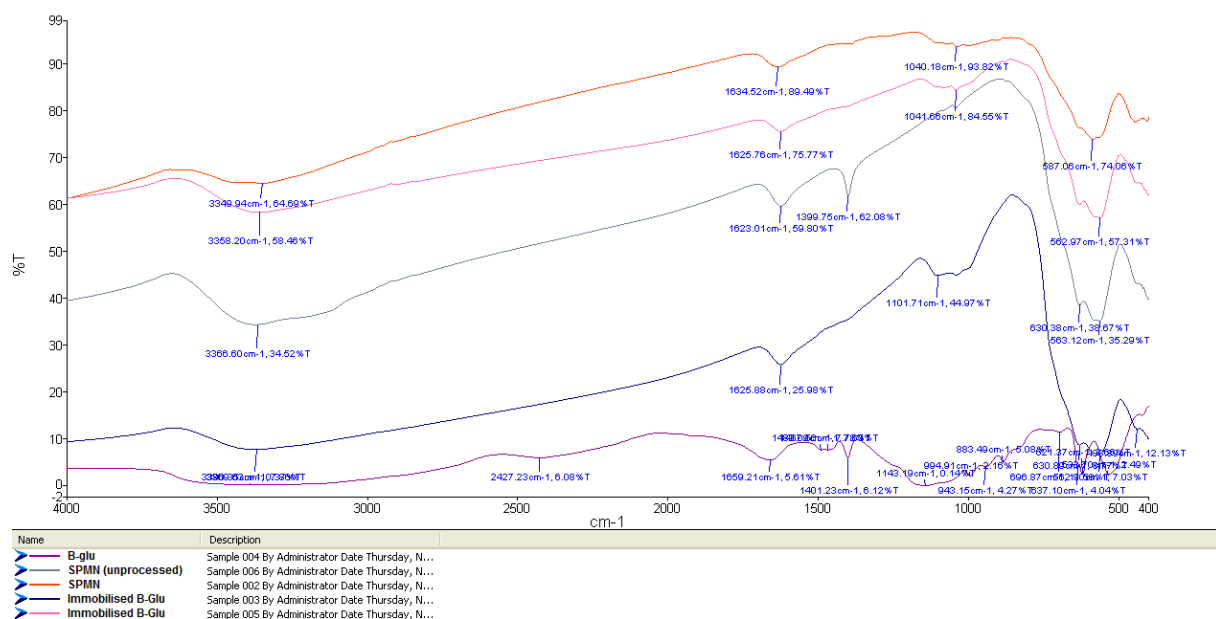


Figure 5. FT-IR spectra of immobilization support material, pure enzyme and enzyme bound nanoparticle.

3.4. Substrate specificity

Enzyme kinetics were studied using p-nitrophenyl- β -d-glucopyranosides (p-NPG) and oleuropein as substrates. The β -glucosidase from olive tissues activates oleuropein by converting the secoiridoid glucoside moiety of the oleuropein when the tissues are damaged by insects or herbivores (KONNO *et al.*, 1999). Olive fruit β -glucosidases that were able to hydrolyse olive glucosides exhibited high substrate specificity to oleuropein (ROMERO-SEGURA *et al.*, 2009). The enzyme was effectively active on p-NPG and oleuropein with the K_m values of 0.37 and 1.7 mM and the V_{max} values of 370.3 and 1000 U/mg, respectively (Table 2). Although the affinity of the olive β -glucosidase for p-NPG was considerably higher than for oleuropein, the activity was lower. The higher β -glucosidase affinity for p-NPG was reported on Sorghum (CICEK and ESEN, 1998), tea leaves (LI *et al.*, 2005), orange (CAMERON *et al.*, 2001) and olive (KARA *et al.*, 2011).

Table 2. Kinetic parameters of purified and immobilised β -glucosidase from olive with different substrates.

β -glucosidase	Substrates	$V_{max}(U)$	K_m	V_{max}/K_m
Purified	p-NPG	370.37	0.37	1001
	Oleuropein	1000	1.7	588.23
Immobilised	p-NPG	384.61	1.34	287.02
	Oleuropein	2000	6	333.3

3.5. Effects of inhibitors

β -glucosidases have been researched with reversible and irreversible inactivators (HE and WITHERS, 1997; REMPEL and WITHERS, 2008). The purified enzyme was incubated in the presence of glucose, lactic acid, sodium hydroxide, sodium chloride, citric acid, some pesticides and Mg^{2+} , Mn^{2+} , Ni^{1+} , Co^{1+} , Cr^{1+} , Zn^{1+} ions using p-NPG as the substrate (1.66 mM) to determine the inhibition kinetics. Although a strong inhibitory effect could not be detected at the studied concentrations, citric acid was more effective with IC_{50} of 2.38 mM than glucose with IC_{50} of 23.33 mM and lactic acid with IC_{50} of 145.7 mM (Fig. 6A-F).

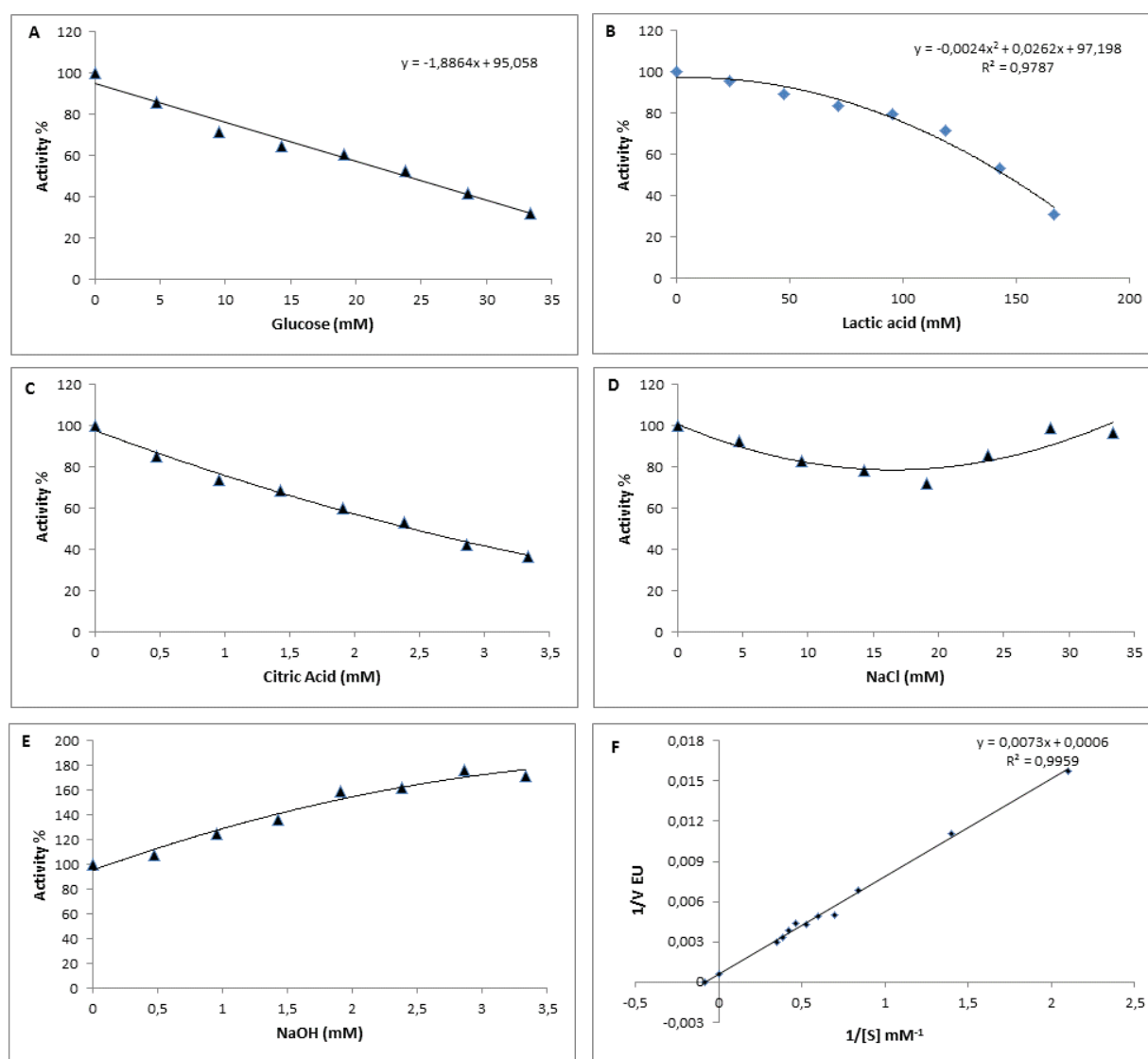


Figure 6. Inhibition of the β -glucosidase purified from olive (*Olea europaea* L.) fruit by p-NPG Activity (%) curve in the presence of different A) Glucose, B) Lactic acid, C) Citric acid, D) NaCl and E) NaOH concentrations, F) Lineweaver-Burk plot with various concentrations of p-NPG for IC_{50} .

It was reported that citric acid is the main organic acid followed by succinic acid in Turkish olive varieties (ASLAN and OZCAN, 2011). It is the most commonly used agent in

olive fermentation for initial acidity in the fermentation medium. It is contemplated that the concentration of the citric acid in the reaction medium will result in a lower pH level than the pH optima of the enzyme.

Glucose is the main compound for enzymatic saccharification of cellulolytic substrates and presents as fermentable sugar in the reaction medium. Similar inhibitory effects of glucose have been reported for β -glucosidase in olive in a previous study (KARA *et al.*, 2011; SAVAS *et al.*, 2018), whereas in another study, it was stated that no inhibitory effect of glucose was determined (ROMERO-SEGURA *et al.*, 2009). Differences in the sequence of amino acids found in the structure of plant β -glucosidases reflect observed quaternary structures and change in the properties of the active site (YU *et al.*, 2007). It is thought that, as a result of this, the kinetic parameters will be changed. These differences in olive β -glucosidases, whose molecular weights and other kinetic parameters are different from each other, are due to the differences in protein structures depending on the variety. It is necessary to study the kinetic parameters of olive β -glucosidases of different varieties and at different maturity levels for future studies.

Generally, plant origin β -glucosidases were reported to be resistant to high glucose concentrations (RAMANI *et al.*, 2012). Unlike other chemicals that are used, the observed slight activation by NaCl and NaOH may be explained by the ions' effect of Na⁺ on enzyme structure stabilization.

β -glucosidases were reported as metalloprotein and required metal ions for action (RAMANI *et al.*, 2012). The inhibition kinetics were determined in the presence of Mg²⁺, Mn²⁺, Ni²⁺, Co²⁺, Cr³⁺ and Zn²⁺ to verify the effects of several metal ions on olive β -glucosidase activity (Fig. 7).

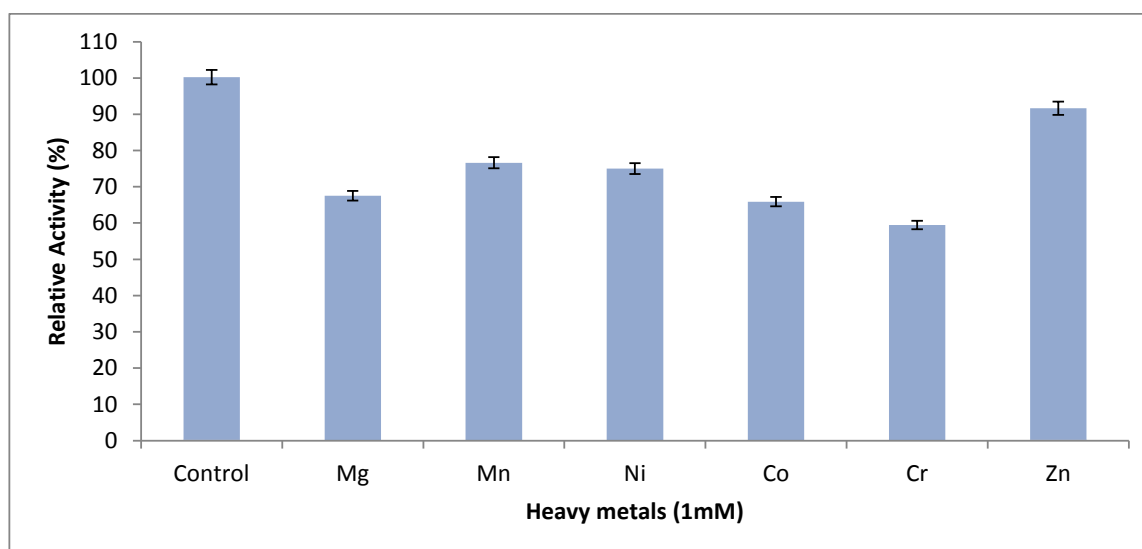


Figure 7. The effects of some heavy metal ions on the activity of β -glucosidase purified from *Olea europea L.* The crude enzyme activity was indicated as the control. The activity of the control was accepted as 100%. Trials were achieved by three replicates.

Metal ion concentration in this study was in the range from 1 to 1.75 mM. The relative enzyme activity was 67.36, 76.46, 74.85, 65.75, 59.33 and 91.4 presence of Mg²⁺, Mn²⁺, Ni²⁺, Co²⁺, Cr³⁺ and Zn²⁺ ions, respectively. The results showed that all metal ions, which were

used showed successful inhibitory effects on olive β -glucosidase. Especially Cr^{+} and Mg^{+} were more effective ions as the inhibitor onto olive β -glucosidase, while Ni^{+} was mentioned in the literature as an inhibitor of olive β glucosidase like Cd^{2+} , Pb^{2+} , Cu^{2+} and Ag^{+} . 1-10 mM of Mg^{2+} and Mn^{2+} were reported as strong activators for fungal β -glucosidases (MA *et al.*, 2011; CHEN *et al.*, 2012; BAI *et al.*, 2013) except for *Neocallimastix patriciarum* W5 (CHEN *et al.*, 2012). Our findings were mostly incompatible with those obtained by other studies. This is thought to be caused by the diversity of the enzyme sources. The metal ions that are mentioned above are found in the chemicals mostly used as antifungal agents.

The IC_{50} values of Deltamethrin, Chlorpyrifos and Alphacypermethrin as the most commonly used pesticides in olive farming were 0.0323, 1.25 and 13.29 mg/L respectively. Deltamethrin is used as insecticide in olive trees. As the reported optimum concentration that was used was 0.31 for olive tree, the obtained IC_{50} value was sufficient for inhibition of olive β -glucosidase.

4. CONCLUSIONS

The olive β -glucosidase was purified from Gemlik variety Turkish olives, and the free enzyme was immobilised onto $\text{Fe}^{2+/3+}$ Superparamagnetic nanoparticles to increase the stability and reusability for industrial applications. This is the main enzyme the is responsible for oleuropein hydrolysis during the maturation period. At the same time, there is also industrial use of it for aroma formation and debittering step in table olive production. In previous studies, basic characterisation of olive β -glucosidase was investigated. However, this is the first time where this enzyme was immobilised onto superparamagnetic nanoparticles and characterization of the immobilised enzyme was studied comparatively. In the study, the potential activator and inhibitor effects of the substances under the process conditions in table olive production were determined for the purpose of developing materials which can be used in the debittering process of table olive production. Because of the nanomaterial that was used is biocompatible and risk-free, it may be safely used in food production. After immobilization, the enzyme became more stable under environmental conditions.

ACKNOWLEDGEMENTS

This study was funded by the Research Fund of the University of Balikesir (BAP.3.2016.0001), and Turkish Research Council Project Grant no 110O778, Turkey and patented under number of PT2013-01335.

ABBREVIATIONS

kDa	kilodalton
HIC	Hydrophobic interaction chromatography
SPMN	Superparamagnetic nanoparticles
EU/mg	Enzyme unit/milligram
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
UV	Ultra Violet
IR	Infra-Red
TEMED	N,N,N',N',- Tetramethylenediamine

pNPG	p-nitrophenyl- α -D-galactopyranoside
oNPG	Ortho-Nitrophenyl-Galactopyranoside
pNPGal	p-Nitrophenyl α -D- Galactopyranoside
oNPGal	Orto-nitrophenyl- β -d-galactopyranoside
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiothreitol
BSA	bovine serum albumin
DMPD	N, N-dimethyl-p-phenylenediamine
DPPH	Di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium
H ₂ O ₂	Hydrogen Peroxide Hydrogen Peroxide
Tris	Tri hydroxymethyl)aminomethane
FTIR	Fourier transform infrared spectroscopy
KBr	Potassium bromide
PE	Purified enzyme
IE	Immobilised enzyme
[S]	Concentration of substrate
[K _m]	Michaelis- Menten Constant
[V _{max}]	Maximum speed
mM	Millimolar
μ M	Micromolar
mg	Milligram
g	Gram
kg	Kilogram

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Paper Received February 15, 2019 Accepted April 15, 2019

A CLUSTERED-BASED SEGMENTATION OF CHINESE WINE CONSUMERS BY MEANS OF KERNAL FUZZY C-MEANS

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ABSTRACT

The aim of this study is to segment Chinese wine consumers based on their preferences, motivations and purchasing behaviors. Data from representative 3420 responses were profiled through Kernel fuzzy c-means (KFCM), and one-way ANOVA analysis was used to define socio-demographic characteristics of each cluster. This study identified four consumer segments: Balanced Consumers, Credulous Consumers, Experiential Consumers and Health Sippers. Each group showed different demographics, eating and purchase habits. These findings reveal a typology of Chinese wine consumers when making the purchase decisions and verify the applicability of KFCM in consumer segmentation. Identification of Chinese consumer segments provides winemakers a better understanding of the various characteristics of wine consumers and their different purchasing behaviors. This study also provides new contributions to research on the segmentation of the Chinese wine market.

Keywords: segmentation, kernel fuzzy c-means, Chinese wine consumer

1. INTRODUCTION

With the increased household income and the growing middle class consumers, the level of wine consumption among Chinese consumers has significantly increased in recent years. During the past twenty years, China's wine consumption has risen from 10.7 million hl in 1999 to 17.9 million hl in 2017, with an annual growth rate of about 10.9% (OIV, 2018). With a population of 1.39 billion and a GDP of 82.7 trillion CNY in 2017 (NATIONAL BUREAU of STATISTICS, 2018), China provides a huge market for food and alcohol sales. The rapid growth of wine demand has not only promoted the development of local wine production, but also enabled a large number of foreign wine products enter to the Chinese market. For winemakers, China has become a very important target market. As reported by International Organization of Vine and Wine, wine consumption in China shows a positive growth trend. In 2017, wine consumption was 1.79 million kiloliters, accounting for about 7% of global consumption (OIV, 2018). In addition, according to a report by Vinexpo, China has become the fifth largest static wine market in the world (VINEXPO, 2018). In other words, wine has become one of the most popular products for Chinese consumers and is increasingly favored by the younger generation. For Chinese wine drinkers, although the per capita alcohol consumption will rise from 1.34 liters/year to 1.53 liters/year, there is still a big gap compared with traditional wine consuming countries – 28 times different from France (IWSR, 2018).

Despite the increase in the production of domestic wine and the growing demand for imported wines, the market potential of Chinese wine consumers is huge. However, the needs and preferences of consumers are heterogeneous which means simple types of wine can't meet the needs of differentiated markets. Because of the competitions in the Chinese wine businesses, it is important to segment the market to attract more potential consumers (ZENG, 2014). There are very few studies on the segmentation of Chinese wine market from the perspective of marketing (CAPITELLO *et al.*, 2015; CAPITELLO *et al.*, 2014; LEE *et al.*, 2015) although market segmentation is an important component and platform for corporate strategic marketing. Market segmentation can help companies accurately distinguish valuable targets and select target markets more effectively, find their own position in market competition, and design unique products to meet the needs of consumers in the market.

Hence, understanding the main drivers and potential motivations for different types of consumers is very important for winemakers. Market segmentation also provides a better framework to customize and distribute products to meet different needs of consumers. In addition, it can provide corresponding marketing strategies for marketers.

2. LITERATURE REVIEW

2.1. Understanding customers in the wine industry

The different attributes and functions of a product are key factors in determining whether a consumer will purchase it (GREEN *et al.*, 2004). Consumers purchase decision can be influenced by intrinsic cues and extrinsic cues as a criterion for judging quality in the process of purchasing products (GRUNERT *et al.*, 2004). Intrinsic cues are properties which are inherent to the products, such as ingredients and sensory attributes. Extrinsic cues mainly refer to physical attributes such as price, brand, packaging, and origin that are not part of the physical attributes AKDENIZ *et al.* (2013). In addition, the marketing

strategies of wine shops and the motivation of consumers to purchase are also important factors (ATKIN *et al.*, 2007; BRUWER *et al.*, 2012; BRUWER *et al.*, 2011).

