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OPINION PAPER

ITALY on the spotlight: EXPO MILAN 2015 and Italian Journal of Food Science

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The year 2015 will certainly be remembered as the Year of the Universal Exposition (EXPO) hosted in Milan, Italy, focusing on a hot theme in the current scenario: "Feeding the Planet, Energy for Life".

This event has drawn a wide international attention towards Italy as a country with peculiar and valuable food traditions, thus strengthening its reputation as "gastronomic capital of the world" rich in protected designation of origin products (PDOs) and characterised by a long-standing food culture.

EXPO 2015 has been a global platform to showcase a vast range of food traditions, as well as a unique opportunity to appreciate different cultures through food practices, thus reflecting on the values that bring people together. EXPO 2015 was a chance for scientists, regulators, industry representatives and buyers, to meet and share cutting-edge knowledge and opinions, and to start fruitful collaborations and networks.

Meetings and conferences scheduled within the EXPO 2015 calendar encompassed various themes, with particular focus on sustainability

- Keywords: Expo 2015, Feeding the Planet, Food Safety, Food Security, Nutrition, Shelf Life, Biodiversity -

of the agri-food system, conservation of biodiversity, protection of environment and its natural resources, such as water, land and soil. Emerging agri-food technologies were also recognised as essential for understanding natural phenomena, monitoring production factors, tracing food products, managing emergency and crisis, ultimately leading to improvement of well-being and prosperity worldwide.

Scientists involved in the fields of agriculture, environment and food processing are challenged by these emerging themes with the task to advance their studies in order to have an impact on education, training and future policies of the agri-food sector.

The Italian Journal of Food Science has been deeply involved, in the last two years, in the diffusion of new ideas and exchange of knowledge on the topics of EXPO 2015.

A primary EXPO 2015 subject was product sustainability, i.e. new and alternative methods of agricultural production aimed to reduce the environmental impact of food production. Accordingly, Lo GIUDICE *et al.* (2013) proposed different approaches to lessen the production impact of Sicilian blood oranges on the environment through the application of the Life Cycle Assessment (LCA) methodology, while GIORDANO *et al.* (2013) studied the influence of cropping conditions on volatile fingerprint and mechanical properties of Muscat blanc (*Vitis vinifera* L.) grapes grown in the north-west mountain region of Italy.

Another relevant EXPO 2015 topic was nutrition and biodiversity, in the awareness that lesser-known species rich in bioactive compounds can play an important role in human health. Thus, the research presented by JURIKOVA *et al.* (2014) tackled at the same time sustainability, nutrition and biodiversity, assaying the antioxidant properties of selected cultivars of interspecific crosses of rowan (*Sorbus aucuparia* L.), a thrifty species with high frost resistance. On the other hand, TRIPALDI *et al.* (2014) combined tradition and new consumer requirements by evaluating traditional and reduced dry salting methods of an Italian valuable PDO product, Pecorino Romano cheese.

The development of new technologies in food science was fostered by MURA *et al.* (2014), which focused on the advances of nanotechnology in food and animal science, as this emerging multidisciplinary field can potentially improve the nutritional value, quality and shelf life of several food products, leading to crucial advantages in terms of consumer safety. The concept of food safety was approached by YASAR and BOSELLI (2015) in a survey on perception and awareness of European Union food safety policy by different socio-demographic groups of consumers.

IJFS strives to promote the advancement of Food Science: therefore, we would like to acknowledge all the Authors for their contribution and the Reviewers for their valuable advice.

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PAPER

DETERMINATION OF THE OPTIMAL FAT AMOUNT IN DRY-RIPENED VENISON SAUSAGE

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ABSTRACT

Six types of salchichon sausage were made using cynegetic venison lean and different amounts of pork meat (40, 30, 25, 20, 15 and 10%) in order to choose the lowest fat content without decreasing the sensory quality of the traditional salchichon sausage. All samples were evaluated using quantitative descriptive sensory analysis, finding significant differences; as the amount of pork meat increased, sausages exhibited a lighter colour and an intense spice and cured odour, as well as being juicier and easier to chew. Furthermore, consumer tests were carried out. All types of sausages were accepted by consumers (scores > 5.5 for all attributes) finding significant differences in the preference test.

- Keywords: consumer test, fat content, quantitative descriptive sensory analysis, venison salchichon sausage -

INTRODUCTION

The production of cynegetic deer in Spain is high, accounting 148195 animals hunted in 2011 (MAGRAMA, 2014); however, its economic value is relatively low because it is considered to be simply a by-product of hunting, oriented to obtain flashy awards. In the autonomous community of Castilla-La Mancha (central Spain), the area designated for hunting spans more than 7 million hectares of which almost 2 million are designated for big game hunting, mainly venison and wild boar (JCCM, 2010). Castilla-La Mancha is also the main venison exporter in Spain, constituting 80% of the total exportation, being Germany its primary destination. Despite the large venison production, its consumption is fairly limited in the region and in Spain generally. Venison is mainly consumed in certain rural areas and restaurants.

Cynegetic venison is a highly nutritive food characterized by a high protein and heme iron content, and a low presence of subcutaneous and intramuscular fat (ZOMBORSZKY *et al.*, 1996; HOFFMAN and WIKLUND, 2006). In addition, this meat has specific organoleptic properties that differ from other species such as its intense and attractive red colour, tenderness and variety of flavours (SORIANO *et al.*, 2009), reflecting the fact that the animals live in the wilderness and nourish on naturally occurring feed.

A wide range of cured products are obtained from cinegetic deer meat, including cecina (dry-cured meat), and dry fermented sausages, as chorizo and salchichon. These are generally labelled "gourmet products" in the international market. To make venison salchichon, an appropriate amount of pork fat has to be added to obtain gradual drying as well as sensory characteristics such as juiciness, tenderness and flavor. In the traditionally produced cynegetic venison salchichon, the fat content is around 40-50% (habitual practices of local manufacturers in Castilla-La Mancha region). Today, this fat amount is considered excessive in terms of the WHO recommendations for a healthy diet (WHO, 2004), which suggest the consumption of low-fat foods. These recommendations are followed by meat manufacturers that are producing meat products containing smaller amounts of fat. On the other hand, an excessive amount of fat can cause an excessive oxidation of the lipids or rancidity (SORIANO *et al.*, 2010).

Several studies have been carried out to characterise the physicochemical and sensory quality of venison (STEVENSON *et al.*, 1992; PEÑA *et al.*, 1993; ZOMBORSZKY *et al.*, 1996; WIKLUND *et al.*, 2001 and 2003). However, very few studies have been reported on microbiological, physicochemical and sensory characteristics of dry sausages made with venison (VIOQUE *et al.*, 2003; SORIANO *et al.*, 2006; GARCÍA RUIZ *et al.*, 2010; SORIANO *et al.*, 2010; UTRILLA *et al.*, 2014). Until now, no study has focused on the reduction of fat in cured sausages made with meat from cynegetic species. However, several scientific studies have been carried out to reduce the fat content in dry-ripened sausages made with pork and/or beef (PAPADIMA and BLOUKAS, 1999; MENDOZA *et al.*, 2001; MUGUERZA *et al.*, 2002; LIAROS *et al.*, 2009; OLIVARES *et al.*, 2010; OLIVARES *et al.*, 2011), foal and pork meat (LORENZO and FRANCO, 2012).

The objective of this study was to obtain a healthier venison salchichon with the lowest fat content that at the same time maintains sensory characteristics of the traditional salchichon.

MATERIAL AND METHODS

Raw materials

Lean venison (*Cervus elaphus*) was obtained from hind legs of male deer obtained during the 2008-2009 hunting season on three neighbouring reserves in Ciudad Real (central Spain). Vegetation in the three reserves was very similar, comprising pine forests, woodlands and scrub. A total of 69 kg of venison was used for each replicate of the experiment. Pork meat with a high fat content was obtained from castrated male pigs (progeny of a Pietrain male x Dalain female cross) raised intensively and slaughtered at the age of seven months. A total of 21 kg of pork meat was used for each replicate of the experiment. A commercial salchichon formula (Ceylamix Salchichón Casero 933, Manufacturas Ceylan S.L., Valencia, Spain) was used, comprising salt, spices, lactose, saccharose, polyphosphates (E-450i, ii), sodium ascorbate (E-301) and potassium nitrate (E-252).

Cynegetic venison salchichon sausage production

Six types of venison salchichon were made, each containing a different proportion of pork meat (40, 30, 25, 20, 15 and 10%) and lean venison (60, 70, 75, 80, 85 and 90%, respectively). Types were labelled from Type 1 (40% pork meat and 60% lean venison) to Type 6 (10% pork meat and 90% lean venison) (Table 1). Venison and pork meat were minced separately in an Unger

Table 1 - Percentages of raw meats used to elaborate the different types of cynegetic venison salchichon.

Type	Venison (%)	Pork meat (%)
1	60	40
2	70	30
3	75	25
4	80	20
5	85	15
6	90	10

W-98 mincer (Andher, Campo de Criptana, Spain) with an 8 mm plate. Venison was then mixed with the appropriate proportion of pork meat and the Ceylamix commercial formula (33 g/kg mixture) previously dissolved in 1 l of cold mineral water, in an AV-80 vacuum mixer (Andher, Campo de Criptana, Spain). The mixture was covered with a cotton cloth, and left to settle for 20 h at 0°C, in order to the whole mass could get the spices and additives. It was then fed through an H52 PAS hydraulic piston-based sausage stuffer connected to a VAE-10 vacuum system (Andher, Campo de Criptana, Spain), into synthetic collagen skins, with a 38-40 mm diameter. Horseshoe-shaped salchichon sausages were then tied off at 60 cm intervals. Average weight of sausages was 737 g. Salchichon sausages were maintained at 20-22°C and a relative humidity of 60% for 2 h, and finally ripened at 11°-12°C and the relative humidity of 75% for 28 days, in a Zanotti curing chamber (Grupo Momplet, Valencia, Spain). After ripening, salchichon sausages were vacuum-packed and stored at 8-10°C for 45 days until its evaluation. The six types were made in duplicate, so the same experiment was repeated on two different dates, in order to maximize reproducibility of the results.

Quantitative descriptive sensory analysis

The quantitative descriptive sensory analysis was carried out in a tasting room that was equipped in accordance with UNE-EN ISO 8589:2010.

Judges

The evaluation of the samples was carried out by a panel test formed by 9 panellists (6 women, 3 men, ages 25-52 years). The panel was previously trained in the evaluation of the attributes and scales employing different commercial venison salchichon. The qualification of the panel members is based on reproducibility verification and concordance between the tasters.

Attributes

A focus group was organized to discuss and choose the most apropiate attributes. The sensory evaluation of the attributes was carried out using non-structured scales of 10 cm and in accordance with UNE-ISO 4121:2006. All the scales were anchored at the extremes with the terms "weak" and "very intense," except for the colour intensity scales in which the colour was indicated at the extremes. The visual attributes evaluated were: amount of fat, fat colour (0=white; 10=yellow) and lean colour (0=light pink; 10=black). The colour scales used were photographs of different types of venison salchichon sausages. The olfactory attributes studied were: black pepper, spices and cured odour as well as odour intensity. The attributes that defined the texture profile of the samples were: hardness, juiciness, chewiness and fat mouth-feel. Finally, taste attributes evaluated were the following: intensity of the taste, salty and pepper taste and intensity of the aftertaste. Data collection was organized on paper.

Preparation of the samples

The samples were presented to the panellists in 3 mm thick slices without skin, at 20°-22°C (room temperature) and tagged (number-letter-number). Three samples were evaluated per sesión at a time. Unsalted crackers and mineral water were provided to the panellists to cleanse the mouth between samples. Samples were presented in all possible orders at each tasting session in order to minimise any effects due to order of presentation. All samples were evaluated in duplicate.

Consumer tests

The consumer tests were carried out in a tasting room equipped in accordance with UNE-EN ISO 8589:2010.

Consumers

A group of 138 habitual consumers of salchichon sausage was used: 42 men aged between 20 and 49, and 96 women aged between 19 and 54. Consumers were recruited from students, professors and staff of the Food Science and Technology Area of the University of Castilla-La Mancha.