Wine consumption is the specific behavior generated by consumers based on complex psychological activities, including psychological cognition and behavioral awareness (PETTIGREW and CHARTERS, 2010). The cognition includes a variety of psychological activities such as stimulation, impression, thinking and imagination. In general, it is the evaluation of the quality, function and image of a certain wine by the consumers. On the basis of cognition, consumers will have needs of wine, which will form the motivation for wine purchase. When the products meet requirements of consumers, the motivation for wine purchase will gradually be translated into specific wine purchase behavior.

Many researchers have carried out studies on wine consumption psychology (cognition, motivation, etc.) and behavior, providing an important theoretical reference for people to understand the behavior characteristics and behavior of wine consumers. Health benefits, auxiliary dining and interpersonal communication are the main motivations of Chinese wine consumers (SOMOGYI *et al.*, 2011). The positive health benefit of wine is that people drink wine in moderation can prevent disease and has anti-aging effect because wine is rich in vitamins and antioxidants (LEE, 2009). Due to the special color, aroma and taste of wine, it helps to improve the taste of the food when eating with wine (WANG, 2016). As a social tool, wine is widely used by Chinese consumers to express emotions or enhance friendship (HALL *et al.*, 2013).

In terms of Chinese wine consumer behavior, previous studies have shown that the attribute of product, individual characteristics of consumers and psychological factors of consumers will affect the behavior of Chinese wine consumers (PICKERING and HAYES, 2017). Various attributes, such as sensory attributes and extrinsic cues including price, brand, and origin are found to be important aspects that influence wine choice (LOCKSHIN *et al.*, 2016).

When consumers make purchase decisions, the sensory attributes of wine are arguably the most important factor in determining whether people drink or not (CHARTERS and PETTIGREW, 2007; KEOWN and CASEY, 1995; SARAR *et al.*, 2010; THOMPSON and VOURVACHIS, 1995). LIU and MURPHY (2007) have shown that the main reason why Chinese wine consumers drink red wines is color, because red symbolizes prosperity and good luck in Chinese culture. CAMILLO (2012) also confirmed that sensory attributes such as taste are the main factors affecting wine purchase and consumption.

Regarding the extrinsic cues of wine, a number of researchers have determined the attributes that have significant effect on wine consumption (XU *et al.*, 2014). They found that the most important factors influencing consumers' choice of wine are previous experiences, quality, brands and recommendations. ARETA *et al.* (2018) believe that the quality ratings provided by experts, the vintage and scale of the winery are important in the process of wine consumers' decision-making. In addition, JOVANOVIĆ *et al.* (2017) empirically demonstrate socio-demographic factors such as region, place of residence (urban and rural areas), family size, age, income and education; and behavioral-cognitive factors such as the importance of market price, place of purchase and product characteristics play a leading role in wine consumers' decision-making process.

Finally, the health function of wine is also an important factor affecting consumer purchases. Nowadays, people pay more attention to their health. The healthy function of wine has been gradually recognized and received more attention by Chinese consumers. SOMOGYI *et al.* (2011) found that the perceived health benefits of wine could influence the behavior of wine consumers. PETTIGREW and CHARTERS (2010) also verified that the health benefit is an important reason for wine consumption.

2.2. Market segmentation theory

Market segmentation is the process of differentiating consumer groups who have different needs in the market according to certain criteria. Market segmentation helps companies meet the needs of each segment more effectively, explore market opportunities, open up new markets, select the target market, and develop effective marketing strategies (MARSHALL and JOHNSTON, 2010). RAAIJ and VERHALLEN (1994) showed that the needs of different segments of consumers could offer new opportunity for enterprises.

Nowadays, researchers mainly segment the wine market from the aspects of personal profile (gender, age, etc.), geographical factors, psychological factors (preference, attitude, etc.) and behavioral factors, like purchase frequency, purchase time, etc. (JAIN, 2012). SPAWTON (1991) classified consumers into four categories: the wine connoisseur, the aspirational wine drinker, the beverage wine drinker and the new wine drinker based on the consumers' location, consumers' attitude towards the brands, and their knowledge and involvement of the wine. A study of Chinese wine consumers from a gender perspective was conducted by BRETHERTON and CARSWELL (2013) and the results showed that wine was considered more masculine rather than feminine. It had also verified the results of LIU and MURPHY (2007) and RITCHIE (2007) at the same time. OGBEIDE and BRUWER (2013) found that older consumers in western countries were more involved in wine related activities from the perspective of age, because they had more time and money to participate in the wine tasting and wine clubs. Regarding the places of purchase, HU *et al.* (2008) showed that 65% of Chinese consumers are accustomed to buying wines in supermarkets or restaurants, and the main motivation for drinking is celebration and accompanying meals. More specifically, SZOLNOKI (2018) used two-step clustering to classify tourists in Rheingau into four classes: Wine and Rheingau lovers, Wine-oriented tourists, First-time tourists and International tourists based on objective variables. MASSON *et al.* (2017) classified wine consumers into six groups from the perspective of drinking occasions. These six groups of consumers were: indifferent occasionals, wine lovers, relaxed amateurs, social networkers, stay-at-home connoisseurs and infrequent money-minded. MALONE and LUSK (2018) segmented consumers into five clusters: Traditional, Mavens, Uninformed, Locavores and Premium by k-means cluster analysis, which was based on consumers' taste perceptions of various brands.

Most business models are based on PCA, factor analysis and K-means method to segment consumers into several groups (DÍAZ *et al.*, 2018; MASSON *et al.*, 2017; YANG *et al.*, 2017). However, the initial point of K-means method is unstable and random, which causes the instability of clustering results and limits the quality of clustering (AKBULUT *et al.*, 2016; LU and YAN, 2015). With the continuous improvement of machine learning methods, cluster analysis based on K-means method are insufficient to meet the requirement of academics and practitioners when segmenting massive data.

With introducing fuzzy set theory, RUSPINI (1969) first adopted the idea of fuzzy in data clustering. Data clustering based on fuzzy set theory has become an important step in data mining and machine learning (AKBULUT *et al.*, 2016). In order to overcome several drawbacks of K-means, Kernel fuzzy c-means (KFCM) was proposed. Compared with the classical clustering algorithm (k-means and factor analysis), KFCM can better distinguish, extract and amplify useful features, so as to achieve more accurate clustering. At the same time, the convergence speed of the algorithm is faster. When the classical clustering algorithm fails, the kernel clustering algorithm can still get the correct clustering (YANG and TSAI, 2008; ZHANG and CHEN, 2003). The KFCM has been widely used in image

segmentation, fault diagnosis and customer segmentation (BASU and SRINIVAS, 2014; CAI *et al.*, 2009; YANG *et al.*, 2011).

The purpose of this paper is to use KFCM to segment Chinese wine consumers by considering the attitudes, purchase motives and purchasing behavior of Chinese wine consumers. The findings will make wine dealers and winemakers better understand the needs of consumers.

3. MATERIALS AND METHODS

3.1. Questionnaire

To achieve the aims of this study, a semi-structured questionnaire was developed and used to classify Chinese wine consumers. The questionnaire was based on geographic, demographic, socioeconomic, psychological and behavioral variables (MASSON and AURIER, 2017). It also includes perception of packaging and labeling, consumer motivation, knowledge and involvement and sensory attributes (BRUWER *et al.*, 2011) which impact on wine purchase (BARBER, 2012). The questionnaire is divided into three parts: consumer's knowledge and his consumption of wine; consumer preferences and motivations for purchasing wine and sociodemographic characteristics. A total of 21 items are divided into six variables: sensory attributes, business marketing, motivation of purchase, consumers' familiarity, product attributes, and where to buy. In this study, the Likert five-point scale method was used to measure the consumers' attitude. The measurement structure was "very disagree=1, very agree=5", which were all ordinal measurement, indicating the attitude of consumers to a certain point of view.

3.2. The sample

In order to improve the representativeness of the sample and the practical application value of the research conclusions, this study conducted a comprehensive sample survey in 24 provinces, municipalities and autonomous regions in the eastern, central and western regions of China from July to August 2018. The samples covers a wide range of regions, thus could represent the purchasing behavior of wine consumers in different regions of China. Before the official investigation, the researchers conducted a centralized and unified training for the investigators and explained the purpose of the survey, the content of the survey, the survey methods and process. The survey was conducted by using convenient sampling because surveys were conducted in shopping malls, streets, residential quarters, parks, farmer's markets, and village markets. The respondents independently filled out the questionnaire according to their actual situation, which would take 20-30 minutes. A total of 4000 questionnaires were collected, of which 2180 were online questionnaires the rest were face-to-face questionnaires. The questionnaires, which had too many missed items and incorrect key information, were removed and finally 3,420 valid questionnaires were actually collected.

The sample distribution of the survey was showed in Table 1 and the sample coverage is considered comprehensive and representative.

Table 1. Samples distribution of the survey.

Location	Number of respondents	Location	Number of respondents
Coastal area		Inland area	
Beijing	240	Anhui province	36
Hebei province	307	Gansu province	100
Shandong province	184	Liaoning province	147
Shanghai	173	Ningxia hui autonomous region	94
Jiangsu province	316	Shanxi province	267
Zhejiang province	198	Guangxi Zhuang Autonomous Region	41
Fujian province	44	Henan province	85
Guangdong province	253	Heilongjiang province	64
Tianjin	90	Hubei province	70
		Hunan province	144
		Jilin province	51
		Shaanxi province	66
		Sichuan province	195
		Yunnan province	147
		Chongqing	108
Total	1805	Total	1615

3.3. Method

3.3.1 Confirmatory factor analysis

Confirmatory factor analysis was used to determine whether items related to wine consumption in literatures could be profiled as general characteristics (HAIR *et al.*, 2011). For this purpose, confirmatory factor analysis was carried out for 21 selected items, and factors were extracted by maximum rotation of variables. Items, which factor loads, were less than 0.5 or items with two or more factor loads greater than 0.5 were excluded from the sampling scale. The results showed that the correlation matrix of the 17 item scales is appropriate (Bartlett's test of sphericity : $\chi^2 = 14653.65$; $df = 435$; $p < 0.000$; the KMO index was 0.861).

Then, the cronbach's α value was tested to measure the reliability of each factor indicating that the cronbach's α values of these factors were higher than 0.7, indicating that the internal consistency between the items was better (HAIR *et al.*, 2011). Finally, a six factor solution using varimax rotation procedure was proposed through SPSS. Confirmatory factor analysis showed that 17 variables accounted for 85.65% of the variance and the KMO index was 0.861 (Table 2).

3.3.2 Cluster analysis

Kernel fuzzy C-means (KFCM) was used to segment Chinese wine consumers based on their purchasing behavior and motivations. The reason for choosing KFCM is that the clustering result of KFCM is more accurate than the general cluster analysis (LU and YAN, 2015). We used Matlab 2014a software for the analysis.

Table 2. Factor loadings and reliability values.

Name	Items	Factor loading	Cronbach's α
Intrinsic cues (MADEIRA <i>et al.</i> , 2009)	IC1: I usually buy wines with famous brand.	0.792	0.929
	IC2: The higher the price, the better the quality of the wine.	0.824	
	IC3: The quality of wines in well-known regions is more reliable.	0.611	
	IC4: The better the packaging, the higher the grade of the wine.	0.792	
	IC5: Good vintage can produce good wines.	0.755	
Extrinsic cues (CHREA <i>et al.</i> , 2011; HALL, 2013)	EC1: The better the color, the better the wine quality.	0.699	0.845
	EC2: The richer the fragrance, the better the quality of the wine.	0.825	
	EC3: The better the taste, the better the quality of the wine.	0.853	
Marketing (MADEIRA <i>et al.</i> , 2009)	M1: Wine advertisement has a big impact on my purchase decision.	0.784	0.932
	M2: Promotion has a big impact on my purchase decision.	0.744	
	M3: The location of the store and its interior style impact my choices.	0.607	
	M4: I trust the recommendation of the sellers.	0.661	
Reference group (FERNANDES <i>et al.</i> , 2018)	RG1: I trust the recommendations of my friends and relatives.	0.755	0.895
	RG2: The more rewards offered by the seller, the better the quality of the wine.	0.899	
Motive (CHREA <i>et al.</i> , 2011; HALL, 2013)	MO1: The health benefits of wine affects my choice.	0.678	0.850
Knowledge and involvement (BRUCKS, 1985)	KI1: The knowledge of wine will influence my choice.	0.711	0.769
	KI2: I have very good knowledge about wine.	0.697	

The principle of KFCM algorithm is to map the points of the original space to the feature space by using the kernel function, then directly or indirectly perform algorithm design, analysis and calculation in the feature space, so as to obtain the clustering of the original space. By mapping the kernel function, it can better distinguish, extract and amplify the features that did not appear before, which makes the clustering results more accurate, and also makes the convergence speed of the algorithm improved.

For traditional fuzzy clustering analysis, fuzzy C-means clustering is the most widely used method. However, the number of clusters in this type of algorithm is unknown in advance and requires artificially determined determination. In order to better determine the number of clusters in the real structure of the data set and improve the accuracy of cluster analysis, this paper based on the cluster validity function proposed by BEZDEK *et al.* (1984) to determine the optimal number of clusters in the data sample set. Among them,

the number of clusters that maximize the value of Cluster Validity Index is the optimal number of clusters.

3.3.3 One-way ANOVA analysis

Descriptive statistical analysis was used to compare the socioeconomic characteristics of each group and wine purchasing and eating habits. In addition, one-way ANOVA was used to compare the mean differences based on age, education and monthly income among the identified groups (LÓPEZ-ROSAS and ESPINOZA-ORTEGA, 2018; SKURAS and VAKROU, 2002). Before testing demographic variables and consumption patterns among groups of consumers, homogeneity test of the variance of the population was conducted. The study found that the variances of sociodemographic backgrounds, purchasing and eating habits are the same among different groups of consumers, then one-way ANOVA can be used to analyze these data. The overall One-way ANOVA analysis using F-test were conducted, and when the Sig is less than 0.05, it can be considered that there are differences among groups. The results were shown in Table 4.

4. RESULTS AND DISCUSSION

4.1. Cluster analysis

BLANCHARD *et al.* (2009) argued that the desires and willingness of the consumer can explain consumer's behavior well and can be used to predict consumers' actual consumption behavior of products. Therefore, it is especially important to understand the factors related to consumer behavior.

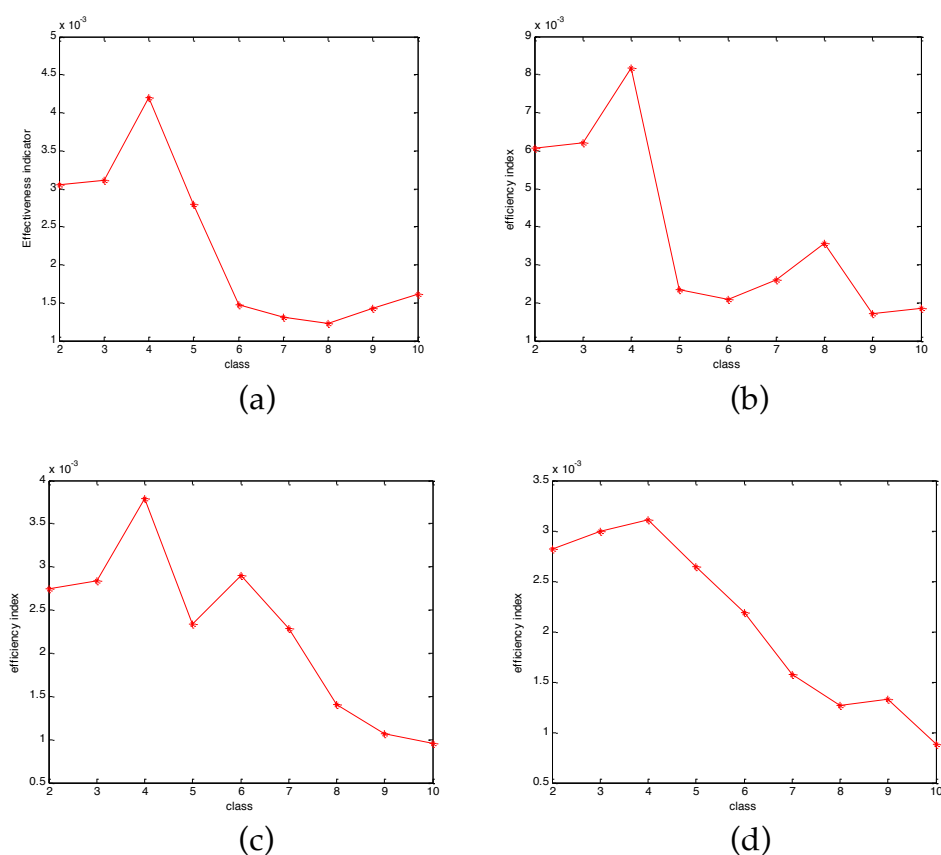
In order to better determine the number of clusters that can reflect the real structure of the data set, it is necessary to calculate the clustering validity function and determine the optimal number of clusters by comparing the index values. The calculation results are shown in Figure 1. According to the definition of clustering validity function, the clustering number corresponding to the maximum value is the optimal clustering number of data sets. Fig. 1 shows that when the fuzzy weighted index is 1.5, 2, 2.5 and 3, the clustering validity index is the largest when class is 4, so the optimal clustering number is 4.

The results of the KFCM revealed four distinct consumer groups that can be named according to the segmenting variables of the consumers (Table 3). The scores of the attributes are shown in Fig. 2: Balanced Consumers, Credulous Consumers, Experiential Consumers and Health Sippers.

Health Sippers: This is the largest group of the four consumer groups, with 39.4% of respondents belonging to this group. What makes it different from other groups is that the main motivation for this group to drink wine is for health. They think that drinking wine is good for health and they are more likely to have a high level of health awareness than other groups. It is worth noting that this group scores the highest on knowledge and involvement, which indicates that they are confident in their knowledge of wine and were care about their health.

Table 3. Categories of wine consumers according to the segmenting variables

Factor	Balanced Consumers (n=1324)	Credulous Consumers (n=237)	Experiential Consumers (n=515)	Health Sippers (n=1347)
Reference group	3.7	3.8	1.9	3.1
Marketing	3.2	4.2	2.1	2.9
Intrinsic cues	3.8	2.9	4.0	3.3
Knowledge and involvement	3.3	3.1	2.8	3.4
Extrinsic cues	3.5	3.2	3.1	2.8
Purchase motive	3.4	3.3	2.1	3.9

**Figure 1.** Diagram of KFCM Validity Function's Change to Data: (a) fuzzy weighted index is 1.5, (b) fuzzy weighted index is 2, (c) fuzzy weighted index is 2.5, (d) fuzzy weighted index is 3.

Balanced Consumers: This group consists of 38.7% of the total respondents. They are "balanced" because consumers in this group consider all aspects of the product in the purchase process, such as the intrinsic and extrinsic cues of wine, the recommendation of family and friends, and the health benefits. The preference scores for marketing strategies such as advertising and shopping guide recommendation are lower compared to the other three groups suggesting that they are more rational. In other words, this group of consumers will evaluate wines by considering the balance of multiple factors during the

purchase process. They don't need to have the highest level of quality for all segmenting variables.

Experiential Consumers: This group of consumers consists of 15.1% of the samples. They show significantly preference for the sensory attributes of wine such as color, taste and aroma. These attributes are usually not confirmed until the product is purchased or used, so consumers can only determine these attributes through "experience." Hence, these consumers may not be influenced by their friends' recommendations and the marketing strategies. For this group, only the score of intrinsic cues are high because they just trust their own experiences. They don't drink wines for its health benefit and they don't know much about the knowledge of wine.

Credulous Consumers: These consumers accounts of 6.9% of the respondents. They are more susceptible to extrinsic cues than other groups of consumers, such as corporate promotions, friends' recommendations, etc. They are more likely to be influenced by others during the purchase process and have less knowledge about the wine. They don't care about the quality and health benefits of the wine itself, but the shopping environment and experience can affect their trust and their purchase decision.

These results are similar to other researchers on wine consumers. For example, MASSON *et al.* (2017) identified six groups of wine consumers using the k-means method. Among them, wine lovers have high product involvement in wine. Similar to Balanced Consumers in this study, when choosing wine, they will mainly consider the recommendation of the independent wine experts. Like Experiential Consumers in this study, relaxed amateurs rely on the taste of wines rather than other variables. They don't have much knowledge of wine and only buy what they like. Finally, consumers belong to the social network cluster have limited knowledge of wine. They buy wines based on recommendations from others like Credulous Consumers in this study and are not interested in the intrinsic and extrinsic cues of wines.

LÓPEZ-ROSAS and ESPINOZA-ORTEGA (2018) also found that there are four groups of wine consumers in Mexico: traditional consumers, consumers in transition, social consumers and consumers linked to the territory. The first group of consumers is similar to Balanced Consumers because they buy wine based on factors such as origin, brand, awards and quality of wines. They are more rational when make purchase decisions. The second group of consumers is similar to the Experiential Consumers; they pay more attention to the sensory attributes of wines. Then, the third group of consumers are not aware of the attributes of the wine and do not pay attention to the product itself. They often do impulse shopping, just like Credulous Consumers.

For Health Sippers, ANNUNZIATA *et al.* (2016) found that Italian consumers who focus on the health warning had a better understanding of the nutritional properties of wines and like wins with detailed nutritional information. These consumers valued wines with health warning followed by nutritional information, when they chose wines. SAMOGGIA (2016) studied the effects of wine consumption on health; they found that health-oriented consumers were willing to spend more money on wines, which could improve their health. They are more concerned about the health benefits of the wines. In addition, YOO *et al.* (2013) have shown that consumers who are more knowledgeable about the health benefit of wines would be willing to spend more money on health-oriented wines. Therefore, winemakers and wine marketers should consider health benefits of wines in order to increase the consumers' desire to buy, especially when developing new wine markets.

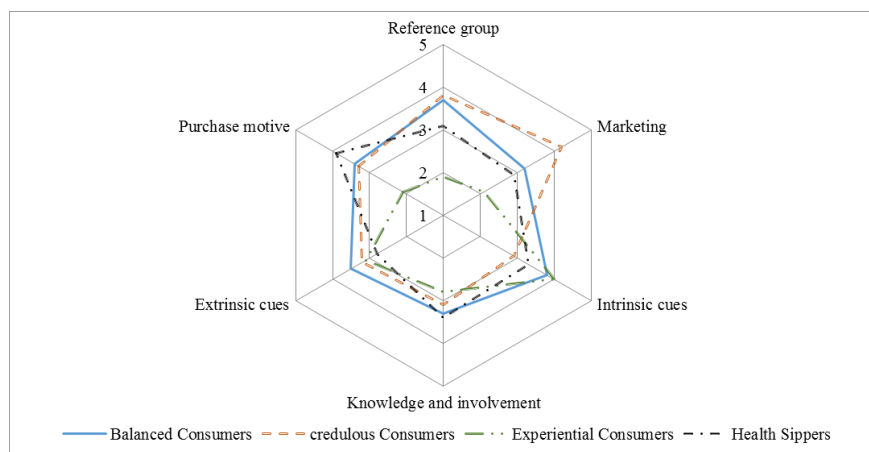


Figure 2. Scores of the groups according to the segmenting variables.

4.2. Socio-demographic backgrounds, purchasing and eating Habits of the groups

The analysis of Table 4 shows that the proportion of males and females of Chinese wine consumers is relatively balanced. The proportion of female consumers is slightly higher than that of men which is in line with MASSON's (2017) study of Chinese consumers.

OGBEIDE and BRUWER (2013) pointed out that the age of consumers is related to the level of wine drinking. The results of one-way ANOVA show the age differences among the four groups of consumers. More than half of all respondents are 26 to 45 years old, similar to the findings of China Wine Barometer – Wave 6 (2016). In addition, more than 24% of consumers belonged to Balanced Consumers. The Credulous Consumers are more than 45 years old. Thus it can be seen that effective marketing can influence purchase decisions of the middle-aged and older consumers.

MAGRI *et al.* (2007) indicated that the main factors affecting consumers' alcohol consumption are consumers' characteristics and the hedonic motivation. However, other motivations, such as health benefits and appropriate labeling content are also important. The results also show that the Health sippers have a higher degree of education. In this study, Health Sippers have the highest percentage of consumers with college and higher education qualification, which is directly related to their knowledge and involvement score.

SANTOS *et al.* (2006) argued that the difference between wine consumer groups is caused by their level of involvement and knowledge of the product. Therefore, the occupation of consumers is also a key factor influencing their purchase. Regarding the occupation of consumers, all four groups of consumers have steady occupations, although there is a considerable proportion of students and retired people. It is worth mentioning that the ratio of retired and independents in Experiential Consumers is higher than in other groups. In this group, wine consumption is related to intrinsic cues, which may be related to their social backgrounds and their belief that drinking wines can to help them to release stress and be more relaxed.

Table 4. Socio-demographic backgrounds, purchasing and eating habits by cluster.

Variable	Sample (%)	Experiential Consumers (15.1%)	Balanced Consumers (38.7%)	Credulous Consumers (6.9%)	Health Sippers (39.4%)	Homogeneity test	F-test	Sig.
Gender								
Male	48.5	49.5	46.9	47.0	49.5	0.61	0.983	0.400
Female	51.5	50.5	53.1	53.0	50.5			
Age								
Below25	13.6	18.6	14.8	15.0	16.2	0.79	27.309	0.000
26-35	32.8	44.5	25.4	32.0	36.5			
36-45	28.2	23.2	31.8	28.2	26.5			
46 or above	25.4	13.7	28.0	24.7	20.8			
Education level								
Secondary/high school or blow	28.7	35.0	18.0	49.0	34.9	0.52	31.297	0.000
College/university	60.4	49.9	70.8	40.4	55.9			
Graduate level or higher	10.9	15.1	11.2	10.6	9.1			
Occupation								
Student	13.1	9.7	16.1	14.4	11.2	0.73	1.192	.311
Independent	16.4	16.3	12.8	15.7	20.2			
Retired	18.9	23.5	18.5	17.4	17.8			
Employed	51.6	50.5	52.6	52.5	50.8			
Per capita monthly income								
below 3000 CNY	12.7	8.3	15.7	13.1	11.3	0.45	17.247	0.000
3001-5000 CNY	13.9	13.6	11.0	26.9	16.3			
5001-7000 CNY	30.1	29.3	23.2	24.7	36.3			
7001-10000 CNY	21.3	23.9	20.9	21.6	20.6			
Above 10000 CNY	22.1	24.9	29.3	13.6	15.5			
Consume bottles of wine in a year? (750ml)								
1-2 bottles	25.2	19.2	24.9	28.0	27.3	0.65	3.043	0.028
3-5 bottles	23.0	24.7	22.5	23.3	22.8			
6-8 bottles	20.8	24.1	20.3	15.3	21.0			
9-11 bottles	12.7	13.6	12.5	13.6	12.4			
12-15 bottles	7.9	5.4	9.6	7.6	7.1			
16-20 bottles	4.6	5.8	3.9	7.2	4.5			
Above 20 bottles	5.8	7.2	6.2	5.1	4.9			
Price of wine (750ml)								
Below 50 CNY	1.0	5.1	8.3	5.4	4.9	0.76	45.317	0.000
51-100 CNY	13.2	20.1	23.2	27.0	21.7			
101-150 CNY	29.1	25.7	33.1	33.2	30.0			
151-200 CNY	23.5	24.7	22.9	18.6	22.0			
201-300 CNY	16.7	14.7	5.1	9.2	12.2			
301-500 CNY	9.5	7.8	4.7	4.0	6.3			
Above 500 CNY	7.0	1.9	1.7	2.5	2.9			
Preferred origin of wine								
Domestic	51.9	39.6	50.6	52.1	57.9	0.82	17.357	0.000
Imported	48.1	60.4	49.4	47.9	42.1			
Dietary preference								
Meat lovers	26.2	23.1	27.6	28.4	25.6	0.25	3.386	.017
Vegetarian lovers	21.4	21.0	16.9	30.5	24.3			
Plasma balance	52.5	55.9	55.5	41.1	50.1			
Preferred type of wine								
Red wine	65.8	60.8	69.9	44.1	67.4	0.47	3.197	.023
White wine	23.9	24.7	20.3	41.5	24.1			
Rose Wine	10.4	14.6	9.8	14.4	8.5			

In terms of monthly income of consumers and the time and price of wine purchased, it can be seen that the proportion of consumers with monthly income of 5001-7000 CNY in all groups is quite large. Consumers with this range of income prefer to buy wines with prices between 101 and 150 CNY per bottle. This finding is similar to that of MU *et al.* (2017). Balanced Consumers group has the largest proportion of high-income respondents. Credulous Consumers accounted for the majority of respondents with a monthly income of less than 5,000 CNY. This income group's purchase price was less than 100 CNY per bottle. The proportion of this group was much higher than the other three groups. The result analysis suggests that these consumers may be more inclined to promotional information when buying wine because they do not know much about wines and have no interest in the product itself.

As for eating habits and frequency of drinking, there is a significant difference in the frequency of consumption by consumers in different groups. Credulous Consumers drink more frequently than Balanced Consumers, Experiential Consumers and Health Sippers. A large percentage of Credulous Consumers consume one bottle of wine per month (750ml). The most frequent drinkers are Credulous Consumers, followed by Balanced Consumers, Experiential Consumers, and finally Health Sippers. In terms of eating habits, Most of sampled respondent are consumers with balanced diet. In particular, the proportion of consumers who prefer vegetarian food in Credulous Consumers is significantly higher than those in other three groups.

Finally, in terms of the types of wines, the findings show that more than 50% of the respondents prefer domestic red wines. Interestingly, more than 60% of Experiential Consumers prefer imported wines, which may be related to the variety and the richer taste of imported wines. In addition, Credulous Consumers believe that there is no big difference between drinking red wine and white wine. This, on the other hand, proves that such consumers are relatively ignorant, easy to be affected by extrinsic factors and unable to make their own judgments.

From the results of the one-way ANOVA, it can be seen that at the level of 0.05, the four groups of consumers have significant differences in terms of age, monthly income, education level, favorite origin of wines and price of wine purchased regularly.

5. CONCLUSION AND IMPLICATIONS

In the past few decades, the global alcohol market has undergone tremendous changes, and wine is no exception. As a result, winemakers have realized that they need to understand their consumers and markets better. In this research, KFCM was used to study the key factors affecting the purchase behavior of Chinese wine consumers. The results show that there are four different groups of wine consumers: Experiential Consumers, Balanced Consumers, Credulous Consumers and Health Sippers. This market-oriented study provides detailed and practical marketing suggestions for each of the four identified groups.

The outcome of this study shows that consumers between the ages of 26 and 45 are the main consumer groups of wine and red wine remains a popular choice of wines. Future researches can measure consumer health awareness trends. Similarly, future research should explore how extrinsic attributes (such as nutrition labels, packaging styles) interact with other identified attributes that affect the consumer wine preferences.

It should be noted that although this study provides useful findings for consumer segmentation in the Chinese wine market, it has certain limitations. For example, the

study is based on the participant's self-description. Future research should supplement provide additional information by observing the actual purchase behavior of the consumers, such as measuring the real amount of purchased wine in the store, and the consumers who go out to eat together with wines. In addition, since China is a very large country, the regional dietetic culture is quite different. It is necessary to increase the number of consumers in different representative regions in order to improve the representativeness of research in the future.

First, for Health sippers, wine makers can add detailed nutrition and health information on the labels attached to bottles to improve the possibility of Chinese wine consumers' purchase. In addition, manufactures can increase the promotion of wine through social media and increase the awareness of wine and its health benefits. This will increase the popularity of wines.

Second, for Experiential Consumers, according to a study by CAPITELLO *et al.* (2015), Chinese consumers tend to try different flavors of wines, therefore the wine makers should conduct more effective market research to ensure the taste and quality of the products and to meet the current changing marketing needs. On the other hand, regular wine tastings and increased communication and understanding of consumers are also effective measures to expand the market and gain more market share. They can also develop and design wines with their own characteristics, create core products and customize different marketing combinations for different products. Moreover, through in-depth understanding of the characteristics and functions of wines, dealers can establish a multi-category bundle of product combinations. Wine stores must have wines of various countries and varieties. Only when consumers have more choices can they be more interested in wines and their consumption.

Third, Balanced Consumers are rational consumers who have certain knowledge of wine and are not affected by the external influence easily. They like wines and have a relatively high income. Therefore, wine makers should provide relevant events to attract these consumers and develop wine tourism projects to increase wine consumption experience to meet the experience needs of wine consumers. By means of drinking cultural propaganda, manufacturers can hold wine related exhibitions of the brand in large and medium-sized cities in China, and cooperate with other media channels such as TV, website, magazines, newspapers and so on to popularize wine drinking experience and culture. Through holding different kinds of wine festivals to establish the image of wine brand and promote the customers' loyalty to increase sales. In terms of publicity and marketing channels, wine producers should also make full use of online sales channels and publicity. Under the traditional mode, television, Newspapers and magazines are the core channels of advertising, but under the influence of the Internet, these traditional media are beginning to be marginalized. Social media has gradually become the main channel for people to communicate and communicate. Mobile media has begun to occupy people's lives and become the most influential media channel.

Finally, in order to cater for the taste of Credulous Consumers, wine makers should highlight the characteristics of the products, because these consumers have little knowledge of the products. Considering that their monthly income is relatively low, low-cost and occasional products discount can be effective for such consumers. In addition, salesmen can be trained to stimulate consumers' interest in products and desire to consume by using different promotion methods, such as free trial drinks, giving gifts and interacting with customers, according to the needs of different sales targets. Lastly, advertising through television, radio and social media to increase product awareness is also an effective way of publicity for wine makers and manufacturers (GENDALL, 2003).

APPENDIX - QUESTIONNAIRE ITEMS

Name	Items
Intrinsic cues (MADEIRA <i>et al.</i> , 2009)	IC1: I usually buy wines with famous brand. IC2: The higher the price, the better the quality of the wine. IC3: The quality of wines in well-known regions is more reliable. IC4: The better the packaging, the higher the grade of the wine. IC5: Good vintage can produce good wines.
Extrinsic cues (CHREA <i>et al.</i> , 2011; HALL, 2013)	EC1: The better the color, the better the wine quality. EC2: The richer the fragrance, the better the quality of the wine. EC3: The better the taste, the better the quality of the wine.
Marketing (MADEIRA <i>et al.</i> , 2009)	M1: Wine advertisement has a big impact on my purchase decision. M2: Promotion has a big impact on my purchase decision. M3: The location of the store and its interior style impact my choices. M4: I trust the recommendation of the sellers.
Reference group (FERNANDES <i>et al.</i> , 2018)	RG1: I trust the recommendations of my friends and relatives. RG2: The more rewards offered by the seller, the better the quality of the wine.
Motive (CHREA <i>et al.</i> , 2011; HALL, 2013)	MO1: The health benefits of wine affects my choice. MO2: The auxiliary purpose of wine affects my choice. MO3: The social purpose of wine affects my choice
Knowledge and involvement (BRUCKS, 1985)	KI1: My knowledge of wine influences my choice. KI2: I have very good knowledge about wine. KI3: I'm very interested in wine. KI4: I select the wines I purchase very carefully

ACKNOWLEDGEMENTS

This research is supported by Chinese Agricultural Research System (CARS-29) and Beijing Food Safety Policy & Strategy Research Base.

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Paper Received February 4, 2019 Accepted March 15, 2019

EFFECTS OF LACTULOSE LEVELS ON YOGHURT PROPERTIES

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ABSTRACT

Therapeutic levels of lactulose were used with commercial starters (Yoflex 801, Yoflex 901 and Yomix 486) in yoghurt. In fact, Yoflex 801 was supplemented with 1.5% lactulose resulting in minor yoghurt quality alterations. This co-culture was retained to study the influence of lactulose levels (0, 4, 6, and 8 %) on yoghurt quality for 28 days at 4°C. Kinetic parameters, syneresis, proteolysis degree, and sensory characteristics were improved by increasing lactulose dose; thus, thixotropic and pseudoplastic gel was shown. Accordingly, functional yoghurt fermented with Yoflex 801, containing 4 to 6 % of lactulose, proved to be the most adequate choice.

Keywords: dose, lactulose, prebiotic, starter, yoghurt

1. INTRODUCTION

Yoghurt is one of the most popular fermented dairy products, widely consumed all over the world, owing to its nutritional and sensory characteristics solicited by consumers (Loveday *et al.*, 2013). It is produced by lactic fermentation of two specific strains: *Streptococcus thermophilus* and *Lactobacillus delbruekii subsp. bulgaricus* (CODEX STAN 243-2003). Yoghurt has nutritional and health benefits, such as improving digestibility and lactose utilization. It promotes gut health and has a hypocholesterolemic action (ADOLFSSON *et al.*, 2004; WEERATHILAKE *et al.*, 2014). Bioactive compounds such as probiotics and prebiotics are usually added in yoghurts to enhance its functionality, quality and therapeutic properties (ÖZER *et al.*, 2005; CRUZ *et al.*, 2013a).

PREBIOTICS ARE SUBSTRATES THAT ARE SELECTIVELY UTILIZED BY HOST MICROORGANISMS CONFERRING A HEALTH BENEFIT (GIBSON ET AL., 2017). Prebiotics cannot be digested by the enzymes of the human gastrointestinal tract, however, they are fermented in the large intestine by colonic microflora, producing lactic acid, short chain fatty acids (acetic, propionic and butyric) and gases (GARCIA *et al.*, 2008; NICHOLSON *et al.*, 2012). Therefore, intestinal pH is reduced and harmful and pathogenic microorganisms proliferation are inhibited (ROLIM, 2015; WANG, 2009). Also, prebiotics prevent diarrhea and other diseases like colon cancer (MANN *et al.*, 2007). Besides, they act in the absorption of calcium and establish favorable mechanisms to immunomodulation as well as beneficial effects on lipid metabolism and various cardiovascular risk factors (DELGADO *et al.*, 2011).

Prebiotics including lactulose, inulin and oligofructose are considered as bifidogenic factors (ROBERFROID, 2000; RAFTER *et al.*, 2007). Thus, they are used in the formulation of dairy products, such as fermented milk (ÖZER *et al.*, 2005), Italian cheese (FERRÃO *et al.*, 2016; BELSITO *et al.*, 2017; FERRÃO *et al.*, 2018), whey beverage (GUIMARAES *et al.*, 2018) and ice cream (BALTHAZAR *et al.*, 2017) in order to add a functional value to these products and improve their technological characteristics.

Lactulose is a prebiotic (FRIC, 2007) used as a drug to treat illnesses, particularly chronic constipation (AIDER and DE HALLEUX, 2007; LEE-ROBICHAUD *et al.*, 2010). Moreover, it stimulates the growth of bifidobacteria (PHARM and SHAH, 2008; OLANO and CORZO, 2009). In this regard, lactulose effects are dose dependent (BOTHE *et al.*, 2017), for instance, 2 g of administrated lactulose would increase the short-chain fatty acid levels of the intestinal content (MIZOTA *et al.*, 2002). Besides, bifidogenic effects of lactulose are acquired when 5 g of lactulose are consumed every day. Therefore, when bacterial counts of *Bifidobacterium*, *Lactobacillus* and *Anaerostipes* increase, subsequently, acetate, butyrate and lactate increase with a decrease of branched-chain fatty acids. Likewise, 7.5 g dose of lactulose, daily, allows decreasing ammonia levels (AGUIRRE *et al.*, 2014).

Accordingly, lactulose appears as an important food ingredient that might be further explored for the production of new functional foods, and thus its future large scale production for food and nutraceutical purposes is anticipated.

For the best of our knowledge, there are few researches about lactulose effects on technological properties of yoghurt starters as well as on yoghurt characteristics.

In this connection, with the present study we intend to formulate new functional yoghurt and explore the possible application of lactulose as a prebiotic agent in this product when varying his concentration.

Then, the first aim of this study is to chiefly evaluate the effect of lactulose on the acidification kinetics and post-acidification, syneresis, proteolysis degree and growth of three different commercial yoghurt starters during storage, in order to select yoghurt

cultures, possessing a low affinity to lactulose, and thus yielding functional yoghurt similar to the conventional one, as requested by consumers. The second aim of this work is to evaluate the effect of the incorporation of lactulose at different doses on the quality of new developed yoghurt inoculated with the selected starter. The lactulose dose effect was determined on biochemical, microbiological, rheological, and sensory yoghurt properties when compared with control that lead to choose the most adequate concentration having the least effects during refrigerated storage.

2. MATERIALS AND METHODS

2.1. Yoghurt manufacture and study design

For yoghurt production, 5% of skim milk powder was added to skimmed milk (not fat solid =10%). Thus, enriched milk was homogenized and heated to 95°C for 3 min. The pasteurized milk was then rapidly cooled down to 43 +/- 1°C and divided into six batches. Three control batches were inoculated with three combinations of frozen starters composed of two strains, *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*, at a concentration of 20 mL/L, which corresponds to an initial count of about 8 log cfu/mL. These technological starters named, respectively, Yoflex 901, Yoflex 801 and Yomix 486 were purchased from Chr. Hansen's Dairy Cultures (Hoersholm, Denmark). They are the most commonly used ones by dairy industries in Tunisia in terms of yoghurt manufacture. Thereafter, 1.5% of lactulose was added to each sample of the remaining batches (Chimica Mugello society, Italy), before inoculation with one of the three co-cultures. Subsequently, milk was distributed in sterilized sealed containers, incubated at 43°C until pH reached 4.6 and acidity reached 75 °D, and then cooled and stored at 4 °C. Finally, Dornic acidity, total solids, proteolysis degree, syneresis and lactic acid bacteria counts were determined after 24 h of production each week, during 4 weeks of refrigeration storage.