Preparation of the samples

Samples were presented at 20°-22°C (room temperature) in 3-mm slices, without skin, using a 3-character alphanumeric code. Samples were presented in all possible orders at each tasting session in order to minimise any effects due to order of presentation. Unsalted crackers and mineral water were provided to the panellists to cleanse the mouth between samples. Consumers were instructed to carry out their evaluation for overall acceptability considering the cross section external appearance, odour, taste and texture of the slices. In one session, consumers evaluated the six samples corresponding to the six different types of venison salchichon sausage.

Acceptance test

To grade the acceptability of each sample, consumers used a non-structured or linear hedonic scale of 10 cm, anchored at either end by the phrases "strongly like" and "strongly dislike", enabling consumers to mark the point which best represented their satisfaction with the sample. Attributes were evaluated in the order: odour,

aspect, texture, taste and overall acceptance. Data collection was organized on paper.

Preference test

A hedonic ranking test was used (UNE-ISO 8587:2010), whereby each consumer was presented with a sample from each type and asked to order the samples by degree of preference, giving 1 point to the least preferred and 6 to the most preferred.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to study the influence of the amount of fat in the attributes evaluated in the quantitative descriptive sensory analysis and the acceptance test. When the interaction was significant, the averages were compared using the Student-Newman-Keuls test. Friedman's (non-parametric) test was performed, following standard UNE-ISO 8587:2010, to check the significance of differences between consumer preferences, and differences between particular sample means were analysed according the Fisher's least significance difference (LSD). All statistical procedures were carried out using the SPSS 19.0 statistical software package for Windows XP (SPSS, Inc, Chicago, Il, USA) with updating rights (License UCLM 7876875).

RESULTS AND DISCUSSION

Quantitative descriptive sensory analysis

Visual attributes of the cynegetic venison salchichon sausage with different amount of pork meat added are shown in Table 2. Significant differences were found for the three studied attributes. The types with the highest fat content exhibited the pinkest colored lean while the types with the lowest fat content were dark brown. The fat colour in all samples was white except for samples bellowing Type 6 (10% fat), which exhibited a more yellowish colour. This was possibly influenced by the darker colour of the lean. Furthermore, all samples showed an amount of visible fat that was directly proportional to the pork meat added during the elaboration.

Attributes that defined the olfactory profile are shown in Table 3. Significant differences were found for all studied attributes. Samples with the highest amount of fat, Types 1, 2 and 3 (40-25%), exhibited a higher intensity of odour (7.6-8.0) and a more pronounced spice, black pepper and cured odour in comparison to Types 4, 5 and 6 (20-10%) which despite having elevated odour intensity (6.8-7.1) exhibited less intensity in all of the attributes studied. These results do not coincide with those obtained by MENDOZA *et al.* (2001), which did not find significant differences in the intensity of odour, obtaining scores between 6.5-7.5 (in a scale of 1-10) with regard to the fat content (6.3, 12.5 and 25% of fat). It should be noted that the authors obtained scores slightly lower than those obtained in this study, possibly due to the higher olfactory intensity of venison in comparison to beef and pork. On the other hand, odour intensity, spice and ripened odour presented similar scores than those obtained by GARCÍA RUIZ *et al.* (2010) in a study about sensory properties of venison sausages made with 50% venison lean and 50% pork meat.

Table 4 organizes the scores awarded by the panellists for the attributes that defined the texture profile of the cynegetic venison salchichon sausage with different quantities of pork meat added. Samples bellowing Type 1 exhibited the lowest

Table 2 - Visual attributes (means ± standard deviations) of the cynegetic venison salchichon with different pork meat added.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Amount of fat	7.19^{a}±0.78	6.58^{b}±0.93	5.95^{c}±0.96	5.07^{d}±0.92	3.86^{e}±0.77	2.32^{f}±0.59
Fat colour	2.03^{a}±0.73	2.28^{a}±0.73	2.26^{a}±0.85	2.23^{a}±0.67	1.98^{a}±0.65	2.85^{b}±1.06
Lean colour	3.12^{a}±0.60	4.36^{b}±1.10	3.92^{b}±0.85	6.02^{c}±1.07	5.76^{c}±0.84	7.24^{d}±1.06

Different superscripts (a, b, c, d, e, f) in the same row denote significant differences (P<0.05).
Type 1 (40% pork meat); Type 2 (30% pork meat); Type 3 (25% pork meat); Type 4 (20% pork meat); Type 5 (15% pork meat); Type 6 (10% pork meat).

Table 3 - Odour attributes (means ± standard deviations) of the cynegetic venison salchichon with different pork meat added.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Odour intensity	7.96^{a}±0.72	7.67^{a}±0.57	7.62^{a}±0.64	6.78^{b}±0.55	6.98^{b}±0.98	7.10^{b}±1.20
Black pepper odour	6.29^{a}±0.74	6.07^{a}±1.06	5.90^{a}±0.72	5.02^{b}±1.29	5.09^{b}±1.48	4.86^{b}±1.93
Spices odour	6.60^{a}±0.73	6.33^{a}±1.03	6.33^{a}±0.87	5.32^{b}±1.66	5.35^{b}±0.93	5.45^{b}±1.20
Cured odour	7.12^{a}±0.75	6.76^{a}±0.91	6.81^{a}±0.96	6.01^{b}±1.71	5.67^{b}±1.40	5.96^{b}±0.98

Different superscripts (a, b) in the same row denote significant differences (P<0.05).
Type 1 (40% pork meat); Type 2 (30% pork meat); Type 3 (25% pork meat); Type 4 (20% pork meat); Type 5 (15% pork meat); Type 6 (10% pork meat).

Table 4 - Texture attributes (means ± standard deviations) of the cynegetic venison salchichon with different pork meat added.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Hardness	4.59^{a}±0.71	5.16^{b}±0.72	5.19^{b}±0.88	5.61^{b}±0.84	5.27^{b}±0.74	5.39^{b}±1.11
Juiciness	6.55^{a}±0.86	5.82^{b}±0.56	6.09^{b}±0.60	4.98^{c}±1.03	4.18^{d}±0.98	3.72^{e}±1.13
Chewiness	4.99^{a}±0.44	4.96^{a}±0.23	5.26^{a}±0.67	5.12^{a}±0.52	6.07^{b}±0.86	6.28^{b}±0.47
Fat mouthfeel	6.43^{a}±0.85	5.81^{b}±1.23	5.76^{b}±1.11	4.46^{c}±1.16	3.32^{d}±0.78	1.80^{e}±0.97

Different superscripts (a,b,c,d,e) in the same row denote significant differences (P<0.05).
Type 1 (40% pork meat); Type 2 (30% pork meat); Type 3 (25% pork meat); Type 4 (20% pork meat); Type 5 (15% pork meat); Type 6 (10% pork meat).

Table 5 - Taste attributes (means ± standard deviations) of the cynegetic venison salchichon with different pork meat added.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Taste intensity	7.46^{a}±0.84	7.32^{a}±0.66	7.76^{a}±0.86	7.50^{a}±0.84	6.71^{b}±0.77	6.52^{b}±1.00
Salty taste	5.10±0.31	5.09±0.67	5.33±0.62	5.14±0.36	5.04±0.57	5.04±0.54
Pepper taste	4.90±1.05	4.23±0.59	4.84±1.09	4.74±1.19	4.27±1.17	4.69±1.21
Aftertaste intensity	7.38^{a}±0.86	7.26^{a}±0.45	7.68^{a}±0.89	7.55^{a}±0.90	7.27^{a}±0.77	6.67^{b}±1.00

Different superscripts (a,b) in the same row denote significant differences (P<0.05).
Type 1 (40% pork meat); Type 2 (30% pork meat); Type 3 (25% pork meat); Type 4 (20% pork meat); Type 5 (15% pork meat); Type 6 (10% pork meat).

hardness (4.59). Samples from Types 2-6 showed values between 5.16-5.61, indicating a good texture for those sausages. Therefore, the reduction in the addition of pork meat to levels below 30% did not negatively affect this attribute. However, samples from Types 5 and 6 presented the lowest juiciness and were more difficult to chew. So the addition of pork meat below 20% to venison salchichon negatively influenced those attributes. Moreover, as the amount of fat increased the juiciness and the fat mouthfeel also increased, highlighting that samples from Types 2 and 3 presented similar values for those attributes. The scores for both attributes were more different between Type 1 with more fat content (6.5 for juiciness and 6.4 for fat in the mouth) and Type 6 with less fat content (3.7 for juiciness and 1.8 for fat in the mouth). Other varieties of dry-fermented sausages evaluated by a trained panel, showed higher scores for texture attributes (mainly hardness and juiceness) as the fat level increased (PAPADIMA and BLOUKAS, 1999; LIAROS *et al.*, 1999; MENDOZA *et al.*, 2001; LORENZO and FRANCO, 2012). In addition, the values found in this study for juiciness, chewiness and fat mouthfeel were similar to those reported by GARCÍA RUIZ *et al.* (2010) in venison sausages (50% lean venison-50% pork meat).

Table 5 shows the means and standard deviations of the taste attributes of the different types of cynegetic venison salchichon sausages. Significant differences were not found for the salty and black pepper taste attributes; all of the samples exhibited a proper salty taste with scores ranging between 5.0 and 5.3 and a pepper taste with scores from 4.2 to 4.9. On the other hand, significant differences were found for the taste intensity which was slightly lower for the Types with the least fat content (5 and 6), and for the aftertaste intensity which was lower for Type 6, with the lowest fat content, owing to the fact that lipids experiment lipolysis and lipid oxidation during the curing process that contributes the flavour (SAMELIS *et al.*, 1993). MENDOZA *et al.* (2001) also found significant differences in the taste intensity of the dry sausages in accordance with the fat content. The samples with the lowest fat content (6.5% and 12.5%) received lower scores ranging between 5.5 and 5.9 while the samples with the highest fat content (25%) received a score of 7.3. These scores are slightly lower than those obtained in this study (6.5-7.8) possibly due to the intense taste of cynegetic venison and its special organoleptics properties. GARCÍA RUIZ *et al.* (2010) found in its study of venison sausages elaborated using 50% lean venison and 50% pork meat, scores of 7.77 for taste intensity, 5.10 for salty taste and 7.38 for aftertaste intensity.

In summary, results obtained in the quantitative descriptive sensory analysis were highly influenced by the fat content of the samples even though all samples were accepted by the tasting panel (whitout sensory defects). These results do not coincide with those obtained by MUGUERZA *et al.* (2002) which determined that the cured sausages made with 10% pork backfat were unacceptable from a sensory point of view because of its winkled surface and excessive hardness.

Consumer tests

Acceptance test

The scores awarded by the consumers for different types of cynegetic venison salchichon sausage with different pork meat added are shown in Table 6. From these results, it can be concluded that all the samples were accepted

Table 6 - Means and standard deviations of the scores obtained for different types of cynegetic venison salchichon in the acceptance test.

	Type 1	Type 2	Type 3	Type 4	Type 5	Type 6
Aspect	7.18^{a}±2.04	6.84^{a}±1.89	7.19^{a}±1.79	5.80^{b}±2.16	5.91^{b}±2.01	5.47^{b}±2.28
Odour	7.39^{a}±1.80	6.66^{b}±2.08	7.25^{a}±1.75	6.22^{b,c}±2.06	6.47^{b,c}±2.09	6.01^{c}±2.23
Taste	7.12^{a}±1.80	6.82^{a}±1.90	7.14^{a}±1.78	6.32^{b}±2.07	6.08^{b}±2.25	5.94^{b}±2.17
Texture	7.22^{a}±1.96	6.86^{a,b}±1.82	7.14^{a}±1.94	6.36^{b,c}±2.12	6.34^{b,c}±2.19	6.22^{c}±2.06
Overall acceptance	7.16^{a}±1.93	6.93^{a,b}±1.85	7.20^{a}±1.81	6.48^{b,c}±2.00	6.11^{c}±2.21	5.90^{d}±2.12

Different superscripts (a,b,c,d) in any row denote significant differences (P<0.05).
Type 1 (40% pork meat); Type 2 (30% pork meat); Type 3 (25% pork meat); Type 4 (20% pork meat); Type 5 (15% pork meat); Type 6 (10% pork meat).

because the average score was above 5.0 (satisfaction threshold). The consumers found significant differences for all the attributes studied. Samples from Types 1, 2 and 3 were awarded higher scores for aspect (6.8-7.2), taste (6.8-7.1) and overall acceptance (6.9-7.2). The score for odour was also higher for Types 1 and 3. An addition of fat between 25% and 40% to elaborate cynegetic venison salchichon sausage therefore appears to provide, at least in the opinion of the habitual sausage consumers, better organoleptics characteristics than a lower addition. OLIVARES *et al.* (2011) found high consumer acceptability for aroma and overall quality in dry-ripened pork sausages elaborated with 20% and 30% pork meat than those with 10% pork meat.