After highlighting the best co-culture for yoghurt with lactulose, in the first part of this study, we focused, in the second part, on the effect of lactulose dose. Accordingly, for yoghurt preparation, the same steps were applied as described above. In fact, four batches were prepared and supplemented with 0, 4, 6 and 8% of lactulose. Each sample was then inoculated with the selected starter. All previous analyses and viscosity measurements were performed on the obtained yoghurt samples. Sensory evaluation was also carried out at days 1, 14 and 28, during refrigeration storage.

2.2. Total solids, pH and Dornic acidity

Total solids, pH values and Dornic acidity (expressed as degree Dornic) were measured according to AOAC (1995) and AFNOR (1980), respectively. Kinetic parameters were also considered in this study: (i) the maximum acidification rate (V_{max}), expressed in 10⁻³pH units/ min, (ii) the time to reach the maximum acidification rate (T_{vmax}), and (iii) the time to complete the fermentation ($T_{pH4.6}$), expressed in hours.

2.3. Syneresis

The gel was stirred at 4°C for 60 s and centrifuged for 20 min at 12,075 g in an ultracentrifuge (Beckman USA) (RINALDONI *et al.*, 2009). Syneresis (%) was calculated as mass of the separated serum from the gel after centrifugation, relating to the total mass of gel that was centrifuged.

2.4. Bacterial enumerations

The enumeration of lactic acid bacteria was performed by using De Man Rogosa and Sharpe agar (pH=6.2±0.2; Oxoid, France) after the incubation of plates at 45°C for 48h (GUIRAUD, 1998).

2.5. Evaluation of the proteolysis

For proteolysis determination, the fractional precipitation method, described by HASSOUNA *et al.* (1999), was used. Total nitrogen (NT) and soluble nitrogen at pH 4.6 (NS) were assayed after mineralization of organic nitrogen followed by distillation according to the kjeldahl reference method (AOAC, 1990). The protein content of yoghurt samples was calculated as follows: TN (g of nitrogen/100 mL of yoghurt) x 6.38 (Duncan *et al.*, 2008). Proteolysis degree = $100 \times [\text{NS (g of nitrogen/100 mL of yoghurt)} / \text{TN (g of nitrogen/100 mL of yoghurt)}]$ as described by BOULARES *et al.* (2011).

2.6. Rheological measurements

The rheological properties were determined according to the method described by NGUYEN *et al.* (2015) with a slight modification. Briefly, flow curves of yoghurt samples were analyzed with a rotary viscometer Rheometric RM180 (Rheomat, Caluire, France), equipped with coaxial cylinders' geometry. The bob and the cup used had 15.18 (R_i) and 21 mm (R_e) radius, respectively, giving a ratio R_i/R_e=0.72. Viscosity measurements at increasing and decreasing strain rates were conducted between 0.01 and 500 s⁻¹. The viscometer was controlled by RSI Orchestrator v6.5.8 software. Flow properties were assessed at temperature 4°C. The regulation of temperature during the rheological measurements was obtained using a circulator bath (Julabo GmbH, Germany). The area of thixotropic hysteresis loop was determined using RSI Orchestrator v 6.5.8 software, which calculates the difference between the area under the up-flow curve and the down-flow curve.

2.7. Sensory evaluation

Throughout the storage period at 4°C (1st, 14th and 28th day), the sensory properties of experimental yoghurts were evaluated by a jury of panelists consisting of 20 trained members (8 male and 12 female, aged between 24 and 45 years). The trained panelists were students from the Tunisian higher Institute of Food Industry and the training was conducted according to the method described by HOOTMAN (1992) and MEILGAARD *et al.* (2006). The test was performed inside a uniformly illuminated room, at approximately 25 °C. The obtained yoghurts were coded with a random six-digit number and served to panelists in a randomized order. The main descriptors, used to evaluate appearance, taste and texture, were sweet taste, bitter taste, mouth feel, granular texture, whey exudation,

white color and overall acceptance, consisted in a 9-point scale (DANTAS *et al.*, 2016; SILVA *et al.*, 2018).

2.8. Statistical analysis

The obtained data were statistically evaluated using a one-way analysis of variance (ANOVA) with Duncan's test for mean comparison to highlight significant differences ($P < 0.05$) among yoghurt samples. All the experiments were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Effect of lactic starter variation and lactulose addition on yoghurt quality

3.1.1 Effects on yoghurt fermentation

Changes in kinetic parameters of acidification during the fermentation of control and lactulose incorporated yoghurts, using three different commercial starters, are shown in Table 1.

Table 1. Kinetic parameters of acidification of yoghurt using different starter co-cultures with and without lactulose at 1.5%.

Yoghurt starters	Lactulose	Vmax (10^{-3} pHunits/min)	Tmax (hours)	TpH4.5 (hours)
Yoflex 801	Control	19.33 $\pm$ 0.02	3.5 $\pm$ 0.19	5 $\pm$ 0.20
	with lactulose	19.33 $\pm$ 0.01	2.5 $\pm$ 0.17	4 $\pm$ 0.22
Yoflex 901	Control	16.61 $\pm$ 0.02	3.5 $\pm$ 0.22	4.5 $\pm$ 0.19
	with lactulose	19.67 $\pm$ 0.03	3 $\pm$ 0.25	4 $\pm$ 0.27
Yomix 486	Control	20.00 $\pm$ 0.01	3 $\pm$ 0.24	4.5 $\pm$ 0.21
	with lactulose	23.33 $\pm$ 0.02	2 $\pm$ 0.23	3.5 $\pm$ 0.22

As expected, on the basis of chemical acidification reaction that underlies the fermentation process, pH dropped during 3.5–5 h (TpH4.6) to values of 4.6 in all experiments. Yomix 486 exhibited the fastest Acidifying kinetics ($V_{\max} = 20 \pm 0.01 \times 10^{-3}$ pH units / min; $T_{\max} = 3$ h); followed by Yoflex 801. In fact, according to Almeida *et al.* (2009), different acidification profiles of LABs depend on their peculiar capacity to use nutritive compounds of milk, which could account for the differences in the kinetic parameters observed amongst the various yoghurts. Thus, LABs capacity to produce lactic acid, which is the main product of the metabolic activity of starter cultures, depends on the strains and their associations (BÉAL *et al.*, 1999). Indeed, it is known that a synergic proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* takes place during yoghurt fermentation (LOURENS-HATTINGH and VILJOEN, 2001). Furthermore, $V_{\max}$ increased by the incorporation of lactulose (1.5%) (Except for Yoflex 801), reaching 19.67 ± 0.03 and $23.33 \pm 0.02 \times 10^{-3}$ pH units/min, in fermented yoghurt, respectively, with Yoflex 901 and Yomix 486. The time for reaching maximum acidification rate ($T_{\max}$) was reduced by 1h, 0.5 h and 1 h, respectively, for Yoflex 801, Yoflex 901 and Yomix 486. Besides, the time for

reaching $T_{pH4.6}$ was reduced by 1h for Yoflex 801 and 0.5h for both Yoflex 901 and Yomix 486. These differences could be attributed to the high rate of *L. bulgaricus* in Yomix 486 starter and/or the differences between strains and species of LABs for lactulose metabolism.

3.1.2 Effects on yoghurt quality during storage

Quality parameters of yoghurts fermented by each of the three different lactic starters with or without lactulose at 1.5%, over 28 days of refrigeration storage, are illustrated in Table 2.

Total solids content of yoghurts were evaluated during refrigeration storage. Initial total solids levels were between 98 ± 0.38 and 102 ± 0.95 g/L for control yoghurts. However, after lactulose incorporation, these values reached 111 ± 0.98 and 114 ± 1.24 g/L. Hence, the presence of prebiotics increased the total solids content of milk bases. These results were in accordance with other studies reporting that the addition of prebiotics in mix increase total solids content (Aryana and MC GREW, 2007). However, no significant differences ($P > 0.05$) between total solids values during storage period were noted.

Postacidification of the yoghurts displayed an increase over the storage period. Dornic acidity values growths were 13.5, 18 and 19°D for control fermented yoghurt, respectively, with Yoflex 801, Yoflex 901 and Yomix 486. The values changed to be 14.5, 20 and 26.5°D, when lactulose was added. Indeed, acid-production trend during storage was similar to other research studies (ÇELİK, 2007). The lowest postacidification was obtained with Yoflex 801, against the highest one noted for Yomix 486 starter, especially in yoghurt, wherein lactulose (1.5%) was added. These findings suggest that *L. bulgaricus* strain of Yomix 486 was able to assimilate more lactulose than the other co-culture strains. Moreover, *L. bulgaricus* produces more lactic acid when lactulose is available (Hernandez-Hernandez *et al.*, 2012).

For syneresis (Table 2), a steady increase in all tested samples was recorded, with the progress of storage time until the 21st day. Syneresis levels increased from 62% to 73% and from 60% to 77%, respectively, for Yoflex 901 and Yomix 486. The use of Yoflex 801 culture was associated with weak whey separation, compared to the other starters, during all storage time. Syneresis values rose from 58% to 69%. These findings could be attributed to the capacity of each strain to produce exopolysaccharides. In fact, AMATAYAKUL *et al.* (2006) reported that syneresis could be reduced by starters producing exopolysaccharides. Besides, there were conflicting findings about fermentation parameters effects on molecular characteristics of exopolysaccharides (MENDE *et al.*, 2016). Furthermore, IBRAHIM (2015) noted that frail gel was obtained when the fermentation time of camel milk was long. The main reasons for syneresis might be ascribed to the structural rearrangements in casein micelles in the gel network and the rate of solubilization of colloidal calcium particles. In this study, a longer fermentation period was achieved by Yoflex 801 culture. However, higher syneresis was observed in yoghurt Yoflex 901 or Yomix 486 (Table 2). Therefore, the primary reason for higher syneresis was considered to be the type of strains in each co-culture.

As indicated in Table 2, at the 28th day, syneresis percentage exhibited little decrease. These results were in accordance with AKGUN *et al.* (2017) findings, pertaining to probiotic yoghurts. As determined by MENDE *et al.* (2016), medium acidity was linked to the interaction between polysaccharides molecules and protein network. Indeed, acidity affects protein network charges, and consequently their joining with polysaccharides would be modified as well.

Table 2. Variations in Total solids, postacidification, syneresis, proteolysis degree and lactic acid bacteria counts of yoghurt fermented using three different starters, with or without lactulose at 1.5%, for 28 days of storage at 4°C.

Parameters	Starters	Lactulose (1.5%)	Storage period (days)				
			1	7	14	21	28
Total solids	Yoflex 801	Control	98±0.38	98±0.88	99±0.84	99.4±0.85	99.18±1.22
		With lactulose	111±0.98**	111.5±0.97**	112±0.94**	113±1.24**	112±0.85**
	Yoflex 901	Control	99±0.99	99.9±0.56	101±0.85	100±0.76	101.1±0.97
		With lactulose	113±0.74**	113.5±0.54**	112±0.65**	112±0.85**	114±1.14**
	Yomix 486	Control	99±1.12	102±0.54	101±0.68	100±0.75	102±0.95
		With lactulose	111±0.98**	112±0.65**	113±0.94**	114±1.24**	113.2±0.85**
Dornic acidity	Yoflex 801	Control	78±0.43	82±0.72*	85±0.45*	89±0.66*	92.5±0.34*
		With lactulose	81.5±0.28**	84±0.63*	90±0.52***	95±0.38***	96±0.71**
	Yoflex 901	Control	76±0.50	84±0.52*	86±0.34*	87±0.35*	94±0.38*
		With lactulose	76.5±0.91	92±0.45***	93±0.34**	94±0.92**	96.5±0.26**
	Yomix 486	Control	79±0.5	90±0.52*	94±0.81*	93±0.73	98±0.90*
		With lactulose	81±0.38**	91±0.27*	96±0.63*	101±0.63***	107.5±0.38***
Syneresis (%)	Yoflex 801	Control	58±0.05	60±0.02*	67±0.1*	67±0.01	66±0.4*
		With lactulose	63±0.01**	63±0.01**	67±0.12*	69±0.02***	68±0.3***
	Yoflex 901	Control	62±0.03	67±0.01*	69±0.1*	73±0.01*	70±0.2*
		With lactulose	63±0.02**	68±0.02***	70±0.08***	74±0.02***	72±0.1***
	Yomix 486	Control	60±0.01	65±0.1*	70±0.01*	77±0.3*	74±0.5*
		With lactulose	61±0.01**	66±0.01***	75±0.5***	79±0.1***	75±0.7*
Proteolysis degree (%)	Yoflex 801	Control	33±0.71	36±0.57*	39.5±0.42*	43±0.57*	44.5±0.71*
		With lactulose	40±0.57**	42.6±0.42***	43.75±0.35***	46±0.71*,**	49.5±0.71***
	Yoflex 901	Control	39±0.28	41±0.99	43.9±0.28	46.8±0.58*	49.2±0.14*
		With lactulose	44±0.71**	45±0.2	49.2±0.14***	50.49±0.58**	52.99±0.56***
	Yomix 486	Control	28±0.42	32±0.56*	38±0.35*	43.9±0.57*	49±0.71*
		With lactulose	30±0.42**	38±0.35*,**	46±0.5*,**	48±0.71*,**	56±0.56*,**
LAB counts (log cfu/mL)	Yoflex 801	Control	8.70±0.3	8.75±0.1	9.08±0.1*	9.39±0.3*	9.60±0.1
		With lactulose	8.85±0.1	9.22±0.24***	9.32±0.2**	9.55±0.2*	9.85±0.1***
	Yoflex 901	Control	9.05±0.1	9.15±0.1*	9.45±0.17*	9.65±0.34	9.80±0.22
		With lactulose	9.15±0.2	9.45±0.3	9.75±0.12**	9.90±0.19	10.10±0.13***
	Yomix 486	Control	9.01±0.2	9.32±0.2*	9.84±0.1*	9.94±0.2	10.4±0.1*
		With lactulose	9.22±0.2	9.58±0.14***	10.1±0.12***	10.12±0.24	10.9±0.16***

Data are presented as the mean±SD of three separate experiments. *, significant differences between storage period ($P < 0.05$); **, significant differences between control and supplemented yoghurt at the same storage time ($P < 0.05$).

Otherwise, yoghurts incorporating 1.5% lactulose had higher syneresis values at each storage period. However, these differences were not significant ($P > 0.05$) compared to control, except for Yomix 486 samples, which recorded the highest syneresis percentage. This might be assigned to lower pH obtained when lactulose was added, which caused an unstable gel network with a continuous changing arrangement, thus, resulting in disturbed protein micells as described by DONKOR *et al.* (2007) and MÖLLER and VRESE (2004).

Yoghurt protein content was 4.6%. This value is in compliance with the standard (CODEX STAN 243-2003), which requires content in minimal equal protein of 2.7 %. Table 2 presents proteolysis degree obtained in yoghurt supplemented with 1.5% and fermented with Yoflex 801, Yoflex 901 and Yomix 486. As expected, proteolysis degrees increased for all yoghurt samples, for 28 days of refrigeration storage. These results induced extracellular proteases activity of lactic acid bacteria through the storage period (YUKSEL and ERDEM, 2010). Besides, NIELSEN *et al.* (2009) proved that proteases are active during refrigeration storage. Further, proteolysis degrees were higher, at each storage period, when 1.5% of lactulose was added. Similar results were obtained by YUKSEL and ERDEM (2010) and DONKOR *et al.* (2007). In fact, they also demonstrated that proteolysis levels depend on the nutrients available to proteolytic microorganisms.

Lactic acid bacteria counts were converted to log scale and reported in Table 2. Even with the addition of lactulose, LAB count was maintained over 10^6 cfu/mL. This result was in good agreement with the Codex Alimentarius Commission (CODEX STAN 243-2003), which established that the counting of lactic acid bacteria must be over 10^7 cfu/mL. It is concluded that LAB counts become higher in yoghurt samples incorporating lactulose. Indeed, over the storage period, their counts increased by 0.9, 0.75 and 1.39 log cfu/mL, in control fermented yoghurts, respectively, with Yoflex 801, Yoflex 901 and Yomix 486. These increases became 1, 0.95 and 1.68 in yoghurts, wherein lactulose (1.5%) was added. These results were consistent with TABATABAIE and MORTAZAVI (2008) who reported that in yoghurt containing lactulose (1 and 3%) during 5 weeks of cold storage, the survival of *L. rhamnosus* LBA and *B. bifidum* CECT considerably improved. RASTALL and MAITIN (2002) found that the highest count of bifidobacteria was noted when adding xyloligosaccharide and lactulose, however, the largest increase in lactobacilli was obtained when adding FOSs. Thus, generally, lactulose was a more effective growth promoter for lactic strains compared to inulin. On the other hand, differences were not significant at each storage period. Indeed, probiotic bacteria metabolise prebiotics more than yoghurt starters.

Furthermore, when lactulose was incorporated, the lowest LAB counts changes were obtained in yoghurts with Yoflex 801. However, greater proliferations were noted for Yoflex 901 and Yomix 486 starters. Therefore, it can be concluded that lactulose had high prebiotic effect on Yoflex 901 and Yomix 486, followed by Yoflex 801. Indeed, it seems that the stimulatory impacts of prebiotics on lactic acid bacteria viability depends on several factors such as strain type and final pH.

Hence, low stimulation of starter bacteria, low postacidification, low proteolysis and low syneresis were sought to obtain functional food, having similar characteristics to the conventional food. Hereinafter, Yoflex 801 is chosen to be used in the rest of the study.

3.2. Characteristics of yoghurt added with different dose of lactulose

3.2.1 Effects of lactulose dose on yoghurt fermentation and post-acidification

Concerning acidification kinetics, shown in Table 3, it was observed that lactulose yoghurts exhibited higher V_{max} than the control; values obtained ranged from 19.33 ± 0.02 to 25 ± 0.02 10^{-3} pH units/min, respectively, for control fermentation milk and samples added with 8% of lactulose. However, the time (T_{max}) to reach V_{max} was 3.5 h for control, 3 h for both 4% and 6% of lactulose and 2.5 h for 8%. Moreover, the time to reach pH= 4.6 was 5 h for control, 4 h for both 4% and 6% of lactulose and 3.5 h for 8%. These findings were in accordance with those of ÖZER *et al.* (2005), revealing that inulin and lactulose addition at different concentrations reduced the incubation period of yoghurt.

In this regard, lactic acidity values increased significantly ($P < 0.05$) during refrigeration storage in all yoghurt samples (Table 4). Indeed, metabolism of yoghurt bacteria continued during the 28 days of storage at 4°C, as shown previously in the first part of the study. Moreover, when lactulose was supplemented, overall postacidification increased weakly (1 to 3°D). This data was in agreement with CRUZ *et al.* (2013b) results, reporting that the supplementation of different doses (2, 4, 6 and 8%) of oligofructose as prebiotic has no significant effect on post acidification. These findings are desirable in modern yoghurt industry, and endorse the choice of Yoflex 801 as starter in this study. However, OLIVEIRA *et al.* (2011) proved that the addition of lactulose in skim fermented milk by probiotic LAB in coculture with *S. thermophilus* decreased pH at the final period of storage, indicating a bifidogenic effect for *Bifidobacterium lactis*.

Table 3. Kinetic parameters of acidification of yoghurt fermented with Yoflex 801 and added with lactulose at different doses (0, 4, 6 and 8%).

Lactulose dose (%)	V_{max} (10^{-3} pH units/min)	T_{max} (hours)	$T_{pH4.5}$ (hours)
Control	19.33 ± 0.02	3.5 ± 0.19	5 ± 0.2
4	20 ± 0.01	3 ± 0.17	4 ± 0.22
6	21.6 ± 0.03	3 ± 0.21	4 ± 0.21
8	25 ± 0.02	2 ± 0.23	3.5 ± 0.19

3.2.2 Effects of lactulose dose on yoghurt quality during storage

The parameters of control yoghurts and those obtained at different lactulose concentrations (4, 6 and 8%) fermented with selected Yoflex 801 starter, for 28 days at 4°C, are presented in Table 4. Total solids content of the four obtained yoghurts displayed an increase ($P < 0.05$) when lactulose concentrations rose. Values varied from 97.5 ± 0.9 g/L (control sample) to 178 ± 0.9 g/L (8% lactulose). Indeed, DE CASTRO *et al.* (2008) reported that the addition of prebiotic was associated with a total dry extract increase. Moreover, these findings outlined that lactulose was still in yoghurts and would be available for consumers as prebiotic, in order to improve health.