Preference test

After having applied the Fischer method to calculate the Least Significant Difference (LSD), the consumer preference scores for each of the samples yielded the following order of preference: Type 3 > Type 1 > Type 2 > Type 4 > Type 5 > Type 6. The samples achieving the greatest consumer preference were those from Types 1, 2 and 3, scores differing significantly from those awarded to Types 4 and 5, which achieved a lower degree of consumer preference; Type 6 samples received the lowest scores. Samples from Types 1, 2 and 3 were preferred over the rest for six reasons: good flavour, appropriate texture, pleasant odour, acceptable fat content, good appearance and attractive colour; Type 6 was the least preferred, due to poor flavour and inappropriate texture.

The results obtained from the preference test coincide with those of the acceptance test leading the authors to conclude that the quantity of fat added to venison salchichon sausage should be at least 25% to achieve a good sensory quality similar to that of the traditional product.

CONCLUSIONS

The results obtained in the quantitative descriptive sensory analysis and the consumer tests perfectly coincide revealing that, from a sensory point of view, using 25% of pork meat and 75% of venison lean is enough. Such a quantity of fat assures proper texture for this type of product helping to attain a satisfactory odour, taste and appearance for the consumer as well as similar attributes to those of traditionally made salchichon sausage with a higher fat content.

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PAPER

EFFECT OF ARTISANAL RENNET PASTE ON THE CHEMICAL, SENSORY AND MICROBIOLOGICAL CHARACTERISTICS OF TRADITIONAL GOAT'S CHEESE

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ABSTRACT

In a study using three replicates, Marzolina goat cheese made with artisanal rennet paste from goat kid was compared with cheese made with commercial liquid rennet from calf. Samples of fresh cheese were subjected to chemical and microbiological analyses. Samples of ripened cheese collected after 50 days of ripening were submitted to chemical and sensory analysis. Results of this study show that cheese made with artisanal rennet pastes did not contain pathogenic micro-organisms and that this kind of rennet provided the enzymatic content necessary to achieve the typical characteristics of traditional cheeses.

- Keywords: rennet paste, chemical and sensory characteristics -

INTRODUCTION

In recent years, there has been an increased interest in the safety and promotion of cheeses prepared according to local traditional processes. The renewed attention is given to artisanal rennet, which is considered one of the most important factors affecting the characteristics of some typical Mediterranean cheeses. ARPs usually originate from lamb or kid abomasa, and farmers directly prepare them for use during cheese making. According to current legislation, the use of ARPs is allowed after a special derogation from Regulation (EC) n. 852/2004 for foods with traditional characteristics (EC Reg. n. 2074/2005). To obtain this derogation, it has been necessary to study their safety and hygienic characteristics. Results of a number of studies indicate that traditional cheese-making does not compromise health and hygiene (COSSEDDU and PISANU, 1980; DEIANA *et al.*, 1980; PISANU and COSSEDDU, 1982; BARZAGHI *et al.*, 1997; CALANDRELLI *et al.*, 1997; IRIGOYEN *et al.*, 2001; MOATSOU *et al.*, 2004; MOSCHOPOULOU *et al.*, 2007; TRIPALDI *et al.*, 2012). Determining the role and influence of ARPs on the sensory characteristics of cheeses is also necessary.

Marzolina is a traditional Italian cheese made from the milk of local goat breeds of the Latium region, in the Centre of Italy and from artisanal kid rennet pastes. Marzolina is characterised by a weigth of about 150 g and high salt content that preserves the cheese for long periods even in a natural room. ARP is traditionally used in the process for MARZOLINA cheese and its particular flavor has been attributed to the use of rennet pastes (Addis *et al.*, 2005). However factors such as the time and the effort required to prepare the rennet at the farm, as well as the lower demand for strongly flavoured cheeses, have contributed to the rapid replacement of ARP with commercial liquid rennet (CLR).

The aim of this trial was to study the hygienic and health characteristics of cheese from ARP so contributing to the approval of the derogation for using artisanal rennet pastes. Moreover this trial was finalised to evaluate the effect of rennet pastes, as compared to liquid rennet, on the chemical, and sensory characteristics of Marzolina goat cheese.

MATERIALS AND METHODS

Marzolina cheese made with ARP from kid was compared with that made with CLR from calf. Both cheeses were produced by farmers. Two kinds of rennet were used and three replicates were performed. The trial was carried out according to the process usually employed by farmers in a small cheese farm. Thirty litres of milk for each kind of rennet in each replicate were processed. Abomasa were removed from suckling kids slaughtered at the age of 30–45 days and they were submitted to a drying phase preceded by a salting phase. Ten abomasa were ground, merged and then utilised in all replicates of the trials. The commercial liquid calf rennet (Naturen®), used in the trial was produced by Chr. Hansen's (Denmark).

Data specifying proteolytic activity of CLR were supplied by Chr. Hansen's. Enzimatic characteristics of ARP were determined according to the following methods.

Total milk clotting activities of the artisanal rennet pastes were determined according to ISO 23058 IDF 199: 2006 method known as REMCAT method. To determine the chymosin and pepsin content, test samples were prepared by dissolving 25 g of rennet paste in 100 g of buffer solution (CH3COOH/CH3COONa) at pH 5.5; the samples were centrifuged at 3000 rpm (2189 g, refrigerated centrifuge ALC 4237R, ALC, Milano, Italy) for 30 min at 4°C. The supernatant was analysed as described in the International IDF Standard 110B: 1997. Chymosin and pepsin enzymes were expressed as a percentage of the sample's total milk clotting activity. Lipase activity was analysed as described in the Food Chemical Codex (1981).

Characteristics of both types of rennet are shown in Table 1. During the study, raw goat milk was utilised and no starter cultures were added. After coagulation at a temperature below 35°C, the curd was cut, reduced to small granules and then packed in a small cylindrical mould. After about 12 hours from the start of cheese-making, the cheeses were subjected to dry salting and then air-dried in a natural room for one week. Finally, they were packaged under vacuum and stored at 4°C.

During each experiment, samples of fresh cheese were collected before dry salting. Sam-

Table 1 - Enzymatic activity of artisanal rennet pastes and commercial liquid rennet.

Rennet	g or mL of rennet on 100 l of milk	total IMCU × g^{-1} or mL^{-1} of rennet	total IMCU on 100 l of milk on 100 l of milk	chymosin (% RU)	chymosin IMCU	ILU × g^{-1} of rennet
ARP	19	129	2493	95,34	2377	35,76
CLR	20	162	3229	80,00	2583	

ARP: Artisanal Rennet paste; CLR: Calf Liquid Rennet.
IMCU: International Milk Clotting Unit; RU: Rennet Unit;ILU: International Lipase Units.

ples of fresh cheese were subjected to chemical analyses, which were conducted in duplicate. Analyses consisted of measurements of moisture (IDF, 1986), total nitrogen (TN) (FIL-IDF, 1993), soluble nitrogen (SN) (FIL-IDF, 1991), fat (FIL-IDF, 2001), salt (IDF, 1988), ash (AOAC, 2000), and free fatty acids (FFAs). FFAs (mmol/kg) were analysed by capillary gas chromatography (De Jong and Badings, 1990) and expressed as mmol/kg to assess each individual FFA independent of its molecular weight. Samples of fresh cheese were also subjected to microbiological analysis. All samples were subjected to qualitative tests for *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* O157, and to quantitative analyses for *L. monocytogenes*, sulphite-reducing clostridia, total microbial count at 30°C, coagulase-positive staphylococci, and β-glucuronidase-positive *E. coli* (Tripaldi *et al.*, 2012). Samples of cheese ripened for 50 days were also collected and then subjected to chemical and sensory analyses.

Descriptive sensory analysis was performed to determine the differences in sensory characteristics of the two kinds of cheeses. Ten panelists (5 males and 5 females; between 20 and 50 years of age) were selected and trained in accordance with ISO 8586-1:1993 e ISO 8586-2:1994 standards.

For the laboratory conditions, UNI ISO 8589 standard was followed. The test was carried out according to UNI 10957:2003 standard based on triplicate analysis of each sample.

Twenty-two descriptors were identified, as follows: 3 visual (color intensity, color omogeneity, rind color), 5 olfactory (smell intensity, stable straw smell, lactic smell, vegetable smell, other smell), 5 basic taste and trigeminal sensations (salty, sweet, sour, bitter, piquant), 6 retro-olfactory (flavour intensity, stable straw flavour, lactic flavour, vegetable flavour, other flavour, persistence), and 3 tactile descriptors (adhesiveness, moisture, firmness). Attributes were rated on a continuous scale of values from 0 to 10 (0 = absence of the intensity, maximum intensity = 10). Sensory descriptors and their rating scale were defined according to UNI 10957:2003 and European Guide for the sensory evaluation of hard and semi-hard cheese standard (BÉRODIER e al.1997; LAVANCHY *et al.*1994).

The GLM procedure of SAS software (SAS Institute Inc., 2007) was used for statistical analysis of chemical parameters by using the model

$$Y_{ijl} = \mu + A_i + B_j + A_i \times B_j + E_{ijl}$$

where
Y_{ijl} = qualitative characteristics of the cheese
A_i = fixed effect of the kind of rennet (i = 1 for ARP; i = 2 for CLR)
B_j = fixed effect of days of ripening (j = 1: 1*d*; j = 2: 50*d*)
E_{ijl} = residual of error

Data processing of sensory evaluations was carried out according to UNI 10957:2003 standard.

RESULTS AND DISCUSSION

Results of proteolytic and lipolytic activity of ARPs and CLR are summarised in Table 1. The clotting characteristics were different: 129.38 total International Milk Clotting Units (IMCU) × g^{-1} and 95.34% of chymosin for ARP and 162.00 total IMCU × ml^{-1} and 80.00% of chymosin for CLR. The latter was subjected to a preliminary test and then added to milk in quantity of 19.93 mL/100 l of milk. This led to coagulation in approximately 60 minutes, the clotting time used by farmers.

During the experiment, the addition of rennet to 100 l of milk resulted in 2493 and 3229 total IMCU for ARP and CLR, respectively. Chymosin IMCU values, 2377 and 2583 per 100 l of milk for ARP and CLR, respectively, were more similar than that of total IMCU. . The lower activity of total clotting of ARP could be due to dilution of enzymes caused by the presence of milk in the abomasa used to produce the rennet (PIRISI *et al.*, 2007). The high chymosin percentage (95.34 and 80.00) in the rennet paste could be attributed to the completely milk-based diet and/or to complete filling of the stomach with milk when the kid was killed (BUSTAMANTE *et al.*, 2000; ADDIS *et al.*, 2005). We observed that in our study the kind of diet given to the kid and the status of the abomasum before the slaughtering agreed with the conditions found by other Authors (BUSTAMANTE *et al.*, 2000; ADDIS *et al.*, 2005). The international lipase units (ILU) of the rennet paste, 35.76 ILU × g^{-1}, was similar to the average value (36.18 ILU × g^{-1}) obtained from other samples of rennet paste from the same region (TRIPALDI *et al.*, 2012).

The chemical characteristics of fresh and ripened goat cheese made using ARP and CLR are reported in Table 2. The moisture values were not largely affected by the kind of rennet in both fresh and ripened cheese (69.65 and 42.30% in ARP cheese and 69.81 and 40.87% in CLR cheese). Generally, the cheese moisture depends on the temperature and relative humidity conditions of cheese-making and the ripening conditions (IRIGOYEN *et al.*, 2002); thus, it is difficult to find differences between the two cheeses in which the only change is the type of rennet.

We can observe slight differences in protein content between the two kinds of cheeses (10.60 *vs* 11.13 and 20.98 *vs* 21.30 in ARP- and CLR-treated fresh cheese and in ARP- and CLR-treated ripened cheese, respectively). The fat content of the two kinds of cheese also differed slightly (13.29 *vs* 14.03% in ARP and CLR of fresh cheese and 28.19 *vs* 30.31% in ARP- and CLR-treated ripened cheese, respectively). On the contrary, SANTORO and FACCIA (1998) observed a significant difference in fat content in CanestratoPugliese cheese

Table 2 - Composition of fresh and ripened Marzolina goat cheese made using artisanal rennet paste (ARP) and commercial liquid rennet (CLR) at 1 day and 1 month of ripening.

	Fresh cheese						Ripened cheese						P		
	ARP	+	++	CLR	+	++	ARP	+	++	CLR	+	++	SE	rennet	ripening
moisture (%)	69,65	ns	a	69,81	ns	a	42,30	ns	b	40,87	ns	b	1,15	NS	*
protein (%)	10,60	ns	b	11,13	ns	b	20,98	ns	a	21,30	ns	a	0,60	NS	*
soluble protein (% of total protein)	4,96	ns	b	3,98	ns	b	9,12	ns	a	10,11	ns	a	0,13	NS	*
fat (%)	13,28	ns	b	14,03	ns	b	28,19	ns	a	30,31	ns	a	0,73	NS	*
NaCl (% of moisture)	0,79	ns	b	0,82	ns	b	7,40	a	a	6,01	b	a	0,44	*	*
ash (%)	1,66	ns	b	1,71	ns	b	5,09	ns	a	4,36	ns	a	0,26	NS	*

ARP: Artisanal Rennet paste; CLR: Calf Liquid Rennet.
+ kind of rennet; ++ ripening time.
a, b, *: $p<0.05$.

made with rennet having different characteristics. They attributed this result to the different aggregation states of the casein micelles in the curd.

The salt in moisture (S/M) content of samples of fresh cheese was similar (0.79% in ARP-treated cheese and 0.82% in CLR-treated cheese), while S/M content was higher in ARP-treated than in CLR-treated ripened cheese (7.40 *vs* 6.01%; $P < 0.05$). The difference in salt content in the two kinds of ripened cheese is probably due to manual dry salting, a practice that is subject to large variations. Our results show that ripened Marzolina cheese has higher salt content in comparison with the majority of ripened cheeses (2.67 in ARP-treated cheese and –3.13% in CLR-treated cheese), corresponding to about 5% of dry matter.

Soluble protein as a percentage of total protein was higher in ARP-treated fresh cheese than in CLR-treated fresh cheese (4.96 *vs* 3.98%). On the contrary, higher values of this proteolysis index were found in ripened CLR-treated cheese in comparison with those obtained in ripened ARP-treated cheese (10.11 *vs* 9.12%). During cheese ripening, the higher salt content of ARP relative to the one of CLR cheese may influence negatively the proteolytic process, as observed in Romano type cheese (GUINEE and FOX, 1984).

Similar values of soluble N/total N at pH 4.6 were found in Protected Designation of Origin (PDO) sheep cheese Canestrato Pugliese (CORBO *et al.*, 2001) at 1 and 35 days of ripening (5.59-6.78% and 8.65-11.50%, respectively). Values of the soluble N/total N at pH 4.6 (7.87%) of the traditional Italian cheese Piacentinu Ennese at 2 months of ripening (FALLICO *et al.*, 2006) are lower than those of ripened Marzolina cheese.

Differences in all parameters between fresh and ripened samples of both kinds of cheese were observed. With ARP and CLR, the moisture of ripened cheese significantly decreased compared with that of fresh cheese. The other cheese components protein, fat, S/M and ash increased significantly during cheese ripening as a result of the decrease in moisture. The soluble protein/total protein ratio was significantly higher in ripened cheese as result of increased proteolysis (UPADHYAY *et al.*, 2004).

Table 3 shows the individual and total FFA (TFFA) content of cheese made with ARP compared with cheese made with CLR. The TFFA content was higher in ARP-treated cheese than in CLR-treated cheese: 8.94 versus 4.09 mmol× kg^{-1} and 39.51 versus 36.56 mmol× kg^{-1} in fresh and ripened cheese, respectively. The difference between fresh and ripened cheese of ARP- and CLR-treated cheeses was significant.

Short chain free fatty acids (SCFFAs) are the most abundant FFAs in ARP and CLR. Similar to TFFAs, SCFFA was present at higher levels in ARP-treated cheese (5.58 *vs* 2.06 mmol× kg^{-1} and 21.46 *vs* 15.75 mmol× kg^{-1}, in fresh and ripened cheese, respectively). The difference was significant between ripened cheeses made with ARP and with CLR and between fresh and ripened cheese of both ARP- and CLR-treated cheeses.

Butyric and capric acids were the most abundant FFAs. Levels of butyric acid were the highest in both kinds of fresh cheese. Levels of capric acid were the highest in both kinds of ripened cheese.. Butyric acid levels were significantly higher in fresh or ripened ARP cheese than in CLR cheese (2.27 *vs* 0.81 mmol × $kg^{-1}$and 4.89 *vs* 3.85 mmol × kg^{-1}, $P \leq 0.05$). Caproic acid only in ripened cheese was significantly higher in ARP-treated cheese than in CLR-treated cheese (4.85 *vs* 3.31 mmol × kg^{-1}). Also Capric acid was higher in ARP ripened cheese than in CLR ripened cheese (8.39 vs 6.05 mmol × kg^{-1}, $P \leq 0.06$)

The content of all individual fatty acids of both groups of ripened cheeses were higher than that of both groups of the fresh ones ($P \leq 0.01$).

Levels of medium-chain FFAs (MCFFAs) and long-chain FFAs (LCFFAs) were higher in fresh cheese made with ARP (1.91 *vs* 1.02 mmol × $kg^{-1}$and 1.44 *vs* 1.00 mmol ×kg^{-1} for MCFFAs and LCFFAs, respectively) and lower in ripened cheese made with ARP (10.58 vs 12.04 mmol × kg^{-1} and 7.48 *vs* 8.77 mmol × kg^{-1}, respectively). The dif-

Table 3 - Free fatty acids (mmol x kg^{-1}) in fresh and ripened Marzolina goat cheese made using artisanal rennet paste (ARP) and commercial liquid rennet (CLR) at 1 day and 1 month of ripening.

	Fresh cheese						Ripened cheese						P		
	ARP	+	++	CLR	+	++	ARP	+	++	CLR	+	++	SE	rennet	ripening
C4:0	2,27	a	B	0,81	b	B	4,89	a	A	3,85	b	A	0,33	*	**
C6:0	1,28	ns	B	0,37	ns	B	4,85	a	A	3,31	b	A	0,31	*	**
C8:0	0,60	ns	B	0,24	ns	B	3,29	ns	A	2,50	ns	A	0,40	NS	**
C10:0	1,42	ns	B	0,62	ns	B	8,39	ns	A	6,05	ns	A	0,80	NS	**
SCFFAs	5,58	ns	B	2,06	ns	B	21,46	a	A	15,75	b	A	1,45	*	**
C11:0	0,01	ns	B	0,00	ns	B	0,04	a	A	0,03	b	A	0,00	*	**
C12:0	0,44	ns	B	0,20	ns	B	2,42	ns	A	1,81	ns	A	0,22	NS	**
C14:0	0,47	ns	b	0,22	ns	B	2,75	ns	a	3,13	ns	A	0,43	NS	*
C15:0	0,03	ns	b	0,02	ns	B	0,18	ns	a	0,23	ns	A	0,03	NS	*
C16:0	0,94	ns	b	0,56	ns	B	5,05	ns	a	6,69	ns	A	0,84	NS	*
C16:1	0,02	ns	b	0,01	ns	b	0,13	ns	a	0,15	ns	a	0,03	NS	*
MCFFAs	1,91	ns	B	1,02	ns	B	10,58	ns	A	12,04	ns	A	1,55	NS	**
C17:0	0,02	ns	b	0,01	ns	B	0,10	ns	a	0,13	ns	A	0,02	NS	*
C18:0	0,45	ns	b	0,33	ns	B	2,15	ns	a	2,73	ns	A	0,34	NS	*
C18:1	0,88	ns	b	0,63	ns	b	4,57	ns	a	5,05	ns	a	0,89	NS	*
C18:2	0,06	ns	b	0,03	ns	B	0,41	ns	a	0,56	ns	A	0,10	NS	*
C18:3	0,03	ns	b	0,01	ns	B	0,23	ns	a	0,30	ns	A	0,05	NS	*
LCFFAs	1,44	ns	b	1,00	ns	B	7,48	ns	a	8,77	ns	A	1,40	NS	*
TFFAs	8,94	ns	B	4,09	ns	B	39,51	ns	A	36,56	ns	A	4,24	NS	**

ARP: Artisanal Rennet paste; CLR: Calf Liquid Rennet.
+ kind of rennet; ++ ripening time.
SCFFAs = short chain free fatty acids MCFFAs = medium chain free fatty acids LCFFAs = long chain free fatty acids. TFFAs = total free fatty acids.
a, b, *: p<0.05; A, B, **: p<0.01

ference was significant for both groups of FFAs only between fresh and aged cheeses.

The higher butyric acid content of cheese made with ARPs compared with cheese made with CLR is confirmed by the high specificity of the enzymatic activity of pregastric lipase for SCFAs, especially butyric acid, esterified to the *sn*-3 position of triglycerides (Pitas and Jensen, 1970; Kim and Lindsay, 1993). FONTECHA *et al*. (2006) found higher butyric acid content in Spanish goat cheese made with rennet paste compared with cheese made with CLR.

The higher concentration of capric acid compared with butyric acid in ripened cheeses was observed in other goat cheeses (BUFFA *et al*., 2001; POVEDA and CABEZAS, 2006; ATASOY and TURKOGLU, 2009). According to BUFFA *et al*. (2001), the capric acid content of cheese from goat milk increased during ripening while butyric acid varied slightly from the start to the end of ripening. The small increase in butyric acid content was probably due to its metabolic conversion to aromatic compounds (BUFFA *et al*., 2001).

Sensory attributes of each kind of cheese are shown in Fig. 1. Mean values of the following sensory descriptors were significantly different

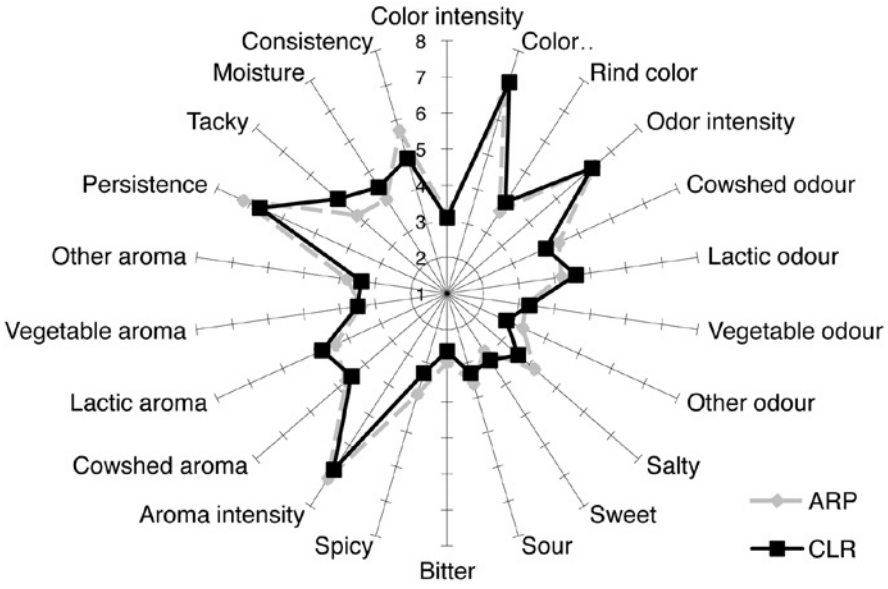


Fig. 1 - Sensory profile of ripened Marzolina goat cheese made using artisanal rennet 14 pastes (ARP) and commercial liquid rennet (CLR).