Table 4. Variations in post-acidification, total solids, syneresis, proteolysis degree and lactic acid bacteria counts of yoghurt containing various doses of lactulose (0, 4, 6 and 8%) and fermented with Yoflex 801, for 28 days of storage at 4 °C.

Storage period (days)	Lactulose dose (%)	Dornic acidity	Total solids (g/L)	Syneresis (%)	Proteolysis degree (%)	LAB counts cfu/mL	(log
1	Control	79±0.13	97.5±0.9	61±0.12	32.38±0.71	8.35±0.14	
	4	80.33±0.2*	125.33±1.2*	58±0.5*	37.1±0.56*	8.56±0.22*	
	6	81±0.2*	155.5±0.8*	56±0.1*	42±0.42*	8.77±0.5	
	8	82±0.2*	178±0.9*	53±0.18*	45.3±0.14*	8.86±0.12	
7	Control	83.66±0.12**	98±0.85	62±0.16**	35.5±0.28**	8.48±0.2	
	4	86.33±0.49***	127±1.24*	60±0.2*,**	38.3±0.78*	8.79±0.18*	
	6	88.66±0.23*,**	155.66±1.22*	59±0.5*,**	44±0.59*,**	9.07±0.1*	
	8	90.33±0.45*,**	168±0.85*	55±0.8*,**	47.5±0.71*,**	9.13±0.1**	
14	Control	86±0.39**	98±0.97	65±0.12	37.8±0.35**	8.86±0.14	
	4	88.33±0.23***	123±1.24 *	62±0.3*,**	40.4±0.58*	9.08±0.22*,**	
	6	89±0.25***	155±1.9*	62±0.4**	45±0.28*	9.3±0.2**	
	8	90±0.13***	170±1.74*	57±0.18*,**	49.5±0.5*	9.38±0.1**	
21	Control	90.66±0.1**	99±0.95	68±0.1	41±0.35**	9±0.1**	
	4	93.33±0.2***	124±0.99*	63±0.24*,**	42.6±0.42*,**	9.11±0.12	
	6	94.6±0.12***	153±1.14*	64±0.1*,**	47±0.14*,**	9.31±0.13*,**	
	8	94.33±0.17***	171±1.41*	59±0.18*,**	50.6±0.58*	9.56±0.1**	
28	Control	94.66±0.39	98.8±0.85	70±0.09	43.8±0.28	9.66±0.14**	
	4	97±0.25***	125±1.9*	65±0.02*,**	45±0.42*	9.87±0.2**	
	6	97.33±0.1*,**	152±1.37*	65±0.01**	48.5±0.71*	10.01±0.2**	
	8	100.66±0.49***	169±1.25*	60±0.03*,**	52.49±0.35*,**	10.16±0.24**	

Data are presented as the mean±SD of three separate experiments.

*, significant differences between lactulose dose at the same storage time ($P < 0.05$); **, significant differences between the same dose of lactulose at different storage periods ($P < 0.05$).

Furthermore, initial syneresis values varied from 53 ± 0.18 % to 61 ± 0.12 %. Besides, whey separation increased significantly ($P < 0.05$) during storage in all samples, and decreased with lactulose dose increase. Syneresis reached 60 ± 0.03 % to 70 ± 0.09 at the 28th day of cold storage (Table 4). These results could be elucidated by the effective role of prebiotics in increasing water-holding capacity in the texture (REID *et al.*, 2003). Moreover, some studies revealed that using prebiotic compounds, such as inulin and lactulose at optimum concentrations, might reduce the percentage of syneresis. In addition, these findings could be related to the total solids. In fact, when dry extracts increased, syneresis decreased (ESTEVEZ *et al.*, 2009). Thus, lactulose levels would improve yoghurt quality by reducing syneresis, which is not sought by dairy industry.

On the other hand, during storage, proteolysis degrees increased significantly ($P < 0.05$). These findings are in line with our previous results and suggest that although lactulose dose did not affect LAB growth, it was involved into their proteolytic activity, as reported by ÖZER *et al.* (2005). However, it is noteworthy that proteolysis would generate free amino-acids, which improve the sensory properties of dairy fermented products.

Further, LAB counts over the storage period (Table 4), increased in all samples. Correspondingly, lactulose dose weakly affected LAB growth. These results were in good agreement with ÖZER *et al.* (2005) findings, who did not note any significant effect of lactulose (2.5%) on the growth of yoghurt starter bacteria. Likewise, those data asserted previous results when different starters were used with 1.5% of lactulose.

3.3. Rheological properties variation

In this study, as shown in Table 5, the results revealed that the increase of lactulose concentration and storage period give rise to the increase of the yield stress values (0.11 ± 0.02 - 0.44 ± 0.01 Pa), consistency coefficients (1.96 ± 0.04 - 3.62 ± 0.03 Pa.sⁿ) and hysteresis area, and the slow decrease of flow index values. This can be explained by the breakdown of the yoghurt structure during storage after shear. Indeed, the increase of consistency values of the formulated samples could be assigned to the increase of the total solid content in lactulose yoghurts, especially when lactulose dose ranged from 4% to 8 % ($P < 0.05$). Therefore, an increase in lactulose concentration was accompanied with an increase in pseudoplasticity. Moreover, lactulose contributed in forming the best structural arrangement in the enriched yoghurts. Thus, its addition increased the rates of aggregation and curd firming reactions in the casein gels, which was in line with the result reported in previous work (ARANGO *et al.*, 2013). The two-way ANOVA test was made to ascertain the effects of the storage period, lactulose concentration as well as the interaction between the storage period and lactulose concentration on rheological parameters (yield stress, flow index, consistency coefficient and hysteresis area). The influence of both factors on each variable tested was clear with P values < 0.05 , except for flow index, which had no significant P values ($P > 0.05$) in terms of storage period and interaction between the storage period and lactulose concentration.

On the other hand, flow curves of control and 8% lactulose enriched yoghurts, shown in Fig. 1, yield hysteresis loops. All samples exhibited thixotropic behavior as illustrated in other studies, covering set yoghurts (CIRON *et al.*, 2012; ESPÍRITO-SANTO *et al.*, 2013 and ILICIC *et al.*, 2014).

Table 5. Variations in Rheological parameters of yoghurt containing various doses of lactulose, for 28 days of storage at 4 °C.

Storage time (days)	Lactulose dose (%)	Yield stress σ_0 (Pa)	Flow index n	Consistency coefficient k (Pa.s ⁿ)	Hysteresis area A (Pa/s)	R ²
1	Control	0.11±0.02 ^a	0.65±0.02 ^a	2.05±0.04 ^a	1170.05±30.10 ^a	0.993
	4	0.15±0.02 ^{ab}	0.63±0.02 ^a	3.13±0.03 ^b	1256.45±24.20 ^b	0.987
	6	0.18±0.01 ^b	0.61±0.01 ^a	3.27±0.07 ^{bc}	1290±20.02 ^b	0.996
	8	0.21±0.01 ^b	0.60±0.01 ^a	3.41±0.05 ^c	1319.30±26.22 ^b	0.975
7	Control	0.12±0.01 ^a	0.66±0.02 ^a	2.13±0.05 ^a	1193.06±40.46 ^a	0.966
	4	0.19±0.02 ^b	0.61±0.02 ^{ab}	3.22±0.03 ^b	1268.21±35.87 ^a	0.973
	6	0.26±0.01 ^c	0.58±0.02 ^{ab}	3.45±0.05 ^c	1277.09±34.91 ^a	0.992
	8	0.32±0.01 ^d	0.57±0.02 ^b	3.52±0.04 ^c	1394.20±30.33 ^b	0.991
14	Control	0.14±0.02 ^a	0.64±0.03 ^a	2.21±0.05 ^a	1292.50±13.70 ^a	0.989
	4	0.25±0.02 ^b	0.61±0.01 ^{ab}	3.38±0.06 ^b	1289.68±19.88 ^a	0.995
	6	0.31±0.03 ^{bc}	0.56±0.01 ^{ab}	3.48±0.05 ^{bc}	1314.59±16.64 ^a	0.970
	8	0.40±0.03 ^c	0.54±0.02 ^b	3.64±0.06 ^c	1349.70±15.50 ^b	0.989
21	Control	0.15±0.0 ^a	0.64±0.02 ^a	2.10±0.04 ^a	1291.18±15.72 ^a	0.964
	4	0.28±0.02 ^b	0.60±0.01 ^{ab}	3.40±0.03 ^b	1296.26±15.93 ^{ab}	0.989
	6	0.34±0.02 ^b	0.57±0.01 ^{bc}	3.48±0.02 ^b	1302.53±113.08 ^{ab}	0.976
	8	0.44±0.01 ^c	0.53±0.01 ^c	3.62±0.03 ^c	1303.46±12.66 ^b	0.985
28	Control	0.13±0.01 ^a	0.68±0.03 ^a	1.96±0.04 ^a	1287.73±14.28 ^a	0.989
	4	0.30±0.01 ^b	0.61±0.01 ^{ab}	3.38±0.04 ^b	1288.34±15.01 ^a	0.974
	6	0.36±0.01 ^b	0.55±0.03 ^b	3.50±0.05 ^{bc}	1299.87±13.53 ^{ab}	0.997
	8	0.43±0.03 ^c	0.52±0.02 ^b	3.64±0.04 ^c	1312.22±14.13 ^b	0.966

Data are presented as the mean±SD of three separate experiments.

Different superscript letters indicate statistically significant ($p<.05$) differences in a column at the same storage time.

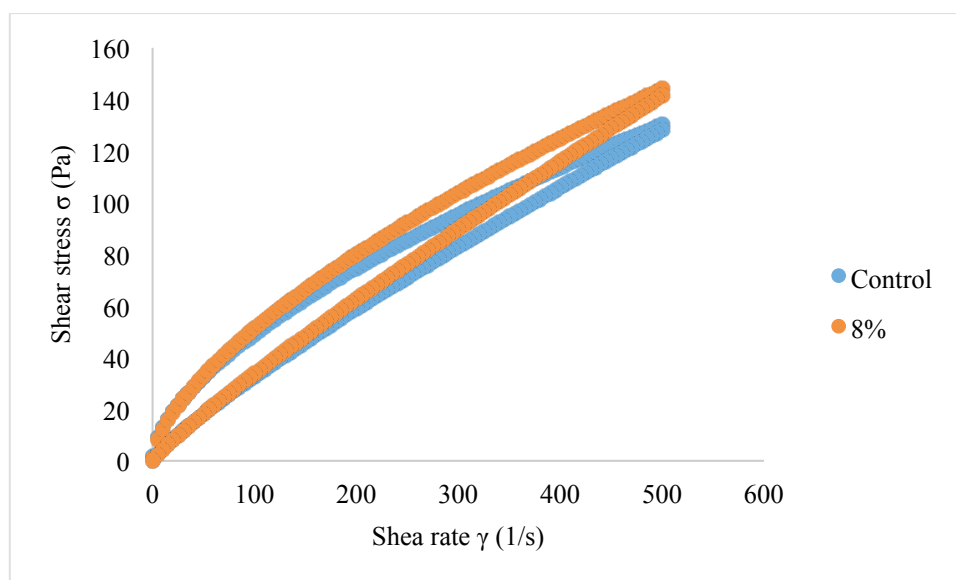


Figure 1. Hysteresis loops of set yoghurts (control and 8 % of lactulose) after 1 day of storage at 4°C.

STEFFE (1996) reported that thixotropic property is observed particularly in fragile structures and the three-dimensional network formed is completely destroyed as in the case of set yoghurts. Accordingly, it is clear that the sample enriched with lactulose has shear stress values, higher than those found in the control. In fact, yoghurts viscosities increased with the increase of lactulose concentration.

A non-linear relationship was detected between shear stress (σ) and shear rate ($\dot{\gamma}$). These findings were in accordance with those of SAHet *et al.* (2016), CUI *et al.* (2014) and CIRON *et al.* (2012). Based on the values of R^2 coefficient, the HERSCHEL-BULKLEY model was found to be a better-fit model for flow curves ($R^2 > 0.96$) and only rheological parameters of this model are presented in this study (Table 5). The obtained data were fitted to HERSCHEL-BULKLEY model according to:

$$\sigma = \sigma_0 + K\dot{\gamma}^n$$

Where σ represents the shear stress (Pa), k is the consistency coefficient (Pa.sⁿ), $\dot{\gamma}$ is the shear rate (s⁻¹), σ_0 is the yield stress and n is the flow behavior index (dimensionless). Moreover, the plot of the shear stress against shear rate of the yoghurt samples under investigation yielded a flow index n of less than 1 (thinning fluid) (0.53 ± 0.01 - 0.68 ± 0.03), indicating that their flow behavior had a non-Newtonian profile.

3.4. Sensory evaluation

Table 6 represents comparative sensory analysis among yoghurts supplemented with different doses of lactulose (0, 4, 6 and 8%) using scoring methodology, after storage for days 1, 14 and 28.

Table 6. Variations in sensory evaluation of yoghurt containing various doses of lactulose (0, 4, 6 and 8 %) for 28 days of storage at 4°C.

*: significant differences between lactulose doses at the same storage time ($P < 0.05$).

Storage period (days)	Lactulose dose (%)	Sweet taste	Bitter taste	Mouth feel	Granular texture	Whey exsudation	White Color	Overall acceptance
1	Control	1.9±0.4	2.28±0.7	5.11±0.5	5.18±0.75	3.9±0.8	2.95±0.8	3±0.35
	4	2.78±0.3*	2.63±0.5	3.15±0.6*	4±0.65	4.56±0.4	3.72±0.6	3.65±0.25*
	6	4.95±0.5*	2.85±0.35	4.88±0.7	2.65±0.75	4±0.3*	5.44±0.5	4.43±0.6
	8	6.84±0.6*	2.48±0.2	6.58±0.25	1.79±0.8	2.9±0.38*	5.31±0.3	6.2±0.39
14	Control	2.45±0.6	3.22±0.3	3.58±0.25	4.2±0.24	4.78±0.32	3.3±0.2	3.11±0.32
	4	5±0.35	3.25±0.25	3±0.7	3.9±0.9	3.75±0.35*	3.25±0.7	3.65±0.62
	6	6±0.5	2.5±0.24*	3.65±0.4	4.45±0.75	4.56±0.56	3.6±0.56	3.4±0.23*
	8	6±0.7	2.99±0.3*	5.15±0.8*	1.72±0.65	3.14±0.38	2.91±0.34	5.65±0.39*
28	Control	2.1±0.2	2.5±0.3	4.29±0.65	4.09±0.9	4.72±0.42	3.34±0.85	3.11±0.8
	4	3±0.35*	2.78±0.24	4.5±0.45	3.5±0.3	3.5±0.6	4.28±0.77	3.5±0.77
	6	3.78±0.4	2.3±0.35	5±0.35*	3.4±0.6	4.52±0.9	4.27±0.36	4±0.65
	8	5.3±0.3*	2.85±0.7	7.1±0.5*	2.9±0.8	4.4±0.42	4.1±0.39	4.34±0.55

The panelists could identify differences ($P < 0.05$) in the sweet taste during storage. Moreover, when lactulose dose increased, sweet taste score increased, being from 1.9 ± 0.4 to 6.84 ± 0.6 at the first storage day, respectively, for control yoghurt and when 8 % of lactulose was added. Indeed, lactulose had a considerable sweetness power (WESTHOFF *et al.* 2000). The bitter taste and color scores of the yoghurt samples were not affected by lactulose addition. Otherwise, lower score of granular texture and whey exudation were obtained in yoghurt with higher lactulose dose. Besides, mouth feel was better, when lactose dose or storage period increased, especially for 6 and 8% of lactulose. The overall appreciation increased when lactulose dose increased. Scores reached, at the first day, 6.2 ± 0.39 for yoghurt with 8% of lactulose against 3 ± 0.35 for control yoghurt. This is probably ascribed to the sweetness power of lactulose. Literatures about the effects of prebiotics on sensory attributes of fermented milk products are rather conflicting. SEYDIN *et al.* (2005) found that yoghurts containing inulin had good flavor and smooth texture. Further, HAYDARI *et al.* (2011) reported that increasing the concentration of prebiotics led to a weaker sensorial gel firmness and scoopability probably ascribed to depletion flocculation of milk proteins during fermentation. Except for inulin, increasing the concentration of prebiotics resulted in less smooth oral texture, and, likewise, higher concentration of prebiotics possessed less flavor acceptability and total acceptability.

4. CONCLUSIONS

Starter type had significant effects on kinetic parameters, postacidification, syneresis and proteolysis degree of yoghurt containing 1.5% of lactulose. Thus, Yoflex 801 was the adequate co-culture to use in prebiotic yoghurt supplemented with lactulose. With respect to lactulose therapeutic dose, increased levels (4, 6 and 8%) reduced syneresis and improved sensory characteristics. However, concerning rheological characteristics, yoghurts supplemented with lactulose had a weak gel, with a thixotropic and pseudoplastic behavior, peculiarly 8% of lactulose. Hence, minor quality alterations were obtained with 4% or 6% of lactulose. Based on the result found in this study, it is concluded that yoghurt fermented with Yoflex 801, and supplemented with 4% or 6 % of lactulose could have interesting outcomes with respect to functional food production and preservation.

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Paper Received January 4, 2019 Accepted April 11, 2019

VARIATION IN MEAT QUALITY CHARACTERISTICS BETWEEN LANDRACE AND SICILIAN PIGS

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ABSTRACT

This study compared the meat quality of Sicilian and Landrace pigs breeds and supported these results with biochemical and histological measurements on the samples collected from the m. *Longissimus Dorsi*, at the level of the 8th thoracic vertebra, before electrical stimulation. Twenty clinically healthy swine, 10 male (5 for each pig breed) and 10 female (5 for each pig breed), were slaughtered at 1 year of age at a body mass of 135±10 kg and 150±10 respectively for Sicilian and Landrace pigs. Particularly on the muscle considered the morphometric characteristics of FG (*fast glycolytic*), FOG (*fast oxidative glycolytic*) and SO (*slow oxidative*) fibre types and their percentage were determined. Measurements related to myofibrillar fragmentation, sarcomere length and connective tissue properties gave convincing support. Sicilian pig produced more tender meat than Landrace, mainly due to favourable calpain-to-calpastatin ratios.

Keywords: connective tissue, meat quality, proteolytic system, sarcomere length

1. INTRODUCTION

Most recently, autochthonous breeds have been recognized as important elements to regional agro-biodiversity and, more specifically, in their relevance to agro-ecosystems that encompass the cultural heritage of a given region (JOVANOVI *et al.* 2011). It is relevant the role that local breeds could play to the regional development of the countries, including social, environmental and economic sustainability issues in a globalized market (BELIBASAKI *et al.* 2012). Furthermore, there has been an increasing interests in local breed because of the high quality perceived by consumers of meat and meat products from indigenous breed (STANIŠIĆ *et al.*, 2015).

Other researchers investigated the characteristics, in term of meet quality, of local pigs breed by comparing to purebred lines and their crossbreed (DEBRECÉNI *et al.*, 2016, STANIŠIĆ *et al.*, 2015; POTO *et al.*, 2007; MORCUENDE *et al.*, 2007) and no relevant literature was found about Sicilian pig breed. This last pig breed is also called Nebrodi or Madonie black swine. The interest in indigenous pig breeds is related to meat products. Particularly among them, the Sicilian pig is widely appreciated for the high quality of its niche products linked to the local gastronomic traditions (VELOTTTO *et al.*, 2007). Precocious and long-lived breed it is characterized by a peculiar fertility and a great number of weaned piglets. This breed is also resistant to diseases and to the inclemency of the weather, being able to appreciate poor food as well. Italian Landrace has become dominant as an improved breed in Italy. They are long pigs with pink skin, white hair and ears that droop over their eyes. The original stock was brought to Italy from Scandinavian sources, but selection has been toward meat characteristics. Landrace pigs are a common second breed in crossbreeding programs in modern production, because of their good meat structure and maternal abilities (VELOTTTO *et al.*, 2014). They are very efficient at converting food to meat, and produce little excess fat. Landraces are known for their rapid growth when young, typically reaching a heavier weight at weaning than other breeds, which is another factor in their lasting popularity in factory farms. Physical and chemical changes occur during storage of meat after slaughtering that affect the meat quality. In particular, tenderness and the mechanism of meat tenderization represent one the most debated subject among the scientific community.

Several researchers investigated the mechanism of meat tenderization and factors responsible for the initiation and course of this process (KOOHMARAI, 1996; ROWE *et al.*, 2004; LI *et al.*, 2012). CHRIKI *et al.* (2012) showed that increased meat tenderness is related lower insoluble collagen and total collagen content, lower cross-sectional area of fibres, and an overall fibre type composition displaying more oxidative fibres than glycolytic fibres. MOHRHAUSER *et al.* (2014) showed that the decrease in m-calpain in the delay-chilled bovine carcasses could explain the improvements of the myofibrillar fragmentation index postmortem. The myofibrillar fragment length (MFL) could represents an indicator of meat tenderness depended on specific proteolytic enzyme activity, such as m-calpain. The activity of μ - and m-calpain is synergistic: μ -calpain contributes to early post-mortem proteolysis, while m-calpain is partially activated and contributes to tenderization during prolonged ageing (VARRICCHIO *et al.*, 2013).