Table 4 - Microbiological characteristics of fresh Marzolina goat cheese made using artisanal rennet paste (ARP) and commercial liquid rennet (CLR).

Sample	Replicate	*Salmonella* spp (in 25 g)	*Listeria monocytogenes* (in 25 g)	*E. coli* (cfu x g^{-1})	*E. coli* O:157 (in 25 g)	Coagulase positive staphylococci (cfu x g^{-1})	Enterobacteriaceae (cfu x g^{-1})	Sulphite reducing clostridia (cfu x g^{-1})	Total mesophilic count (cfu x g^{-1})
ARP	1	negative	negative	<10	negative	<10	<10	<2	$2.6x10^7$
CLR	1	negative	negative	<10	negative	<10	<10	<2	$3.0x10^8$
ARP	2	negative	negative	<10	negative	<10	$3.5x10^3$	<2	$9.3x10^5$
CLR	2	negative	negative	<10	negative	<10	$4.8x10^3$	<2	$1.3x10^6$
ARP	3	negative	negative	100	negative	<10	$6.2x10^3$	<2	$4.9x10^6$
CLR	3	negative	negative	<10	negative	<10	<10	<2	$1.5x10^5$

(P<0.05) in the two kinds of cheese (ARP vs CLR, respectively): color omogeneity (6.6 vs 7.1), rind color (3.7 vs 4.0), stable straw smell (4.4 vs 4.0), lactic smell (4.2 vs 4.6), other smell (3.3 vs 2.8), salty (4.2 vs 3.6), sweet (2.9 vs 3.2), sour (3.6 vs 3.3), bitter (2.9 vs 2.6), piquant (3.9 vs 3.3), flavour intensity (7.1 vs 6.8), stable straw flavour (4.7 vs 4.5), lactic flavour (4.4 vs 4.8), other flavour (3.8 vs 3.4), persistence (7.2 vs 6.7), adhesiveness (4.3 vs 5.0), moisture (4.1 vs 4.5) and firmness (5.7 vs 4.9).

There was no significant interaction between assessor and replicate, suggesting high repeatability of panellist assessment in the three replicates. Sample-replicate and sample-panellist interactions were not significant, showing either homogeneity of samples in the three replicates or good agreement among panellist assessments during sensory evaluation. Analysis of the eyes and slits in cheese showed a higher number of samples made with ARPs having these defects compared with those in cheese samples made with CLR (82 vs 57).

The basic tastes salty, acid, bitter and piquant were more pronounced in cheeses made with ARP than in cheeses made with CLR. In addition, the smell and flavour of cowshed were more marked in cheese made with ARP than in cheese made with CLR. Its texture was firmer, less tacky and less moist than the latter kind of cheese. The odour and flavour given by lactic acid to cheeses made with CLR are more dominant than in cheeses made with ARP. Generally, the sweet, salty and sour attributes of taste were less pronounced. The texture was tackier, more moist and less firm than that of cheese made with ARP.

It is noteworthy that basic tastes salty and piquant, which are more pronounced in cheese made with ARP than in cheese made with CLR, agree with the chemical results. As reported by Addis *et al*. (2005), cheese made with ARPs has a major amount of butyric acid, which may be responsible for piquancy in cheese (Rennet paste has been associated with piquancy or pungency and with characteristic flavours of certain cheeses from the Mediterranean basin (ANIFANTAKIS, 1976; NELSON *et al*., 1977; WOO and LINDSAY, 1984; BATTISTOTTI and CORRADINI, 1993; BARZAGHI *et al*., 1997; CALANDRELLI *et al*., 1997). In a study on Idiazabal cheese (ETAYO *et al*., 2006), cheese made with lamb rennet pastes showed higher butyric acid content and received higher scores compared to cheese made with commercial liquid lamb rennet. .

A larger number of eyes and slits in cheese made using ARPs was also observed by FERRANDINI *et al*. (2012). This could be attributed to different textural properties (FERRANDINI *et al*., 2011) of cheese made with the two kinds of rennet. In fact, results of microbiological analyses carried out on ARP used in this study (TRIPALDI *et al*., 2012) exclude the presence of microorganisms markers of hygiene characteristics including germs causing microbiological spoilage in cheese.

Table 4 displays the microbiological characteristics of the cheese. *Salmonella* and *L. monocytogenes*, pathogens considered as health markers (Reg. CE 2073/2005), were undetected by qualitative analyses. β-Glucuronidase-positive *E. coli* and coagulase-positive staphylococci, which include *Staphylococcus aureus*, are considered as hygiene markers (Reg CE 2073/2005). The maximum count tolerated for coagulase-positive staphylococci in cheese from raw milk is 10^5cfu × g^{-1}. Samples with more elevated counts must be

analysed for staphylococci enterotoxins. Counts of coagulase-positive staphylococci in all samples were lower than the detection limit of the method (10 cfu $\times$ g^{-1}). The count of β-glucuronidase-positive *E. coli* was 100 cfu $\times$ g^{-1} in only one sample, but lower than the detection limit in other samples (10 cfu$\times$ g^{-1}). Qualitative analysis for *E. coli* O157 in our samples gave negative results. Sulphite-reducing clostridia is another group of microbial pathogens considered as hygiene markers, but legislation has not established permissible levels for these in food. Their levels in all samples were lower than the detection limit of the method (2 cfu $\times$ g^{-1}).

The mean total mesophilic count in our samples was 5.6×10^7cfu$\times$ g^{-1}. Microbiological analyses of the cheese samples confirmed the results obtained during the monitoring of some ARPs collected in the same region of Marzolina production (TRIPALDI *et al.*, 2012). Similar results were obtained in three PDO raw ewe milk cheeses from Spain, as Manchego, Idiazabal and Zamorano cheese (ETAYO *et al.*, 2006), where the hygienic quality of cheeses made with lamb rennet paste is comparable to that of cheeses manufactured with non-paste commercial rennet. Another study on Idiazabal did not detect *E. coli*, *Clostridium*, *Salmonella* or *L. monocytogenes*, and levels for other microorganisms were below the limits of the European legislative standards for cheese manufactured with raw milk (GIL *et al.*, 2007).

The results of our study show that treatment with ARPs did not favour the growth of microbial pathogens and that ARPs provided the enzymatic content necessary to achieve the typical characteristics of traditional cheeses.

CONCLUSIONS

Butyric acid was the main marker of cheeses made with ARPs because of the high specificity of enzymatic activity of pre-gastric lipase for butyric acid. Results of sensory evaluation show that the piquant flavours as well as the odour and flavour of cowshed were more pronounced in cheeses made with ARP, confirming the results for other Mediterranean cheeses made with rennet paste. Therefore, the use of ARPs provided the enzymatic content necessary to achieve the typical characteristics of traditional cheeses. At the same time, this kind of rennet did not favour the growth of microbial pathogens in cheese.

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PAPER

EFFECT OF SOLID-STATE FERMENTATION WITH *RHIZOPUS OLIGOSPORUS* ON BIOACTIVE COMPOUNDS AND ANTIOXIDANT CAPACITY OF RAW AND ROASTED BUCKWHEAT GROATS

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ABSTRACT

The effect of solid-state fermentation with *Rhizopus oligosporus* on the changes in the total phenolic compounds, rutin, vitamin B and C, tocopherol, phytic acid and antioxidant capacity of raw and roasted buckwheat groats was studied. The roasted groats contained reduced level of studied bioactive compounds as compared to raw groats. In this study was evidenced that the solid-state fermentation with *Rhizopus oligosporus* enhanced water soluble vitamins (thiamine, pyridoxine and L-ascorbic acid) as well as tocopherols contents. In contrast the decrease of the inositol hexaphosphate, phenolic compounds, the rutin content and antioxidant capacity determined by ACL and ABTS methods was noticed.

- Keywords: antioxidant capacity, bioactive compounds, buckwheat groats, *Rhizopus oligosporus*, solid-state fermentation -

INTRODUCTION

Globally, there is a growing interest in buckwheat products as healthy foods. The major producers of buckwheat are China, Russia, Ukraine and Kazakhstan, but it is also cultivated in Slovenia, Poland, Hungary, Brazil and Austria (BONAFACCIA *et al.*, 2003). Rutin (quercetin-3-rutinoside) is the main buckwheat flavonoid, which poses antioxidant, anti-inflammatory and anticarcinogenic properties. Buckwheat is also rich in other antioxidant compounds such as phenolic acids, tocopherols, reduced glutathione, inositol phosphates and melatonin (WIJNGAARD and ARENDT, 2006). This pseudocereal is characterised also in a high content of thiamine (vitamin B1) and riboflavin (vitamin B2), proteins with a well balance amino acid composition, including a high lysine content, phytosterols, soluble carbohydrates, D-chiro-inositol, fagopyritols and thiamin-binding proteins. Its fatty acid composition is superior to that of cereal grains, with typically 80% unsaturated fatty acids, including more than 40% of linoleic acid, an essential polyunsaturated fatty acid (STEADMAN *et al.*, 2001).

Raw and roasted buckwheat groats are particularly popular in Central and Eastern Europe. Roasted buckwheat groats are usually served like rice after cooking, while raw buckwheat groat or flour is used as a substitute for wheat flour in products for people with allergy to gluten and can be a valuable ingredient in diets or food products for coeliac patients (WRONKOWSKA *et al.*, 2010). Roasting affects the chemical composition and functional properties of buckwheat groats. The reduction of parent antioxidants as well as the formation of Maillard reaction products after roasting was observed (ZIELIŃSKA *et al.*, 2007a).

Tempeh, or "tempe", if we use authentic Indonesian spelling, a traditional product originating from Indonesia, is usually made from soybeans. The traditional tempeh process involves soaking and cooking, cooling and dehulling of the beans, followed by 20-30 hours of solid-state fermentation with *Rhizopus oligosporus*. Tempeh products have high protein contents of 40-50% and can be serve as tasty protein complements to starchy staples, and can substitute meat or fish (NOUT and ROMBOUTS, 1990). The solid-state fermentation with *Rhizopus oligosporus* has several beneficial effects, for example enhances antioxidant properties such as free-radical scavenging activity. HANDOYO *et al.* (2006) used fermentation with *R. oligosporus* to produce a fermented buckwheat flour that not only had a higher content of amino acids and minerals than non-fermented buckwheat samples, but also a lower content of allergic proteins. However, the production of tempeh-like products from buckwheat groats themselves has not been investigated. Therefore, in order to promote the utilization of raw or roasted buckwheat groats as well as to create a new type of healthy food, the effects of solid-state fermentation with *Rhizopus oligosporus* on the changes in the total phenolic compounds, rutin, vitamin B and C, tocopherol, phytic acid and antioxidant capacity of raw and roasted buckwheat groats was addressed in this study.

MATERIALS AND METHODS

Chemicals

Acetonitrile and methanol (HPLC-grade) were provided by Merck (Darmstad, Germany). Rutin (quercetin-3-rutinoside), L-ascorbic acid, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiamine, riboflavin, pyridoxine, Taka-diastase from *Aspergillus oryzae* (EC No 232-588-1) and inositol hexaphosphoric acid (dodecasodium salt) from corn were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, U.S.A.). Other reagents of reagent-grade quality were from POCh, Gliwice, Poland. Water was purified with a Milli-Q-system (Milipore, Bedford, USA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

Solid-state fermentation (SSF)

The raw (B) and roasted common (*Fagopyrum esculentum Moench*) buckwheat groats (RB) were purchased from a local healthy food shop (Olsztyn, Poland). The SSF was performed according to the modified method of HANDOYO *et al.* (2006). Briefly, 50 grams of raw or roasted buckwheat groats were cooked with 200 mL of deionized water for 5 min at 100°C. After cooling to room temperature, the excess of water was discarded. The drained samples were then inoculated with *Rhizopus oligosporus* NRRL 2710 (approx. 10^4 spores/g, Northern Regional Research Laboratory, Peoria, Illinois, USA) and incubated at 37°C for 24 h. The fermented raw groats (SSF-B) and fermented roasted groats (SSF-RB), as well as unfermented materials, were lyophilized using a Labconco Co. (Kansas City, Missouri, USA) laboratory freeze-dryer at a pressure of 13 Pa at -60°C for 24 h.