In this study variation in meat tenderness between Landrace and Sicilian pig breeds were investigated. Particularly, supportive histological and biochemical evidence was provided to explain the variation in these quality characteristics. The aim of this work was to compare the Landrace breed with Sicilian pig. The study focused attention on size and distribution of fibre types of *Longissimus Dorsi* (LD), one of the most studied muscles.

2. MATERIAL AND METHODS

2.1. Slaughter and sampling procedures

Muscle samples were collected from pigs of the pure autochthonous breed “Sicilian black swine” and the breed “Italian Landrace” slaughtered in an abattoir. Animals were reared in an extensive system and raised using the most traditional farming methods, the diet of swine shifted gradually from woodland forage and scraps to corn. Twenty clinically healthy swine (10 male and 10 female: 5 male and 5 female Landrace; 5 male and 5 female Sicilian pig) from farms located in south and north Italy, were slaughtered at 1 year of age at a body mass of 135 ± 10 kg and 150 ± 10 respectively for Sicilian and Landrace pigs. Carcasses were electrically stimulated for 60 s after exsanguinations (400 V peak, 5ms pulses at 15 pulses/s) and entered the cold rooms 45 min after killing. Animals handling followed the recommendations of the European Community (Reg. CE 1/2005; directives 74/577/EEC; Law 439 2 August 1978) concerning animal care. Samples for muscle fibre typing were collected from the *m. Longissimus Dorsi* (LD), at the level of the 8th thoracic vertebra, before electrical stimulation. Samples were collected for measurements of sarcomere length, muscle fibre type, collagen properties, and proteolytic enzyme activities. Sarcomere length was performed on fresh samples (2, 9 and 16 days post mortem). Samples for the remaining tests were vacuum packaged and frozen in liquid nitrogen (-196°C) and stored at -80°C .

For each of the following methods the tests were performed in triplicate.

2.2. Muscle biochemistry

Samples collected for enzyme studies (24 h post mortem) were frozen in liquid nitrogen and preserved at -80°C . Calpastatin in combination with m-calpain and m-calpain was extracted from 5 g of the LD frozen samples as described by DRANSFIELD (1996) and separated by means of the two-step gradient ion-exchange chromatography method according to GEESINK and KOOHMARAI (1999). Calpain assays were determined by using azocasein as substrate according to DRANSFIELD (1996). Data were expressed as units/g of muscle. Frozen samples of the LD were analysed for collagen content and solubility according to the method described by BERGMAN and LOXLEY (1963), HILL (1966) and WEBER (1973).

2.3. Histological measurements

Samples for sarcomere lengths of fresh LD samples (24 h post mortem), were prepared according to the method of HEGARTY and NAUDÉ (1970) by using distilled water instead of Ringer – Locke solution (DREYER *et al.*, 1979). Myofibrils were extracted according to CULLER *et al.* (1978) as modified by HEINZE and BRUGGEMANN (1994). The sections were stained histochemically for myosin ATPase (myosin ATPase reveals muscular contraction) and succinic dehydrogenase (SDH reveals metabolism) simultaneously on the same myofibres (SOLOMON and DUNN 1988; VELOTTO *et al.*, 2010).

2.4. Statistical analyses

The data were subjected to analysis of variance (Gen Stat - RVSN International, Hemel Hempstead, UK; PAYNE *et al.*, 2007). Treatment means were separated using Fisher's protected t-test least significant difference at the 5% level of significance.

3. RESULTS AND DISCUSSION

Variation in MFL in the two pigs showed that Sicilian pig exhibited lower MFL compared to Landrace pig (Table 1).

Table 1. Breed comparison for histological measurements.

Myofibrillar fragment length (μm)	Sicilian pig	Landrace
2 days of aging	33.9 ^A	41.4 ^B
9 days of aging	28.1 ^A	34.4 ^B
16 days of aging	28.1 ^A	28.3 ^A
2 vs. 16 days	10.8 ^A	13.2 ^B
Sarcomere length (μm)	1.57 ^A	1.92 ^B

Different letters in the row indicate significant level of $p < 0.001$

Particularly at 16 days post mortem, the differences among breeds were not significant, whereas the changes in MFL between 2 and 9 days post mortem were higher for Landrace samples indicating a higher degree of fragmentation over the length of aging. Landrace had longer sarcomere lengths than Sicilian pig ($p < 0.001$). The fibre type composition was delineated histochemically in pig muscle samples, identifying three fibre types: FG, with high myosin ATPase activity and low oxidative activity; FOG, with moderate myosin ATPase activity and intermediate oxidative activity; and SO, with low myosin ATPase and high oxidative activity. The differences between Landrace and Sicilian pigs for the LD muscle, with regard to fibre area were 18% for FG, 18% for FOG and 22% for SO. Sicilian pig had the smallest fibre area for all fibre types and differed significantly from Landrace (Table 2).

Regarding the histological features of muscle LD, it is confirmed that is a light muscle due to the higher percentage of FG both in Landrace than Sicilian pigs (Figs. 1 and 2).

The proteolytic enzyme activity was analysed at two days post-mortem and gave the following results: landrace had higher calpastatin activity and calpastatin: m-calpain ratio and lower m-calpain activity than Sicilian pig ($p < 0.001$; Table 3), Sicilian pig had a non-significantly higher m-calpain than Landrace pig.

About collagen content were found some differences among breeds, particularly Sicilian pig had less total collagen and soluble collagen ($p < 0.001$) than Landrace.

Most of the tenderness-related results in this study clearly distinguish Sicilian pig from Landrace breed. It is important to know the physical and chemical changes that occur during storage of meat after slaughtering to develop a classification system for both the tenderness of the meat rather than optimize the same tenderness process. The importance of μ -calpain for tenderization process was reported by numerous authors (KOOHMARAIE, 1996; ROWE *et al.* 2004; LI *et al.* 2012). The mechanism of meat tenderization is complicated. Numerous investigations are being conducted which are

aimed at explaining the mechanism of meat tenderization and factors responsible for the initiation and course of this process. The activity of μ - and m-calpain is synergistic: μ -calpain contributes to early post-mortem proteolysis, while m-calpain is partially activated and contributes to tenderization during prolonged ageing (VARRICCHIO *et al.* 2013). In this study, favourable lower calpastatin (inhibitor) and higher calpain activities characterized the Sicilian pig, the lower activity of m-calpain in Sicilian pig revealed a probably activation of this enzyme in prolonged ageing. Variation in MFL among breeds, especially at 2 days post mortem, corresponded with the variation in proteolytic enzyme activity showing less fragmentation for Landrace pig in early post mortem compared with the Sicilian one. The relatively higher rate of fragmentation (measured as differences between 2 and 16 days MFL) for Landrace pig breed with prolonged aging suggested delayed aging for the tougher breeds.

Table 2. Mean value and coefficient of variation (cv,%) of morphometric characteristics of fibre types in the considered muscles.

Muscle*	Fibre Types					
	Sicilian pig			Landrace		
	FG ^a		FOG ^a		SO ^a	
	mean	v.c.,%	mean	v.c.,%	mean	v.c.,%
LD ^b	5400 ^A	36	3803.80 ^A	37	6058.42 ^A	36
			Area/ μm^2			
LD ^b	45		35		20	
			Percentage (%)			
LD ^b						
			Area/ μm^2			
LD ^b	6600 ^B	44	4649.09 ^B	45	7404.73 ^B	44
			Percentage (%)			
LD ^b	48		35		17	

*SO: red; FOG: intermediate; FG: white. ^bLD = m. *Longissimus Dorsi*. Different letters in the column indicate significant level of $p < 0.001$ (A vs. B)

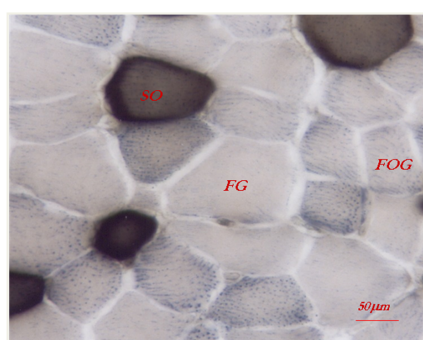


Figure 1. *Longissimus Dorsi* muscle Sicilian Pig. FOG = fast oxidative glycolytic, FG = fast glycolytic, SO = slow oxidative.

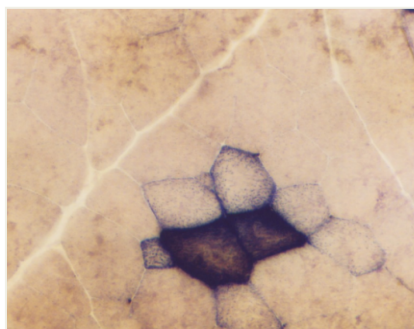


Figure 2. *Longissimus Dorsi* muscle. Landrace.

Table 3. Breed comparison for biochemical characteristics of *m. Longissimus Dorsi*.

	Sicilian	Landrace
Proteolytic enzyme activity		
Calpastatin ^a	4.3 ^B	5.3 ^A
m-calpain ^b	1.2 ^A	0.9 ^A
m-calpain ^b	0.8 ^A	0.9 ^A
Calpastatin: m-calpain ratio	4.11 ^B	5.02 ^A
Calpastatin: m-calpain ratio	5.49 ^B	6.72 ^A
Calpastatin: total calpain ratio	2.08 ^B	2.29 ^A
Connective tissue properties		
Total collagen (mg/g)	3.55 ^B	4.34 ^A
Soluble collagen (%)	18.8 ^B	22.9 ^A

^aCalpastatin activity: one unit of calpastatin is defined as the amount that inhibited one unit of m-calpain activity.

^bOne unit of calpain activity is defined as an increase in absorbance at 366 nm of 1.0 absorbance unit per gram of muscle per hour at 25°C. Different letters in the row indicate significant level of $p < 0.001$.

The activity of m-calpain was highly correlated with that of calpastatin and MFL and this finding supports the hypothesis that m-calpain initiates the breakdown of myofibril proteins. The negative correlation between m-calpain and MFL indicated that fewer proteolysis occurred in muscle when myofibril particles are large, and therefore, MFL as a determinable indicator of meat tenderness depended on activities of m-calpain. The same conclusion was found by MOHRHAUSER *et al.* (2014) according to which in their study on bovine the decrease in μ -calpain activity in the delay chilled carcasses could explain the improvements in myofibrillar fragmentation index postmortem. Several studies have demonstrated that muscle fibre type composition contributes to the variation in meat quality (VELOTTI *et al.* 2014). However, according to Table 2, the fibre characteristics of Sicilian pig and Landrace presented some differences as confirmed by their tenderness measurements supported by proteolytic enzyme activities that showed differences in favour of Sicilian pig. With the histochemical staining procedure used in this study three muscle fiber types (SO, FOG, and FG) can be differentiated. The ratio of slow-twitch oxidative (type I) and fast-twitch glycolytic (type II) muscle fibers also evidently influence

meat tenderness. The ratio varies among individual animals of the same breed, breeds, and crosses. CHRIKI *et al.* (2012) in their results showed that increased meat tenderness is related lower insoluble collagen and total collagen content, lower cross-sectional area of fibres, and an overall fibre type composition displaying more oxidative fibres than glycolytic fibres. It is possible to identify light and dark muscles in the pig. Particularly our study shows that LD muscle had a high percentage of FG fibre types in both species. Muscles harboring a higher percentage of type IIB fibers tend to result in a generally poorer meat quality than muscles harboring a higher percentage of type I fibers (CHOI *et al.* 2013). But it is possible to notice the presence of more developed SO fibres in LD in both breeds. Landrace sarcomere lengths were significantly shorter ($p<0.05$) than that of Sicilian pig. Tenderness is also positively related to the sarcomere length. Within sarcomeres, the contractile filaments actin and myosin act together in the cross-bridge cycle, determining the state of contraction post mortem. In this study sarcomere length could have contributed to the slightly tougher meat of the Landrace together with other factors discussed previously. Sicilian pig had significantly less soluble collagen than Landrace ($p<0.05$). According to WOJTYSLAK (2013) the decrease in soluble collagen content during growth of pigs are important factors influencing shear force value, and thus raw meat tenderness. Therefore, other factors also accounted for the variation in tenderness. Variation in colour and water-binding characteristics can be related to variation in the glycolysis rate and muscle temperature decline. In conclusion, the parameters analysed provided the best explanation for variation in meat tenderness and particularly it seems that the Sicilian pig has a slight disposition in terms of tenderness when compared with Landrace. Meat quality is the result of a multi-factor process which begins from the moment of slaughter and to a large extent depends on the breed and species of the animal from which the meat comes.

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Paper Received November 27, 2018 Accepted April 10, 2019

PRODUCT CARBON FOOTPRINT: STILL A PROPER METHOD TO START IMPROVING THE SUSTAINABILITY OF FOOD AND BEVERAGE ENTERPRISES

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ABSTRACT

Given the complexity of food production, supply chains and distribution, this paper sustains that the mere assessment of the product carbon footprint might still be regarded as a first trial in the field of improving the sustainability of the food and drink industry.

After having reviewed the greenhouse gas (GHG) emissions associated with the agro-food system in industrialized countries, and summarized the main direct environmental impacts of the food industry, the pros and cons of the Life Cycle assessment (LCA) methodology were briefly examined together with the current standard methods used to assess the environmental impact of food and drink products. Once a cradle-to-grave product carbon footprint modelling had been developed, some mitigating actions might be tested with the final goal of reducing the GHG emissions associated with the most impacting product life cycle stages. As an example, such a procedure was applied to approximately halve the cradle-to-grave carbon footprint of two cereal-based products (i.e., dry pasta and malt beer). A cost/benefit analysis is required to relate the marginal increase in the product processing costs to each reduction in the product environmental load.

Keywords: beer, carbon footprint, dry pasta, environmental impact, GHG emissions

1. INTRODUCTION

The current food system is regarded as ecologically unsustainable (CHURCH, 2005; FOODDRINKEUROPE, 2012; WRI, 2013), since fossil fuels are essential requirements for running crop production, animal husbandry, food production and distribution, as well for the construction and maintenance of machinery and processing equipment, transportation vehicles, and infrastructures.

The greenhouse gas (GHG) emissions associated with food production and consumption were evaluated to constitute 19-29% of the global GHG emissions (VERMEULEN *et al.*, 2012). By referring to the major environmental impact categories, such as climate change (CC), ozone depletion (OD), photochemical ozone creation (POC), acidification (A), eutrophication (NP), resource depletion, human toxicity, and eco-toxicity, the food, drink, tobacco and narcotics area of consumption in the EU-25 was estimated to generate up to 20-30% of the main impact categories (including 22-31% for CC), with the exception of 59% for NP (TUKKER *et al.*, 2006).

An increasing number of studies has dealt with the long-term sustainability of the current trends in the production and consumption of food. In particular, the EU Standing Committee on Agriculture Research (SCAR) observed that food production is near to exceed environmental limits; land use change and land degradation, as well as the dependence on non-renewable fossil energy sources, contribute about one-fourth of the GHG emissions; agriculture, including fisheries, is the single largest driver of biodiversity loss (EUROPEAN COMMISSION, 2011). The average USA and EU diet, being rich in meat, fat and sugar, is a risk for individual health, social systems and the environment. Since the world population is expected to grow from about 7 billion to 9.6 billion people in 2050, as well as the global meat and milk consumption, especially in China and India, the promotion of healthy diets can reduce the environmental footprint of food consumption (EUROPEAN COMMISSION, 2011; FAO, 2018; MORESI and VALENTINI, 2010; WRI, 2013). In addition, food processing and retail industries are asked to stimulate the necessary changes in production and consumption patterns (WRI, 2013).

The food and beverage industry is a major contributor to the EU economy (FOODDRINKEUROPE, 2018), followed by the automotive, machinery and equipment, and chemical industries. As of 2015, it was the major driver of the economy, with turnover of € 1.109 trillion, employment of 4.57 million employees with 294,000 total number of companies. Actually, 99.1% (i.e., 280,000) of the companies are small and medium-sized enterprises (SMEs), these generating 48.1% (i.e., €538 billion) of the overall turnover, 48.4% (i.e., €107 billion) of the value added and 61.3% (i.e., 2.8 million employees) of employments. Owing to its environmental and economic importance, an intergovernmental set of 17 Sustainable Development Goals has already been identified in the food sector (FOODDRINKEUROPE, 2019), this being a core part of the 2030 Agenda for Sustainable Development (UN, 2015). Beyond the general statement of decreasing environmental burdens, such as GHG emissions, waste generation, as well as water and energy consumption, Goal 9 aims at building resilient infrastructures, promoting inclusive and sustainable industrialization and fostering innovations. The complex relation between innovation and agro-food sustainability was deeply analyzed by EL BILALI (2018) in order to identify what type of innovation should be promoted to foster transition towards a more sustainable food system.

Given the complexity of food production, supply chains and distribution, this paper aimed to present how the mere assessment of the product carbon footprint might effectively help food and drink industries to improve their sustainability. Section 2

focused on the GHG emissions associated with the agro-food system in industrialized countries, and especially in Italy. Section 3 summarized the main direct environmental impacts of the food industry. Section 4 briefly reviewed the basic of the Life Cycle Analysis (LCA) methodology with the pros and cons of the main standard methods used to assess the food and drink environmental impact. Section 5 further discussed if the key elements for sustainable food processing are a priori identifiable or should be considered on a case-by-case basis. Finally, the importance of prioritizing the life cycle stages with the highest environmental impact as derived from business-to-consumer LCA studies was addressed in Section 6. More specifically, by resorting to the cradle-to-grave carbon footprint (CF_{cc}) modelling for two typical cereal-based food and drink products (i.e., dried pasta and malt lager beer), several mitigation options were selected in order to reduce their climate change impact. In spite of assessing the effect of such options on other environmental impact categories, the only estimation of the CF_{cc} was regarded as intrinsically sufficient to promote a first improvement in the sustainability of the great majority of the food and drink enterprises.

2. GHG EMISSIONS FOR THE AGRO-FOOD SYSTEM IN INDUSTRIALIZED COUNTRIES

Although from the millenary climate observations the warming since the middle of the 20th century might be primarily attributed to natural causes, such as solar activity and random variations (DE LARMINAT, 2016), the human contribution cannot be considered negligible (IPCC, 2013). The human population has grown from about 3.0 to 7.7 billion people since 1960 (ANONIMOUS, n.d.), and in all probability has exerted a primary impact on the environment. It is, indeed, responsible for the huge release of the so-called greenhouse gases (GHG), namely CO_2 , CH_4 , N_2O , hydrochlorofluorocarbons (HFCs), perfluorinated chemicals (PFCs) and SF_6 , in the atmosphere. Since 1980 the volumetric concentrations of CO_2 , CH_4 and N_2O in the atmosphere over marine surface sites have definitely increased from about 380 to 405 ppm (NOAA, n.d.), 1566 to 1835 ppb and 301 to 328 ppb (EEA, 2017), respectively.

To allow any person now living on the Earth and those expected to live until 2100 the same rights to emit GHGs, the GHG emission space per capita and in a year should be limited to 2400 kg of CO_2 , 59 kg of CH_4 , and 0.67 kg of N_2O , provided the atmospheric concentration of CO_2 is less than 450 ppm with CH_4 and N_2O emissions kept at the same levels measured in 1995 (CARLSSON-KANYAMA, 1998; IPCC, 1996). Thus, the per capita GHG emissions permitted each year within a 20-yr time perspective, as estimated by summing the mass of each GHG times its corresponding global warming potential (IPCC, 2013), would amount to $(1 \times 2400 + 84 \times 59 + 264 \times 0.67 =) 7533 \text{ kg } CO_{2e} \text{ yr}^{-1}$.

By referring to the national inventory reports (NIR) published by UNCC (2018), it is possible to assess whether such permitted GHG emissions are congruent with the ones currently in several countries. In 2007, the direct per capita emissions ranged from 24.0 to 1.6 $Mg \text{ } CO_{2e} \text{ yr}^{-1}$ for the USA and India, respectively (BERNERS-LEE, 2010).