Sample preparation for measurement of rutin, total phenolic compounds and antioxidant capacity

The lyophilized buckwheat samples were extracted in triplicate with 80% aqueous methanol (1/10; w/v) for 2 h, with shaking at 37°C (1400rpm, Comfort R, Eppendorf, Germany). Samples were then centrifuged at 2600xg at 4°C for 15 min in a Beckman GS-15R centri-

fuge (Beckman Instruments, Inc., Palo Alto, CL, USA). The samples were preserved at -20°C prior to further analysis.

Preparation of hydrophilic and lipophilic extracts for the measurement of antioxidant capacity by photo-induced chemiluminescence (PCL) assay

For the hydrophilic extract, about 100 mg of lyophilized buckwheat samples were extracted for 3 min with 1 mL of HPLC-grade water using a Genie-2 type vortex (Scientific Industries, USA). Next, samples were centrifuged for 5 min at 4°C, at 16100xg (5415 R, Eppendorf, Germany) and the fresh supernatants were directly used to determine the antioxidant capacity formed by water-soluble antioxidants (ACW). For the lipophilic extracts, about 100 mg of lyophilized buckwheat samples were extracted for 3 min with a mixture of 200 μL of n-hexane and 800 μL of methanol using a Genie-2 type vortex (Scientific Industries, USA). Next, samples were centrifuged for 5 min at 4°C, at 16100xg (5415 R, Eppendorf, Germany) and the fresh supernatants were directly used to determine the antioxidant capacity formed by lipid-soluble antioxidants (ACL).

Determination of total phenolic compounds

The content of total phenolic compounds (TPC) was determined according to SHAHIDI and NACZK (1995). Buckwheat extracts (0.25 mL) were mixed with 0.25 mL of Folin-Ciocalteu reagent (previously diluted with water, 1:1 v/v) and 0.5 mL of Na_2CO_3 solution, and 4 mL of water. The mixture was allowed to stand at room temperature for 25 min and then centrifuged at 2000xg for 10 min. The absorbance of the supernatant was measured at 725 nm using a spectrophotometer (UV-160 1PC, Shimadzu, Japan). The data were calculated as rutin equivalents.

Determination of rutin content by HPLC

Rutin content was analyzed in a HPLC system (Shimadzu, Kyoto, Japan) comprising two pumps (LC-10 AD), a UV detector (SPD-10A) set at 330 nm, an autosampler set for 5 μL injection (SIL-10 ADVP), a column oven (CTO-10 ASVP), and a system controller (SIL-10 ADVP) according to method described by ZIELIŃSKA *et al.* (2010).

Determination of thiamine (B1) and riboflavin (B2) by HPLC

To determine the content of thiamine and riboflavin, the modified method of PRODANOV *et al.* (1997) was used. The 500 mg of lyophilized buckwheat were extracted by acid hydrolysis with 15 mL of 0.3 M HCl in autoclave (15 min at 120°C) and, after cooling, the pH was adjusted to 5-5.4 using 4 M sodium acetate. Then 5 mL of an aqueous solution of Taka-Diastase enzyme (100 U/mg) was added to the samples and incubated for 3 h at 45°C, in a water bath with shaking (120rpm, HS-B20, IKA Labortechnik, Germany). After enzymatic hydrolysis, the extracts were filtered by Whatman No. 40 filters and water was added to complete the volume to 25 mL. The samples were preserved at -20°C prior to HPLC analysis.

Determination of pyridoxine (B6) and L-ascorbic acid by HPLC

Analysis of pyridoxine and L-ascorbic acid content was made by the modified method of ESTEVE *et al.* (1997). Approximately 100 mg of lyophilized buckwheat samples were added to 1 mL of 5% aqueous solution of metaphosphoric acid. The samples were extracted in triplicate, and then centrifuged at 12000xg for 10 min at 4°C (GS-15 R Beckman Instruments, Inc., Palo Alto, CL, USA). The supernatant were mixed with 100 μL of dithiotreitol (DTT), incubated for 1 h (without light) and then centrifuged at 13000xg for 5 min at 4°C. The samples were preserved at -20°C prior to further HPLC analysis.

Determination of tocopherol content

Tocopherol (α-T, β-T, γ-T, δ-T) was extracted with methanol (0.5 g of sample/7 mL). After evaporation, extracts were redissolved in *n*-hexane. The HPLC analysis was run with a Shimadzu system (LiChrospher® Si-60, 5-μm particle size, 4 x 250-mm column) according to the method described by PEGG and AMAROWICZ (2009). The tocopherols contents were calculated from the peak areas using standard curves of tocopherols.

Determination of inositol hexaphosphates and its lower forms

The analysis of the content of tri-, tetra-, penta-, and hexaphosphate inositols was made according to the method of HONKE *et al.* (1999). Inositol hexaphosphate was determined as follows: exactly 0.5 g of the buckwheat samples were extracted with 20 mL 0.5 M HCl for 5 h using a BM1 magnetic stirrer (IKA, Staufen, Germany). The extract was centrifuged at 3500xg for 40 min (Centrifuge MPW-360, Factory of Precise Mechanics, Warsaw, Poland) and the supernatants were decanted, frozen overnight (-20°C), thawed at room temperature and recentrifuged at 3500xg for 40 min. The supernatants (15 mL) were evaporated under reduced pressure to dryness at 40°C and dissolved in 15 mL of 0.025 M HCl. The samples were transferred to minicolumns filled with Dowex AG 1-X8 resin, from which the inositol phosphates were eluted using 2 M HCl (5 x 4 mL). After removal of the sol-

vent by evaporation with an air stream, the dry residue was dissolved in a mobile phase. Then the samples were analysed by HPLC. The inositol hexaphosphates contents were calculated from the peak areas using standard curves of inositol hexaphosphates.

Measurement of the antioxidant capacity of buckwheat products against $ABTS^{+\bullet}$ and $O_2^{-\bullet}$

The antioxidant capacities of the 80% aqueous methanol extracts from lyophilized buckwheat samples were determined against $ABTS^{+\bullet}$ radical cation using a spectrophotometric assay. The $ABTS^{+\bullet}$ radical cation was prepared by mixing $ABTS^{+\bullet}$ stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture remained for 12-24 h until the reaction was complete and the absorbance was stable. Antioxidant capacity was determined following the procedure described by RE *et al.* (1999) with a minor modification. The $ABTS^{+\bullet}$ solution was diluted with 80% methanol to an absorbance of 0.700 ± 0.020 at 734 nm. For the photometric assay, 1.48 mL of the $ABTS^{+\bullet}$ solution and 20 µL of the buckwheat extracts or Trolox standards were mixed and measured immediately and again after 6 min at 30°C and 734 nm using a spectrophotometer (UV-160 1PC, Shimadzu, Kyoto, Japan). Appropriate solvent blanks were run in each assay. The antioxidant capacity was calculated on the basis of percentage inhibition of absorbance at 734 nm using a Trolox standard curve and was expressed as µmol Trolox/g of dry matter (d.m.).

The Photo-Induced Chemiluminescence Assay (PCL), carried out according to the method of POPOV and LEWIN (1999), was used to measure the antioxidant capacity of unfermented and fermented groat extracts against superoxide anion radicals ($O_2^{-\bullet}$), which were generated from luminol, a photosensitizer, under exposure to UV light. The antioxidant capacity of buckwheat water or methanol extracts were determined using ACW (antioxidative capacity in water-soluble substances) and ACL (antioxidative capacity in lipid-soluble substances) kits (Analytik Jena, Leipzig, Germany) reported in details by ZIELIŃSKA *et al.* (2010). Antioxidant capacity was expressed in terms of µmol Trolox/g d.m.

Statistical analysis

The measurement were performed in triplicate for each of two independent fermentation batches. The data are the mean results with the standard deviation. The effects of the two parameters, type of product (P) and fermentation process (F) or their interactions (P x F) were tested using a two-way ANOVA (Statistica, ver. 7.1, USA). Fisher's Least Significant Difference Test at a significance level of $p<0.05$ was performed for *post-hoc* comparison.

RESULTS AND DISCUSSION

Total phenolic compounds (TPC) and rutin (Ru) contents

Table 1 shows total phenolic compounds (TPC) and rutin (Ru) contents of unfermented and fermented raw and roasted buckwheat groats. Raw buckwheat groats (7.2 mg Ru equiv/g d.m.) was almost two times richer in phenolic compounds

Table 1 - The phenolic, vitamin B1, B2, B6, C and α-, γ- and δ-tocopherol contents of SSF raw and roasted buckwheat groats.

	B	SSF-B	RB	SSF-RB	ANOVA		
					P	F	P x F
The phenolic contents							
rutin [µg/g d.m.]	205.84±0.94a	164.54±24.59b	158.84±3.42b	142.09±1.81b	ns	***	***
total phenolic compounds [mg Ru equiv/g d.m.]	7.19±0.84a	5.15±0.23b	3.54±0.02c	3.41±0.16c	***	***	***
Content of vitamin B1, B2, B6 and C							
thiamine (B1) [µg/g d.m.]	9.70±0.29b	10.91±0.50a	5.41±0.13d	7.56±0.29c	***	***	***
riboflavin (B2) [µg/g d.m.]	1.65±0.12a	1.70±0.08a	1.29±0.09b	1.18±0.01c	***	***	***
pyridoxin (B6) [mg/g d.m.]	0.12±0.01b	0.33±0.01a	0.11±0.02b	0.12±0.01b	***	***	***
L-ascorbic acid [mg/g d.m.]	0.05±0.01c	0.11±0.02a	0.05±0.01c	0.09±0.01b	***	***	***
Content of α-, γ- and δ-tocopherol							
α-tocopherol	0.73±0.06b	1.10±0.12a	0.27±0.17c	0.64±0.07b	***	***	***
γ-tocopherol	106.20±1.97b	126.88±3.18a	60.40±0.59d	95.08±1.01c	***	***	***
δ-tocopherol	2.93±0.30b	3.45±0.28a	3.78±0.32a	2.10±0.07c	ns	***	***

Control buckwheat groats: raw (B) and roasted (RB); fermented buckwheat groats: raw (SSF-B) and roasted (SSF-RB). Data expressed as mean±standard deviation (n=6). Different letters within the same line indicate statistically significant differences at $p<0.05$ in NIR Fisher test (for interactions PxF).
*** significant effects by kind of products (P), fermentation process (F) or their interactions (PxF) at $p<0.05$; ns, not significant.

than the roasted groat (3.5 mg Ru equiv/g d.m.). Our results confirms results of ZIELIŃSKA *et al.* (2007b).

SSF led to a statistically significant decrease in the rutin content of raw groats (by 20%) but did not affect the rutin content of roasted groats. Performed two-way ANOVA analysis showed that the content of rutin was closely associated with the used SSF process.

It is well known that buckwheat groats contain mainly rutin and a little amounts of isovitexin, depending on the cultivar and growth conditions (WIJNGAARD and ARENDT, 2006). Isovitexin was not detected in our buckwheat samples. The rutin content of raw buckwheat groats was 205.8 μg/g d.m. and 23% lower ($p<0.05$) for roasted groats. It is connected with the hydro-thermal processes used for roasting. DIETRICH-SZOSTAK and OLESZEK (1999) found that rutin content in buckwheat groat was affected by temperature and heating time adversely. WANG *et al.* (2011) found the reduction of rutin content after the fermentation of peanut flour with the different strains of lactic acid bacteria.

SSF led to a statistically significant decrease in TPC for raw groats (by 28%), but did not affect the TPC of SSF roasted groats. DORDEVIC *et al.* (2010) showed that the fermentation of buckwheat by *S. cerevisiae* and *L. rhamnosus* caused the increasing of TPC compared to non-fermented samples. DUEÑAS *et al.* (2012) found significant increase of phenolic acid content in the soybean fermented with different microorganisms (*Aspergillus oryzae, Rhizopus oryzae* and *Bacillus subtilis*).

Vitamins B and C contents

The contents of vitamins B1, B2, B6 and C before and after fermentation of raw and roasted buckwheat groats are presented in Table 1. The raw and roasted groats used for fermentation had different thiamine and riboflavin contents, but the levels of pyridoxine and L-ascorbic acid were similar. The level of vitamin C in whole buckwheat flour ranges from 3.9 to 7.3 mg/l00 g, our results are similar to those presented by other authors (WIJNGAARD and ARENDT, 2006). The concentrations of B1 and B2 in the raw groats were 80 and 28%, higher, respectively, than those of roasted groats. This finding is in accordance with reports regarding the presence of vitamins B in buckwheat (WIJNGAARD and ARENDT, 2006).

The SSF of raw groats caused a statistically significant increase of thiamine, pyridoxine and L-ascorbic acid ($p<0.05$) compared to the control samples. The pyridoxine content increased almost three-fold, the L-ascorbic acid content more than two-fold, whereas the thiamine content by 12%. Fermentation did not change the riboflavin content of raw groats. SSF of roasted groats caused the increasing of thiamine content by 40%, the L-ascorbic acid content almost doubled, the pyridoxine contents did not change and riboflavin content decreased.

The increase of the contents of some B-group vitamins obtained in this study is similar to those of previous reported by other authors. The increases in thiamine content that we obtained after fermentation of the raw and roasted buckwheat groats are in contrast to results reported for SSF of other substrates. NOUT and ROMBOUTS (1990) found that tempeh fermentation of soya caused the increase of content of vitamins, except for thiamine for which decrease of the content was observed. FADAHUNSI (2009) showed the effect of *Rhizopus oligosporus* on the vitamin content in the flour obtained from bambara nut. After the 24 h fermentation this author found the significant reduction in the thiamine content, while riboflavin, folacin, niacin and biotin content increased significantly. In traditional Turkish fermented wheat-flour-yoghurt mixture (tarhana) EKINCI (2005) found no significant differences in thiamine and pyridoxine contents with the increase of fermentation period. While, he observed significant increases of riboflavin, niacin, pantothenic acid, ascorbic acid and folic acid during the fermentation.

Tocopherol content

Table 1 shows the tocopherol content in raw and roasted buckwheat groats before and after fermentation. In buckwheat, γ-tocopherol is the major tocopherol homologue and the β-form is present in only trace amounts. In our study α-T, γ-T, δ-T were found in the raw and fermented buckwheat materials, whilst β-T was not detected. The raw groats had almost three- and two-fold higher contents of α-T and γ-T, respectively, compared to the roasted groats. GEMROT *et al.* (2006) found for gourd seeds the decrease of tocopherol content under the influence of roasting process. In the literature data there are no information concerning the tocopherol level in fermented buckwheat groats.

The SSF of raw and roasted groats caused a statistically significant increase of the α-T and γ-T. The content of δ-tocopherol was significantly associated with the used fermentation process, but not with the type of the product. In contrast to results obtained in this study the literature data showed the decrease of tocopherols content under the influence of fermentation. For soya fermented with *A. oryzae* decreased of α-T content was observed by ESAKI *et al.* (1994), but they do not observed a modification of tocopherol content for soya fermented by *B. subtilis* or *R. oligosporus*. DENTER *et al.* (1998) found that in soybean tempeh the tocopherols content slightly decreased as a consequence of fermentation with 14 varieties of *Rhizopus* studied.

Contents of inositol hexaphosphate and its lower forms

Table 2 shows the contents of inositol hexaphosphate (IP-6) and its lower forms (IP-5, IP-4 and IP-3) in raw and roasted buckwheat groasts before and after fermentation. Phytic acid may be classified as a prohealthy or antinutritional compound, depending on its action. It can form an iron chelate that inhibits iron-mediated oxidative reactions and limits site specific DNA damage. Phytic acid inhibits tumor growth by suppressing the formation of damaging hydroxide free radicals and other reactive oxygen species (VUCENIK and SHAMSUDDINY, 2003).

The raw groats contained 18.3 mg/g d.m. of IP-6. After roasting, a decrease by 43% was noted. This finding was in accordance with the previously reported by other authors (ZIELIŃSKI *et al.*, 2006). In our study, IP-5, IP-4 or IP-3 were not found in raw groats, but they were detected after roasting.

After 24 h fermentation with *Rhizopus oligosporus*, the formation of IP-3, IP-4, and IP-5 was noted in fermented raw groats. Moreover, SSF caused the significant increase of IP-3 and IP-4 in fermented roasted groats (Table 2). These findings were related to the three- and two-fold decreased content of IP-6 in fermented raw and roasted groats. Fermentation, steaming and extrusion cooking were identified as processes causing the degradation of IP-6 to the lower forms (ZIELIŃSKI *et al.*, 2006). EGOUNLETY and AWORH (2003) showed that fermentation with *R. oligosporus* reduced the phytic content by 30.7% in soybean, 32.6% in cowpea and 29.1% in ground bean.

Antioxidant capacity

In this study, the antioxidant capacity of raw and roasted groats before and after fermentation was measured against the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+\bullet}$) and by the photo-induced chemiluminescence assay (PCL) against the superoxide anion radical ($O_2^{-\bullet}$).

The PCL method can be conducted by two different protocols, ACW and ACL, which allowed for measurement of antioxidant capacity of the water- and lipid-soluble components, respectively. Finally, it was possible to calculate the total antoxidant capacity as a sum of ACW and ACL values. The results are compiled in Table 3. The antioxidant capacity of raw groats against $ABTS^{+\bullet}$ and $O_2^{-\bullet}$, expressed as ACW+ACL, was 34 and 20% higher compared to the roasted groats. It should be also noted that lipophilic antioxidants (ACL) were the main contributor (up to 80%) to the total antioxidant capacity of raw and roasted groats. ZIELIŃSKA *et al.* (2007a) observed decrease by 27% of ABTS value for buckwheat groats after using roasting process. Also ZHANG *et al.* (2010) found that the scavenging activity of tartary buckwheat flour against $O_2^{-\bullet}$ was reduced by roasting.

The SSF of raw and roasted groats caused statistically significant decreases ($p<0.05$), by 32 and 15%, respectively, of antioxidant capacity measured against $ABTS^{+\bullet}$. Also decrease of total antioxidant capacity evaluated by PCL (ACW+ACL) was noticed under the influence of SSF. Similar finding were presented by BERGHOFER *et al.* (1998) for the fermented product obtained from faba bean, soybean and oat. In our study lipophilic antioxidants were significantly reduced after fermentation of raw and roasted groats (by 35 and 13%). On the other hand, these lipophilic antioxidants highly contributed, up to 75%, to the total antioxidant capacity of the both fermented buckwheat products.

The observed decrease in antioxidant capacity of fermented raw and roasted groats could be connected with the increasing of water soluble

Table 2 - Content of inositol phosphates of SSF buckwheat groats.

Sample	Inositol phosphates [mg/g d.m.]			
	IP-3	IP-4	IP-5	IP-6
B	nd	nd	nd	18.33±0.24a
SSF-B	0.33±0.04b	1.65±0.15b	2.01±0.18b	5.65±0.19c
RB	0.29±0.01b	2.22±0.07a	3.80±0.08a	10.51±0.07b
SSF-RB	0.44±0.05a	1.86±0.18b	3.62±0.09a	5.66±0.10c
ANOVA effects				
P	***	***	***	***
F	***	***	***	***
P x F	***	***	***	***

Control buckwheat groats: raw (B) and roasted (RB); fermented buckwheat groats: raw (SSF-B) and roasted (SSF-RB). Data expressed as mean±standard deviation (n=6). Different letters within the same column indicate statistically significant differences at $p<0.05$ in NIR Fisher test (for interactions PxF).
*** significant effects by kind of products (P), fermentation process (F) or their interactions (PxF) at $p<0.05$; ns, not significant; nd, non detected.

Table 3 - The antioxidant capacity of SSF buckwheat groats provided by ABTS and PCL assay.

Sample	Antioxidant capacity (μmol Trolox/g d.m.)			
	against $ABTS^{+\bullet}$	against $O_2^{-\bullet}$		
		ACW	ACL	ACW + ACL
B	22.93±1.24a	3.97±0.59b	17.32±0.59a	21.29±0.11
SSF-B	15.68±0.94b	4.57±0.59a	11.18±0.59d	15.75±0.12
RB	15.15±2.34b	3.76±0.59c	13.19±0.59b	16.95±0.74
SSF-RB	12.82±0.51c	3.84±0.59c	11.50±0.59c	15.34±0.20
ANOVA effects				
P	***	***	***	***
F	***	***	***	***
P x F	***	***	***	***

Control buckwheat groats: raw (B) and roasted (RB); fermented buckwheat groats: raw (SSF-B) and roasted (SSF-RB). Data expressed as mean±standard deviation (n=6). Different letters within the same column indicate statistically significant differences at p<0.05 in NIR Fisher test (for interactions PxF).
*** significant effects by kind of products (P), fermentation process (F) or their interactions (PxF) at p<0.05; ns, not significant.

antioxidants (e.g. vitamins B, L-ascorbic acid) and lipid-soluble antioxidant (e.g. increased tocoperols level) and decreasing of the phenolic compounds, including rutin. In our study, the total content of vitamins B was positively correlated with the antioxidant capacity of buckwheat groats before and after fermentation, when evaluated by ABTS test (r = 0.43) and by ACW assay (r = 0.86). The level of ascorbic acid was also positively correlated with antioxidant capacity, as measured by ACW assay (r = 0.74). Moreover, a very high correlation was calculated between rutin contents and antioxidant capacity, as determined by the ABTS test (r = 0.99). The same observation was made in relation to TPC contents and values provided by ABTS. Phenolic compounds, including rutin, could be extracted by medium used for the ACL assay since a high correlation was also noted between Ru and TPC vs ACL (r = 0.90 and r = 0.77, respectively). In our study, no correlation existed between total tocopherols and ACL values, what could indicate that this group of compounds has no impact on the formation of antioxidant capacity of non-fermented and fermented buckwheat groats, both raw and roasted. Therefore, the antioxidant capacity of fermented groats clearly depended on the antioxidant activity of vitamins B, vitamin C and rutin. It is a known that vitamins B have little or no antioxidant activity (GLISZCZYŃSKA-ŚWIGŁO, 2006). On the other hand, the antioxidant activity of rutin provided by ABTS and PCL ACL assay (1.16±0.02 and 1.38±0.07 mmol Trolox, respectively) as reported by ZIELIŃSKA *et al.* (2010), was higher than antioxidant activity of L-ascorbic acid (1.05±0.02 mmol Trolox by ABTS and 1.0 mmol Trolox in PLC ACW) (RE *et al.*, 1999). Therefore, the noted decrease of the antioxidant capacity evaluated by ABTS and PCL assay in SSF raw and roasted groats was mainly related to the decreased rutin content and could not be ameliorated by increased content of both vitamins B and C.

General remarks

Two-way ANOVA used for statistical analysis of the obtained data indicated that both analysed parameters: type of product (raw and roasted), fermentation process (fermented and unfermented) and their interactions significantly influenced on the obtained results. Only in the case of rutin and δ-tocopherol content, the type of product had not significant effect. Also principal component analysis (PCA) was performed on the covariance matrix of the samples with no rotation (data not showed). Two principal components were extracted (PC1 and PC2) and together explained 83.66% of the total variance. The PC1 was differentiated by almost all investigated compounds except rutin, IP-5, IP-6, δ-tocopherol and antioxidant capacity determined by ACL and ABTS methods.