As shown in Table 1, in 2016 the Italian GHG emissions (including those adsorbed by land use, land use change and forestry, LULUCF) amounted to circa 398 Tg CO_{2e} (ISPRA, 2018), equivalent to the Italian per capita CF of about 6.7 $Mg \text{ } CO_{2e} \text{ yr}^{-1}$. Altogether, these emissions were mainly composed of CO_2 , followed by CH_4 and N_2O , while the contribution of the halogenated compounds (i.e., HFCs, PFCs, NF_3 , and SF_6) was negligible. The main GHG emissions were from the energy sector (347.1 Tg CO_{2e}), this was followed by the industrial

(32.1 Tg CO_{2e}), agricultural (30.4 Tg CO_{2e}), and waste (18.3 Tg CO_{2e}) sectors, while the category LULUCF was the main GHG sink (-29.9 Tg CO_{2e}). More specifically, the agriculture sector mainly emitted CH₄ from animal husbandry [i.e., enteric fermentation (14.0 Tg CO_{2e}) and manure management (3.1 Tg CO_{2e})] and rice cultivation (1.7 Tg CO_{2e}), and N₂O from agricultural soils (8.9 Tg CO_{2e}) and manure management (2.1 Tg CO_{2e}). The industrial processing ones were mainly due to the iron and steel industry, followed by the chemical, and pulp, paper and print ones. The food processing, beverages and tobacco sector emitted ~3.7 Tg CO_{2e} (ISPRA (2018)).

The contribution of the agro-food sector to the overall direct GHG emissions cannot be directly extracted from any NIR. In fact, most of its subsectors (namely, agro-food product transportation; production and transportation of packaging materials; food transport from retailer to consumer's house; electric energy consumed to preserve foods in the home freezer, fridge, etc.; gas and/or electric energy consumed to cook foods; disposal of food losses or wastes) are aggregated in other sectors. The Italian contribution without the consumer and post-consumer phases was found to be about 19% of the overall GHG emissions (MORESI, 2014), this falls within the range estimated by TUKKER *et al.* (2006).

The main direct impacts of food processing are derived from waste generation, water use, and energy use (DIEU, 2009). Food waste is intense in farms due to spoilage (~21% of supply), but limited to ~7% throughout food processing. Food waste may be the loss of inedible materials or rejected products from sorting, grading, peeling, trimming, and squeezing. It may amount to the 50-70% of fresh citrus fruits or crab and shrimp processed (DIEU, 2009). Packaging materials (i.e., paper- and card-board, plastics, glass, metals, and wood) are largely used to protect processed foods not only from deterioration and/or contamination (primary packaging), but also from mechanical damage through the distribution and retailing operations (secondary and tertiary packaging).

In food processing large volumes of water are used as the main ingredient, particularly in drink production, as the initial and intermediate cleaning source, transportation conveyor of raw materials, and principal agent used in sanitizing plant areas and machinery (DIEU, 2009). The water consumption in fruit and vegetable processing ranges from 4 to 32 m³ per Mg of product treated, of which approximately 50% is used just for washing and rinsing. The water used to make beer or milk products may vary from 9 to 18 m³ Mg⁻¹. The resulting wastewaters are generally rich in organic matter, and sometimes are contaminated with pesticide residues from raw material treatments. Up to 50-60% of the water might be reclaimed and reused after screening, filtering or dilution with fresh water.

Air emissions during food processing may contain fine particles, combustion products (CO, CO₂, NO_x), volatile organic compounds, and in the case of fish by-products may contain unpleasant odorous contaminants, such as H₂S, and (CH₃)₃N (DIEU, 2009).

The energy needs of food industry are of low or medium intensity. Some sectors (e.g., wet corn milling, beet sugar, soybean oil mills, malt beverages, meat packaging, canned and frozen fruits and vegetables, bread, and baked products) are however high-energy users (DIEU, 2009). The 38% of all the energy consumed by the Italian agro-food industry is electric, while the remainder is thermal (MISE, n.d.). The total impact of energy use might be lessened by minimizing the energy needs of production, producing energy from waste, and using renewable energy sources.

Table 1. Summary report for the overall Italian direct CO₂ equivalent emissions, including or excluding the net GHG emissions adsorbed from Land Use, Land-Use Change and Forestry (LULUCF), as referred to the main GHG sources (i.e., CO₂, CH₄, N₂O, and halogenated compounds, HC, as HFCs, PFCs, NF₃, and SF₆) and sink categories in the year 2016, as extracted from ISPRA (2018).

GHG Source Sink Categories	CO ₂	CH ₄	N ₂ O	HC	Subtotal 1 Tg CO _{2e}	Subtotal 2	Total
1. Energy	334.93	7.66	4.49	0			347.08
A. Fuel combustion	332.44	2.93	4.48	0		339.86	
1. Energy industries	103.79	0.13	0.44	0	104.36		
2. Manufacturing industries and construction	46.96	0.28	0.71	0	47.94		
3. Transport	103.38	0.22	0.91	0	104.51		
4. Other sectors	77.81	2.30	2.41	0	82.52		
5. Other	0.52	0.00	0.02	0	0.53		
B. Fugitive emissions from fuels	2.48	4.73	0.01	0		7.22	
1. Solid fuels	0.00	0.04	0.00	0	0.04		
2. Oil and natural gas	2.48	4.69	0.01	0	7.18		
2. Industrial processes and product use	14.76	0.05	0.57	16.72		32.10	32.10
A. Mineral industry	10.61	0.00	0.00	0.00	10.61		
B. Chemical industry	1.46	0.00	0.12	1.49	3.08		
C. Metal industry	1.71	0.04	0.00	0.01	1.76		
D. Non-energy products from fuels and solvent use	0.98	0.00	0.00	0.00	0.98		
E. Electronic Industry	0.00	0.00	0.00	0.22	0.22		
F. Products used as substitutes for Ozone-depleting substances	0.00	0.00	0.00	14.66	14.66		
G. Other product manufacture and use	0.00	0.00	0.46	0.33	0.79		
3. Agriculture	0.54	18.87	10.98	0.00		30.39	30.39
A. Enteric fermentation	-	14.04	-	0.00	14.04		
B. Manure management	-	3.11	2.12	0.00	5.23		
C. Rice cultivation	-	1.71	-	0.00	1.71		
D. Agricultural soils	-	-	8.86	0.00	8.86		
E. Prescribed burning of savannas	-	-	-	0.00	0.00		
F. Field burning of agricultural residues	-	0.017	0.004	0.00	0.02		
G. Liming	0.01	-	-	0.00	0.01		
H. Urea application	0.53	-	-	0.00	0.53		
4. LULUCF	-31.08	0.40	0.76	0.00		-29.93	-29.93
A. Forest land	-36.08	0.28	0.001	0.00	-35.80		
B. Cropland	2.46	0.002	0.03	0.00	2.49		
C. Grassland	-6.64	0.12	0.04	0.00	-6.48		
D. Wetlands	-	-	-	-	-		
E. Settlements	9.01	-	0.68	0.00	9.69		
F. Harvested wood products	0.17	-	-	0.00	0.17		
5. Waste	0.09	16.29	1.90	0.00		18.29	18.29
A. Solid waste disposal	-	13.62	-	0.00	13.62		
B. Biological treatment of solid waste	-	0.12	0.53	0.00	0.65		
C. Incineration and open burning of waste	0.09	0.06	0.02	0.00	0.18		
D. Waste water treatment and discharge		2.49	1.35	0.00	3.84		
Total CO ₂ equivalent emissions without LULUCF							427.86
Total CO ₂ equivalent emissions with LULUCF							397.94

3. THE ENVIRONMENTAL IMPACT OF FOOD PROCESSING

The complete supply chain of the food industry from the production of raw materials via food processing to the consumption and disposal by the consumer is quite complex and is schematically sketched in Fig. 1.

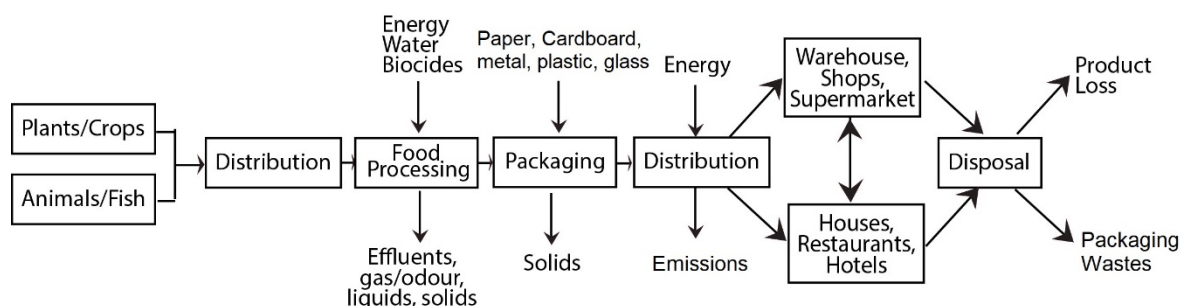


Figure 1. Simplified flow sheet of the supply chain of the food industry, as adapted from MORESI (2014).

4. LIFE-CYCLE ASSESSMENT: PROS AND CONS

Life-cycle assessment (LCA) is a technique capable of assessing the environmental impact associated with a product, process or activity during its life cycle from raw material extraction via material processing, packaging, distribution, use, repair and maintenance to the final disposal, that is from cradle to grave (MINKOV *et al.*, 2016). Its procedure is standardized by the International Organization for Standardization (ISO, 2006ab) and is performed in four different phases:

- i) *Goal and scope* of the study to set the functional unit (i.e., the reference unit), system boundaries, allocation methods and impact categories of choice, as well as the assumptions and limitations used.
- ii) *Inventory analysis* by constructing a flow chart including all the activities involved in the system boundaries and a flow model to relate all input and output data to and from the environment in order to account for 99% of the mass and energy used in the system under study.
- iii) *Impact assessment* to convert the inventory analysis results into specific environmental impact categories. These may be also categorized under the development, manufacture, use, and disposal phases of the product examined.
- iv) *Interpretation* to discuss the outcomes of the above stages, identify the data elements contributing most significantly to each impact category and measure their sensitivity, assess the completeness and consistency of the study, and provide a basis for conclusions and recommendations.

Several impact categories are used to measure the potential impacts to the natural environment, human health or depletion of natural resources. Table 2 lists the main ones together with their characterization models, as derived from MANFREDI *et al.* (2012) and MORAWICKI (2012). Thus, by summing up any release to air, water or soil Y_i (expressed in mass, energy, mass-km basis) associated to the system boundaries times its corresponding science-based conversion factor, called characterization factor ($F_{i,j}$), it is possible to estimate the score of the generic impact category (IC_j) as:

$$IC_j = \sum_i (\Psi_i F_{i,j}) \quad (1)$$

In particular, the environmental impact of climate change can be directly calculated by using the 100-year time horizon Global Warming Potentials (GWP) relative to the CO₂ of the GHGs, which were recently reassessed by IPCC (2013).

Table 2. Main impact categories used in several LCA standard methods, as extracted from MANFREDI *et al.* (2012) and MORAWICKI (2012).

Impact category	Category definition	Indicator Unit	Ref.s
Climate Change (CC)	The potential change on the Earth climate is due to human activity and GHG release.	kg CO _{2e}	IPCC(2007)
Ozone Depletion (OD)	The industrial gas concentrations accelerating O ₃ decomposition in the Earth's stratosphere affect living organisms	kg CFC-11 _e	WMO (1999)
Acidification (A)	The release of NO _x and SO ₂ which combine with water in the atmosphere forms HNO ₃ and H ₂ SO ₃ .	mol H ⁺ _e	SEPPÄLÄ <i>et al.</i> (2006)
Eutrophication- aquatic (NPA)	The release of N- and P-rich nutrients in surface waters results in excessive plant growth.	Fresh water: kg P _e ; Marine water: kg N _e	STRUIJS <i>et al.</i> (2009)
Eutrophication- terrestrial (NPT)	The deposition of N from the emissions released by N-rich nutrients affects terrestrial ecosystems too.	mol N _e	SEPPÄLÄ <i>et al.</i> (2006)
Photochemical Ozone Creation (POC)	The formation of ground-level O ₃ , as due to the reaction of NO _x and volatile organic compounds, causes irritation for humans and damage for plants.	kg NMVOC _e	VAN ZELM <i>et al.</i> (2008)
Ecotoxicity-aquatic, freshwater (ET)	Interaction among chemical compounds and organisms in the environment.	CTU _e	ROSENBAUM <i>et al.</i> (2008)
Human Toxicity- cancer effects (HTC)	Chemical compounds may cause several types of cancer in humans or	CTU _h	ROSENBAUM <i>et al.</i> (2008)
non-cancer effects (HTNC)	chronic non-cancer effects including mutagenicity, toxicity, etc.	CTU _h	ROSENBAUM <i>et al.</i> (2008)
Particulate Matter (PM)	Particulate matter causes respiratory problems.	kg PM _{2.5e}	HUMBERT <i>et al.</i> (2011)
Ionizing Radiation- human health effects (IR)	Ionizing radiation affects the risk for human cancer incidence and mortality increase.	kg U _{235e}	DREICER <i>et al.</i> (1995)
Resource Depletion- water (RDW)	Use and depletion of fresh water, minerals and fossil resources impact ecosystems and many species survival.	m ³ of water related to local water scarcity	FRISCHKNECHT <i>et al.</i> (2008)
mineral/fossil (RDMF)		kg Sb _e	VAN OERS <i>et al.</i> (2002)
Land Transformation (LT)	The extent of changes in land properties and effects on the area affected.	kg Soil Organic Matter	MILÀ I CANALS <i>et al.</i> (2007)

The environmental performance of food and drink production may be currently assessed by various standard methods, such as those listed in Table 3. Some of them (i.e., Product Carbon Footprint; PAS2050; Bilan Carbone®, BC; GHG Protocol) make use of only the impact category of climate change and give no hint about the overall environmental impact of the products, even if the emissions from direct land-use changes over the previous 20 years are generally included (Table 3). Other standard methods evaluate from seven (i.e., LCA, and Environmental Product Declaration, EPD®) to 14 (Product Environmental Footprint, PEF) impact categories. Their scores are estimated using a series

of LCA data sources and characterization factors, which obviously are strongly dependent on the LCA databases used. There is thus a strong need for reliable databases to achieve a trustworthy assessment of a product life cycle environmental performance, as observed by the food and drink companies involved in several PEF pilot tests (FOODDRINKEUROPE, 2017).

Table 3. Brief description of some international standard methods for product and service environmental assessment together with the impact categories (IC) accounted for (same labels as in Table 2).

Standard method	Description	Impact categories chosen	Ref.s
Life Cycle Assessment (LCA)	Specifies requirements and provides guidelines for LCA studies.	CC; OD; A; NP; POC; RD; LU.	ISO (2006ab)
Carbon Footprint of Product	Allows the calculation of PCF, based on LCA specified in ISO (2006ab).	CC; LUC.	ISO/TS (2013)
PAS 2050	Provides a standardized guidance for calculating the PCF of goods and services.	CC; LUC.	BSI (2008)
Bilan Carbone®	Tool developed by the French Environment & Energy Management Agency GHG to assess GHG emissions.	CC; LUC.	ADEME (2010)
Environmental Product Declaration (EPD®)	Tool supported by the Swedish government.	CC; OD; A; NP; POC; RD; LU.	ISO (2006c)
GHG Protocol	Defines how measuring, and reporting GHG emissions in the USA.	CC	BHATIA <i>et al.</i> (2011)
Product Environmental Footprint (PEF)	Novel European Community methodology under development.	CC; OD; A; NPA; NPT; POC; ET; HTC; HTNC; PM; IR; RDW; RDMF; LT.	MANFREDI <i>et al.</i> (2012)

The greater the number of impact categories accounted for, the more precise the environmental profile of the product under study will be. Nevertheless, the estimation with as many as 14 impact categories (Table 3) was harshly criticized by numerous stakeholders, such as academia (CIMINI and MORESI, 2018a; FINKBEINER, 2014; LEHMANN *et al.*, 2016), industry (ACEA, 2013; BDI, 2015), policy-makers (BMUB/UBA/TUB, 2014), and consumer associations (ANEC, 2012), for being uselessly complex and very expensive. In fact, the Federation of German Industry (BDI, 2015) estimated an average cost of about 100 k€ for assessing the PEF profile of a single product. Furthermore, some critical issues were identified to ensure that the LCA delivered robust results (NOTARNICOLA *et al.*, 2017). In particular, the intrinsic variability of the agricultural system affected the inventory analysis, impact assessment, and interpretation phases. The higher the output per hectare the higher will be the eco-efficiency of the final product. However, long-term sustainability of food production in a given production area is not considered in the current LCA method. Many LCA studies give no details about the soil, climate and weather conditions, timescale adopted, transport distances and modes used to deliver raw materials and final products, as well as the use phase and related wastes. A more meaningful functional unit for food products was also proposed by SONESSON *et al.* (2017) in order to relate the nutritional function of foods to their LCA results and account for the sustainable food consumption and food security. How to represent such variability in LCA studies without having to collect an enormous number of extra data that would make such studies disproportionately expensive is a primary

challenge for LCA researchers and practitioners. Thus, to allow small- and medium-sized food and drink enterprises to improve their sustainability in the most direct and economical method, the assessment of the Product Carbon Footprint (PCF) appeared to be more useful. In fact, not only was the climate change impact category with the lowest levels of uncertainty (CIMINI and MORESI, 2018a), but also was the major contributor to acidification ($r=0.82$), eutrophication ($r=0.66$), and photochemical ozone formation ($r=0.86$) categories (HUIJBREGTS *et al.*, 2006).

5. IDENTIFICATION OF THE KEY ELEMENTS FOR SUSTAINABLE FOOD PROCESSING

The food and beverage industry is seeking to improve its environmental performance and identify which actions are suitable for a more sustainable production (MORESI, 2014).

No food processing nowadays is 100% sustainable owing to the lack of energy, ingredients and packaging materials derived from renewable resources; excessive water use; the inherent CH₄ and N₂O emissions associated with crop production and animal husbandry; and lack of biodegradable packaging materials (MORAWICKI, 2012). Nevertheless, by accounting for only the impact category of climate change, MORAWICKI (2012) suggested a simple and progressive approach to relieve the environmental impact of a food company. First, food processing plant efficiencies for energy, water, and raw and packaging material consumption should be improved and fossil energy usage replaced with renewable one by purchase or self-generation. Second, the GHG emissions associated with the transportation of raw materials and final products should be reduced. Third, the GHG emissions resulting from the field phase should be minimized. Fourth, the impact of the post-consumer disposal of packaging materials, as well as food loss, is to be reduced.

Despite being firm-oriented, such an approach might result in mitigation actions exerting a minimum reduction in the product carbon footprint. Thus, the mitigation opportunities should be prioritized starting from the life cycle stages with the highest contribution to PCF, as previously assessed (CIMINI and MORESI, 2018b). This procedure was specifically applied to improve the sustainability of two typical cereal-based food and drink products, as detailed in the following cases studies.

6. CASE STUDY NO. 1: LAGER BEER PRODUCTION

The cradle-to-grave carbon footprint (CF_{cg}) of a malt lager beer was previously estimated (CIMINI and MORESI, 2016, 2018c) by applying the PAS 2050 standard method (BSI, 2008). All the aforementioned four LCA canonical stages were referred to a functional unit consisting of 1 hL of malt beer, as produced in a large-sized brewery with an annual beer capacity of 3x10⁶ hL and packed in 66-cL glass bottles. The system boundaries for this case study are shown in Fig. 2. According to PAS 2050 (Section 7.2), the geographical and time scopes of this LCA study are the Western Europe and from the years 2006-2016. Main process data were of the primary type (CIMINI and MORESI, 2016).

By using all the essential data previously given (CIMINI and MORESI, 2018b), the LCA model was able to estimate the CF_{cg} as 127 kg CO_{2e} per hL of beer. The contribution of the different life cycle stages are shown in Fig. 3.

BEER PRODUCTION SYSTEM BOUNDARIES

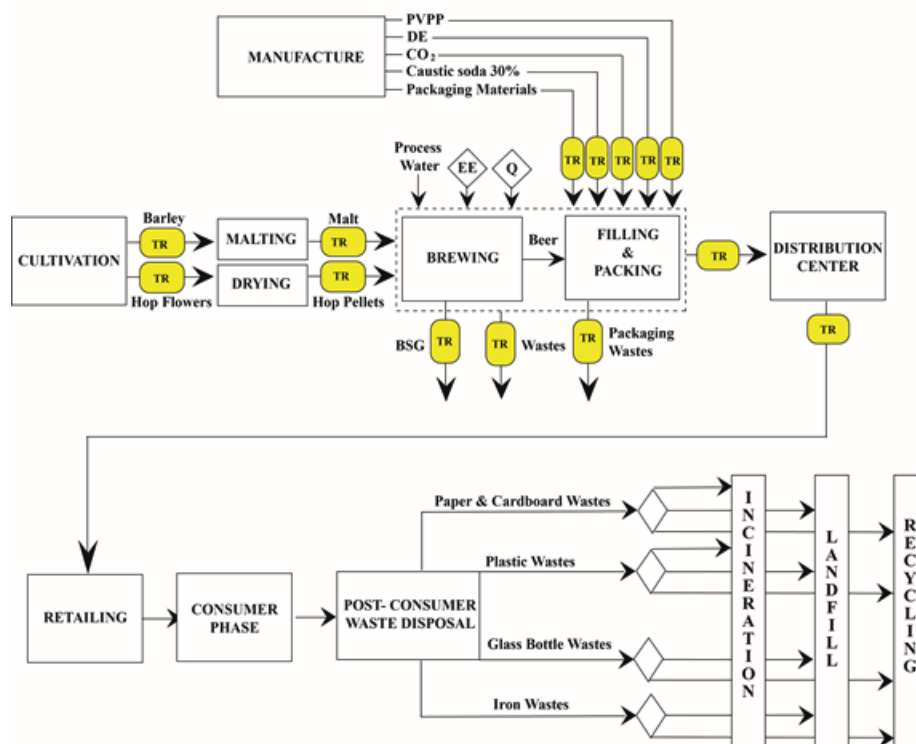


Figure 2. Beer system boundaries, as adapted from CIMINI and MORESI (2018c). The main identification items are listed in the *Abbreviations and Nomenclature* section.

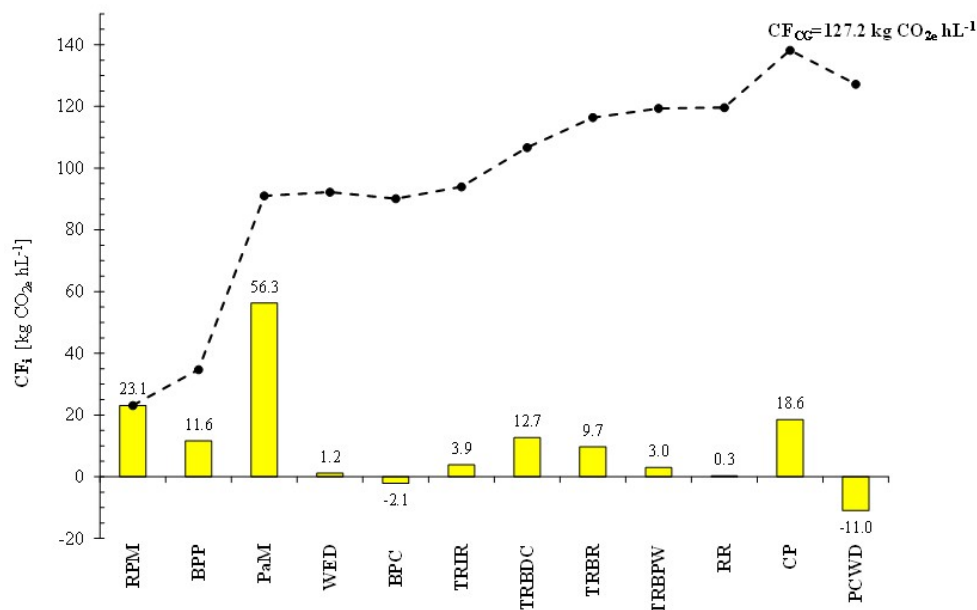


Figure 3. Contribution of the different life cycle phases to the cradle-to-grave carbon footprint (CF_{cc}) of 1 hL of beer packed in 66-cL glass bottles in a large-sized brewery, as estimated from the LCA model previously developed (CIMINI and MORESI, 2018b), and its cumulative score (see broken line). For the identification items refer to the *Abbreviations and Nomenclature* section.

The life cycle phases contributing mostly to the CF_{cg} , in descending order, were associated with packaging material manufacture ($\sim 56 \text{ kg CO}_{2e} \text{ hL}^{-1}$), overall transportation ($\sim 29 \text{ kg CO}_{2e} \text{ hL}^{-1}$), production of malted barley and processing aids ($\sim 23 \text{ kg CO}_{2e} \text{ hL}^{-1}$), consumer use ($\sim 19 \text{ kg CO}_{2e} \text{ hL}^{-1}$), beer production and packaging ($\sim 12 \text{ kg CO}_{2e} \text{ hL}^{-1}$), and waste disposal ($1.2 \text{ kg CO}_{2e} \text{ hL}^{-1}$). CO_{2e} credits derived from the use of spent grains and surplus yeast as animal feed ($2.1 \text{ kg CO}_{2e} \text{ hL}^{-1}$) and from recycling of glass bottles, paper and cardboard wastes ($11 \text{ kg CO}_{2e} \text{ hL}^{-1}$).

Instead of adopting the aforementioned MORAWICKI's approach to sustainability, a series of improvement opportunities was scheduled to sequentially reduce the contribution of the most impacting life cycle phases of the above reference case.

Firstly, the replacement of 10% recycled glass bottles with 100% recycled ones reduced the CF_{cg} by about 21 % with respect to the reference case. By shifting the transportation mode from 100% of road freight to 100% of rail freight to manage logistics flows, an additional 10% decrease in CF_{cg} was achieved. The use of organic instead of conventional barley grown locally had the effect of decreasing the CF_{cg} by another 9%. A quasi zero-carbon alternative for electricity generation is solar-photovoltaic electricity. Such a shift further lessened the CF_{cg} by 13%. On the contrary, by reducing the delivery distance of malted barley from 500 to 250 km, no significant change was observed in the CF_{cg} ; hence, reducing distance had a negligible effect.

Table 4 shows all the emission factors (EF) that were varied and how the above sequential series of mitigation options practically halved the beer carbon footprint from about 127 to 60 $\text{kg CO}_{2e} \text{ hL}^{-1}$.

Since the per capita consumption of beer in Italy is about 31.8 L yr^{-1} (ASSOBIRRA, 2018) and the current Italian population is 59,228,336 (WORLDOMETERS, 2019), the GHG emissions associated with the Italian consumption of beer would be reduced from 2.39 to 1.13 $\text{Tg CO}_2 \text{ yr}^{-1}$. The application of the aforementioned mitigating actions had the effect of limiting the contribution from beer to 0.28% of the overall Italian GHG emissions (Table 1).

Table 4. Effect of the sequential mitigation strategies used to minimize the cradle-to-grave beer carbon footprint (CF_{cg}) and its cumulative percentage variation with respect to that pertaining to the reference case ($\sum \frac{\Delta CF_{cgj}}{CF_{cg}^*}$). The sequential stepwise procedure started from the most impacting life cycle phase as resulting from Fig. 3.

Mitigation strategy	Parameter varied		Unit	CF_{CG} [kg CO _{2e} hL ⁻¹]	$\sum \frac{\Delta CF_{CGj}}{CF_{CG}^*}$ [%]
Beer reference case (*)				127.2	0
100% recycled glass bottles	EF _{RB}	1.08→0.48	kg CO _{2e} kg ⁻¹	100.3	-21
Malt & beer rail transport	EF _{RT}	0.168→0.039	kg CO _{2e} (Mg km) ⁻¹	88.2	-31
Organic malt	EF _{OC}	1.143→0.546	kg CO _{2e} kg ⁻¹	76.6	-40
Local malt	d _{RM}	500→250	km	76.5	-40
Photovoltaic electric energy	EF _{PEE}	0.324→0.055	kg CO _{2e} kWh ⁻¹	60.2	-53

7. CASE STUDY NO. 2: DRY PASTA PRODUCTION

The cradle-to-grave CF_{cg} of an organic durum wheat semolina pasta was previously estimated (CIBELLI *et al.*, 2017; CIMINI *et al.*, 2019c) using the PAS 2050 standard method (BSI, 2008). All the LCA canonical stages were referred to a functional unit consisting of 1

kg of dry pasta, produced in a medium-sized pasta factory with a capacity of approximately 125 Gg yr⁻¹ and packed in 0.5-kg polypropylene (PP) bags. The system boundaries for this case study are shown in Fig. 4. According to the PAS 2050 (Section 7.2), the geographical and time scopes of this LCA study were the Western Europe and from the years 2006-2016. Finally, the process data were of the primary type, as reported by CIMINI *et al.* (2019c).

The estimated dry pasta CF_{CG} was about 1.8 kg CO_{2e} kg⁻¹, the contribution of all the life cycle phases being plotted in Fig. 5. Their impacts were therefore ranked as follows: field phase (~0.67 kg CO_{2e} kg⁻¹), home pasta cooking (0.65 kg CO_{2e} kg⁻¹), pasta production and packaging (~0.20 kg CO_{2e} kg⁻¹), transportation (~0.15 kg CO_{2e} kg⁻¹), packaging material manufacture (~0.11 kg CO_{2e} kg⁻¹), durum wheat milling (~0.05 kg CO_{2e} kg⁻¹), end of life of packaging materials (~0.03 kg CO_{2e} kg⁻¹) and pasta losses (~0.02 kg CO_{2e} kg⁻¹). CO_{2e} credits resulted from using wheat milling by-products, and pasta making and packaging wastes for animal feed (~0.07 kg CO_{2e} kg⁻¹) as an alternative to soybean meal fodder (CIMINI *et al.*, 2019c).

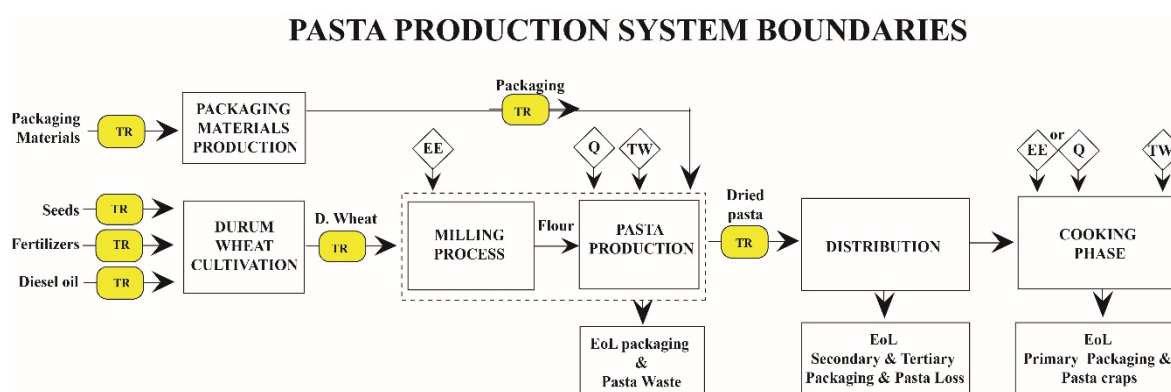


Figure 4. Dry pasta system boundaries, as adapted from CIMINI *et al.* (2019c). The main identification items are listed in the *Abbreviations and Nomenclature* section.

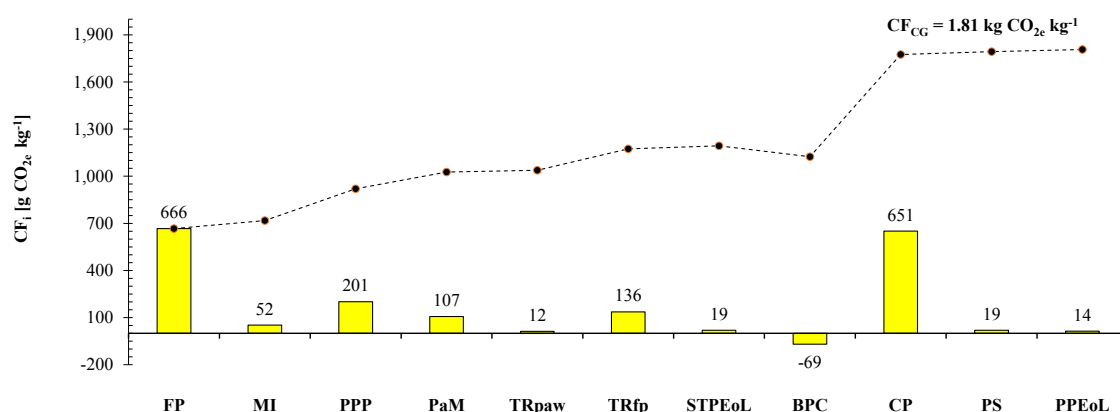


Figure 5. Contribution of the different life cycle stages to the cradle-to-grave carbon footprint (CF_{CG}) of 1 kg of dried organic pasta packed in 0.5-kg PP bags in a medium-sized pasta factory, as estimated from the LCA model previously developed (CIMINI *et al.*, 2019c), and its cumulative score (see broken line). The main identification items are listed in the *Abbreviations and Nomenclature* section.

To improve the sustainability of such product, a series of mitigating actions were programmed to reduce the contribution of the most impacting life cycle phases of the above reference case. In particular, to limit the impact of the primary hotspot (i.e., the consumer and post-consumer ones), the eco-sustainable pasta cooking procedure suggested by CIMINI *et al.* (2019ab) was applied by setting the cooking water-to-dry pasta ratio at 2 L kg⁻¹ and the nominal cooking power at 0.4 kW. In this way, the CF_{cg} was cut by 29% with respect to the reference case. Use of organic crop rotation enabled the CF_{cg} to be decreased by another 13%. By replacing the methane needed for the steam generating boilers with biogas, the CF_{cg} was further reduced by 7%. Use of solar-photovoltaic electricity also lessened the CF_{cg} by an extra 9%. Similarly, by shifting from road to rail freight transport, a supplementary 2% reduction in the CF_{cg} was obtained. Finally, when the final product or grain delivery distance was shortened from 900 or 150 km to as low as 250 or 50 km, respectively, the CF_{cg} still reduced by 2 or 1%. In total, such a sequential series of mitigating options allowed the dry pasta carbon footprint to be reduced from 1.81 to 0.68 kg CO_{2e} kg⁻¹ (Table 5).

Table 5. Effect of the sequential mitigation strategies used to minimize the cradle-to-grave dry pasta carbon footprint (CF_{cg}) and its cumulative percentage variation with respect to that pertaining to the reference case ($\sum \frac{\Delta CF_{cgj}}{CF_{cg}^*}$). The sequential stepwise procedure started from the most impacting life cycle phase as shown in Fig. 5.

Mitigation strategy	Parameter varied		Unit	CF _{cg} [kg CO _{2e} kg ⁻¹]	$\sum \frac{\Delta CF_{cgj}}{CF_{cg}^*}$ [%]
Dry pasta reference case (*)				1.81	0
Eco-sustainable cooking	P _C	2.3→0.4	kWh kg ⁻¹	1.28	-29
Organic rotation cropping	EF _{OC}	0.534→0.36	kg CO _{2e} kg ⁻¹	1.06	-42
Thermal energy from biogas	EF _{BG}	0.231→0.029	kg CO _{2e} kWh ⁻¹	0.92	-49
Photovoltaic electric energy	EF _{PEE}	0.513→0.055	kg CO _{2e} kWh ⁻¹	0.77	-58
Pasta rail transport	EF _{RT}	0.168→0.047	kg CO _{2e} (Mg km) ⁻¹	0.72	-60
Pasta regional distribution	d _P	900→250	km	0.70	-62
Durum wheat local supply	d _{RM}	150→50	km	0.68	-63

Since the per capita consumption of pasta in Italy is about 23.5 kg yr⁻¹ (UNAFPA, 2015), the GHG emissions associated with the Italian consumption of dry pasta would reduce from 2.52 to 0.95 Tg CO₂ yr⁻¹. The aforementioned mitigating actions had the effect of reducing the impact of the dry pasta sector to the 0.24% of the overall Italian GHG emissions (Table 1).

8. CONCLUSIONS

In this work, the main direct environmental impacts of the food industry and GHG emissions for the agro-food system in industrialized countries were analyzed together with the main advantages and disadvantages of the standard methods currently used to assess the food and drink environmental impact.

Owing to the great deal of money needed to characterize the whole environmental profile of a single product, and the fact that the climate change impact category was by far more reliable than all the other ones used in the EPD® and PEF standard methods made the assessment of the product carbon footprint a cheaper tool to identify the major hotspots of the food supply chain. Thus, it is probably the best method to start improving the sustainability of the 99% of the food and beverage SMEs. It was used here to select a sequential series of mitigating actions in order to reduce the cradle-to-grave product carbon footprint (CF_{cc}) of 1 hL of beer packed in 66-cL glass bottles from about 127 to 60 kg CO_{2e} hL⁻¹, and that of 1 kg of dry organic pasta packed in 0.5-kg PP bags from 1.81 to 0.68 kg CO_{2e} kg⁻¹. A cost/benefit analysis might help SMEs to relate the marginal increase in the overall final product costs to each reduction in the product environmental load.

Since only the assessment of GHG emissions might result in burden shifting, a further step should investigate the effect of the selected mitigating actions on other environmental impact categories.

ABBREVIATIONS AND NOMENCLATURE

A	Acidification
BC	Bilan Carbone®
BPC	CO _{2e} credits from by-product use as cattle feed;
BPP	Brewing and packaging processing
BSG	Brewer's spent grain
CC	Climate Change
CF _{cc}	Cradle-to-grave product carbon footprint [kg CO _{2e} hL ⁻¹ or kg ⁻¹]
CFC	Trichlorofluoromethane or Freon-11
CO _{2e}	Carbon dioxide equivalent
CP	Consumer phase
CTU _e	Comparative Toxic Unit for ecosystems
CTU _h	Comparative Toxic Unit for humans
DE	Diatomaceous earth
DC	distribution centers
d _p	Distribution distance of packed dry pasta [km]
d _{RM}	Supply distance of raw materials [km]
EE	Electric energy
EF _{BG}	Emission factor for biogas [kg CO _{2e} kWh ⁻¹]
EF _{OC}	Emission factor for organic crop [kg CO _{2e} kg ⁻¹]
EF _{PEE}	Emission factor for photovoltaic electric energy [kg CO _{2e} kWh ⁻¹]
EF _{RB}	Emission factor for 100% recycled glass bottles [kg CO _{2e} kg ⁻¹]
EF _{RT}	Emission factor for rail freight transport [kg CO _{2e} (Mg km) ⁻¹]
EOl	End of life
EPD	Environmental Product Declaration
ET	Ecotoxicity – aquatic, freshwater
EU	European Union
F _i	Generic i-th characterization factor of the j-th impact category
FP	Field phase
GHG	Greenhouse gas
HC	Halogenated compound
HFC	Hydrochlorofluorocarbon
HTC	Human toxicity - cancer effects
HTNC	Human toxicity - non-cancer effects
IC	Impact category
IR	Ionizing radiation – human health effects
LCA	Life cycle assessment
LT	Land transformation
LULUCF	Land use, land use change and forestry
MI	Milling
NIR	National Inventory Report
NMVOc	Non-methane volatile organic compound

NP	Eutrophication
NPA	Eutrophication- aquatic
NPT	Eutrophication- terrestrial
OD	Ozone depletion
PaM	Packaging materials
PAS	Publicly available specification
P_c	Specific food cooking power [kWh kg ⁻¹]
PCF	Product carbon footprint
PCWD	Post-consumer waste disposal
PEF	Product environmental footprint
PFC	Perfluorinated chemical
PM	Particulate matter/respiratory inorganics
POC	Photochemical ozone creation
PP	Polypropylene
PPEoL	Primary packaging end of life.
PPP	Pasta production and packaging
PS	Pasta scraps
PVPP	Polyvinylpyrrolidone
Q	Thermal energy
RD	Resource depletion
RD MF	Resource depletion – mineral/ fossil
RDW	Resource depletion – water
RPM	Raw and processing materials
RR	Retailer refrigeration
r^2	Coefficient of determination
SME	Small- and medium-sized enterprise
STPEoL	Secondary and tertiary packaging end of life
TR	Transportation
TRBPW	Transportation of byproducts and wastes
TRBR	Transportation of beer in cartons from DCs to retailers
TRBDC	Transportation of palletized beer to distribution centers
TRfp	Transport of final product
TRIR	Transportation of input resources to the brewery gate
TRpaw	Transport of packaging and auxiliary materials, and wastes
TW	Process water
WED	Waste and effluent disposal
$\Delta CF_{cc}/CF_{cc}$	Relative percentage variation of CF_{cc} with respect to that pertaining to the reference case [%]
Ψ_i	Generic i-th activity, expressed in mass, energy or mass-km basis.

ACKNOWLEDGMENTS

This research was supported by the Italian Ministry of Instruction, University and Research, special grant PRIN 2015 – prot. 2015MFP4RC_002.

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Paper Received February 8, 2019 Accepted May 15, 2019

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Bills D.D. 1982. Private communication. USDA-ARS. Eastern Regional Research Center, Philadelphia, PA.

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Gratitude is expressed to the following entities for contributing to the realization of the Journal by being supporting subscribers for 2019.

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ITALIAN JOURNAL
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Rivista Italiana di Scienza degli Alimenti
DIRETTORE RESPONSABILE: Alberto Chiriotti
AUTORIZZAZIONE: n. 3/89 in data 31/1/1989
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