CONCLUSIONS

Solid-state fermentation with *Rhizopus oligosporus* was used to obtain tempeh-type products from raw and roasted buckwheat groats. The used SSF enhanced water soluble vitamins (thiamine, pyridoxine and L-ascorbic acid), as well as and α-, δ- and γ-tocopherol contents. After fermentation, a decrease in total phenolic compounds as well as rutin contents was observed. These changes had an impact on $ABTS^{\bullet+}$ radical cation and superoxide anion radical ($O_2^{-\bullet}$) scavenging activity of fermented raw and roasted buckwheat groats. Based on the correlation studies and knowledge on the antioxidant activ-

ity of analysed bioactive components it should be noted that rutin content was the main factor responsible for the antioxidant capacity of fermented products. On the other hand, the fermented products were rich source of vitamins B and C and therefore SSF with *Rhizopus oligosporus* can be recommended for production of tempeh-like functional buckwheat-based foods with reduced antinutritional factor.

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PAPER

COMPARISON OF FATTY ACID PROFILES OF MALE AND FEMALE GIANT RED SHRIMPS (*ARISTAEOMORPHA FOLIACEA* RISSO, 1827) OBTAINED FROM MEDITERRANEAN SEA

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ABSTRACT

This study was carried out to detect the concentration of fatty acid in female and male specimens of commercially important giant red shrimp (*Aristaeomorpha foliacea*) obtained from (including 20 male shrimps and also 20 female shrimp) Mediterranean Sea. In fatty acid composition, the saturated fatty acid fraction was dominant, followed by polyunsaturated fatty acid and monounsaturated fatty acid for both sexes. The analyses indicated that PUFAs, and the MUFAs content were higher in female shrimp than in those of males and they were statistically significant differences in fatty acid profile between females and males ($p<0.05$).

- Keywords: *Aristaeomorpha foliacea*, fatty acids, giant red shrimp, Mediterranean Sea -

INTRODUCTION

The giant red shrimp (*Aristaeomorpha foliacea* RISSO, 1827) belongs to the family Aristeidae, which includes other important species such as the blue and red shrimps (*Aristeus antennatus* RISSO, 1816) and the scarlet shrimp (*Plesiopenaeus edwarsianus* JOHNSON, 1868) (RAGONESE *et al.*, 1997). *A. foliacea* is widely distributed in the eastern and western Atlantic, Indian Ocean and western Pacific, in the waters of Japan, Australia, New Zealand and in the Mediterranean Sea. In the Mediterranean Sea, the species inhabits muddy bottoms of the continental slope approximately between 100 and 1200 m depth. This species plays an important role in the overall biomass of the Mediterranean Sea and represent an important commercial resource among the other shrimp species since 1959 (D'ONGHIA *et al.*, 1998; DESANTIS *et al.*, 2003; FERNANDEZ *et al.*, 2011). Due to its economic relevance, recently there are many studies on this species from Mediterranean Sea and there has been a considerable amount of research on nutritional value of various species of shrimp. However there is not any data on the nutritional and fatty acids composition of *A. foliacea.*

Seafoods are important source of nutrients in the human diet. Crustaceans such as shrimps have high nutritive value, are low in fat, especially saturated fatty acids; contain high amount of polyunsaturated fatty acids (omega-3 and omega 6) (OKSUZ *et al.*, 2009; TAG EL-DIN *et al.*, 2009; TURAN *et al.*, 2011; SHALINI *et al.*, 2013). These fatty acids could not be synthesised by the human body and must be obtained through the diet are crucial for normal brain structure and function (ALASALVAR *et al.*, 2002; RICHARDSON, 2003). In addition to this, these fatty acids have great importance to humans for prevention of coronary artery diseases, diabetes, hypertension and cancer (VISENTAINER *et al.*, 2007; CENGIZ *et al.*, 2012). The levels of these fatty acids are low in many modern diets, particularly those in which highly processed foods predominate. The omega-3 polyunsaturated fatty acids (PUFAs) that the human needs (EPA and DHA) are found in appreciable quantities only in oily fish, seafood, aquatic invertebrates and algae (RICHARDSON, 2003; GÖKCE *et al.*, 2011). Therefore, this study was carried out to determine the nutritive value and fatty acid content of giant red shrimp collected from the Mediterranean Sea of Turkey.

MATERIALS AND METHODS

Collection and preparation of samples

The samples were caught by bottom trawlers between 450 and 500 m of depth, during in 2013 from Mediterranean Sea of Turkey (36° 22' 707"N-24° 25' 941" E /36° 14' 919" N- 34° 19' 163" E).

Immediately, after collection, shrimps were stored in a container, preserved in crushed ice and transferred to the laboratory, where the heads, shells and intestines were separated and placed in labeled polyethylene bags respectively and stored at -20°C until processing for analysis. For each season, 20 female and 20 male samples of *A. foliacea* were obtained by random sub-sampling.

Fatty acid analysis

The samples were transported with dry ice to the Accredited Industrial Services Laboratory of Turkey/Istanbul. The methyl esters of fatty acids of samples were prepared according to IUPAC Methods II. D. 19 (1979). The analyses were carried out by using a Perkin Elmer Autosystem. XL Gas Chromotography and Flame Ionization Detector (FID) equipment and a Supelco 2330 fused silica capillary column (30 m x 0.25 mm x 0.20 μm film thickness) for determining the fatty acid composition.

Data analysis

For data analysis independent samples t-test was used to identify significant differences in fatty acid concentration. Statistical significance was defined at $p<0.05$. The mean values were obtained from 3 experiments and reported as means±SD (DINÇER and AYDIN, 2014).

RESULTS AND CONCLUSIONS

Table 1 shows mean weights (g) of female and male species of shrimp (*Aristaeomorpha foliacea*) obtained from Mediterranean Sea.

Table 1 - The Nutritional value and mean weights (g) of female and male shrimps.

Parameter	Female	Male
Weight (g)	29.96^{a}±6.53	12.26^{b}±2.07
Protein (%)	15.72^{a}±1.02	18.0^{b}±0.80
Lipid (%)	0.72^{a}±0.11	0.51^{b}±0.13

Different letters (a,b) in the same row represent significant statistical differences ($p<0.05$).

The mean weight for female shrimps was found to be higher than the mean weights for male shrimps. Similar results were reported by YILMAZ and YILMAZ (2007) for *Penaeus semisulcatus* collected from Mediterranean Sea of Turkey and also by TURKMEN (2012) and CEVIK *et al.* (2008) for *Penaeus kerathurus* and *Parapenaeus longirostris* respectively. Our findings are consistent with prior research. The levels of protein and lipid vary depending upon season, age, maturity, sex, water temperature, spawning cycle and availability of food, types of diet and

feeding system of organism (OKSUZ *et al.*, 2009; TURAN *et al.*, 2011; ROSLI *et al.*, 2012).

In a study on *Crangon crangon* protein and lipid content were 18.47 and 0.95% respectively (TURAN *et al.*, 2011). SAGLIK and IMRE (1997) determined total lipid as 0.93% for *Parapenaeus longirostris* and 0.58% for *Penaeus semisulcatus*. YANAR *et al.* (2011) found that protein and lipid of *Penaeus semisulcatus* ranged between 22.76-23.53% and 0.76-1.44% respectively. FATIMA *et al.* (2012) reported that lipid in the muscle tissue of *Fenneropenaeus penicillatus* varied from 0.92 to 1.0% and of *F. merguiensis* from 0.87 to 0.98%. Protein and lipid were also reported as 20% and 1.1% for *Parapenaeus longirostris* and 14.2% and 2.6% for *Plesionika martia* by OKSUZ *et al.* (2009). DINCER and AYDIN (2014) determined that protein and lipid of *Metapenaeus affinis* ranged between 18.4-19.1% and 1.07-1.30% respectively. In the present study the content of protein and lipid were identified as slightly lower than those reported previously for some shrimp species. The main reason for this is thought to be related to variation in seasonal feeding habits (different types of diet and feeding system) and habitats. In the study, the protein content for male shrimps was found to be higher than the protein content for female shrimps whereas the lipid content was found to be lower in male shrimp ($p<0.05$). Similar results were reported by DINCER and AYDIN (2014) for female and male species of *Metapenaeus affinis*.

The ratios of PUFA/SFA and *n*-6/*n*-3 and the fatty acid compositions of the investigated shrimp are presented in Table 2.

The fatty acids analyzed were grouped as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). In the present study, in both groups, SFA was the highest followed by PUFA and MUFA. These results were in agreement with that obtained by TURAN *et al.* (2011) who reported highest levels of SFA followed by PUFA and MUFA for brown shrimp (*Crangon crangon*) from Sinop Region, Black Sea. Similar results were also reported by OURAJI *et al.* (2011) for wild Indian white shrimps (*Fenneropenaeus indicus*); by OKSUZ *et al.* (2009) for rose shrimp (*Parapenaeus longirostris*) and red shrimp (*Plesionika martia*); by YANAR *et al.* (2011) for *Penaeus semisulcatus*; by FATIMA *et al.* (2012) for *Fenneropenaeus merguiensis* and *F. penicillatus* and by DINCER and AYDIN (2014) for *Metapenaeus affinis*. According to the results, C16:0 (Palmitic acid) and C18:0 (Stearic acid) were the main saturated fatty acids in both shrimp species. In both sexes, the predominant monounsaturated fatty acids were found as C18:1 (Oleic acid). The principal acids in PUFA group were eicosapentaenoic acid (C20:5, EPA), docosahexaenoic acid (C22:6, DHA) and linoleic acid (C18:2) for female and male shrimp species. These results agree with studies on fatty acids found in other shrimp species (OKSUZ *et al.*, 2009; TAG EL-DIN *et al.*, 2009; SAGLIK and IMRE 1997; OURAJI *et al.*, 2011; TURAN *et al.*, 2011; YANAR *et al.*, 2011; FATIMA *et al.*, 2012). In the present study, the rate of SFAs, PUFAs and MUFAs were determined as 43.69%, 29.33% and 24.37% for female shrimps and as 47.15%, 25.41% and 17.34% for male shrimps respectively. However, different percentage compositions of fatty acids obtained from various species and subspecies of sea and freshwater shrimps were also reported by several Authors. These differences among species might be associated with the different characteristics of the shrimp species (KARUPPASAMY *et al.*, 2013). In a study, TURAN *et al.* (2011) reported SFA, MUFA and PUFA rates in brown-color shrimp at 33.04, 22.17 and 29% respectively. OKSUZ *et al.* (2009) reported the MUFA rate in *P. longirostris* and *P. martia* at 26.09% and 34.47% respectively. OURAJI (2011) reported the rate of SFA in wild white Indian shrimp and its cultured specimen at 32.88 and 33.79% respectively. EMAMI *et al.* (2014) reported that the rate of SFA in *Penaeus vannamei* at 37.26%, in *Penaeus semisulcatus* at 49.12% and the rate of MUFA in *P. vannamei* at 24.9%, in *P. semisulcatus* at 33.76% and the PUFA in *P. Vannamei* at 37.84%, in *P. semisulcatus* at 16.9% respec-

Table 2 - Fatty acid composition of female (F) and male (M) shrimps.

Parameters	Female (%)	Male (%)
C6:0	1.23^{a}±0.03	2.70^{b}±0.01
C8:0	0.11±0.01	n.d
C14:0	3.43^{a}±0.03	2.16^{b}±0.10
C16:0	27.59^{a}±1.20	27.29^{a}±0.60
C18:0	11.10^{a}±0.04	14.37^{b}±0.75
C24:0	0.23^{a}±0.04	0.63^{b}±0.03
∑SFA	43.69^{a}	47.15^{b}
C16:1	2.69^{a}±0.20	1.66^{b}±0.23
C18:1	21.68^{a}±0.70	15.68^{b}±1.00
∑MUFA	24.37^{a}	17.34^{b}
C18:2*n*6	6.26^{a}±0.02	4.41^{b}±0.60
C20:3*n*3	0.11±0.01	n.d
C20:5*n*3(EPA)	13.36^{a}±0.50	11.47^{b}±0.60
C22:6*n*3(DHA)	9.60^{a}±0.50	9.53^{a}±0.05
∑PUFA	29.33^{a}	25.41^{b}
PUFA/SFA	0.67	0.54
∑*n*3	23.07	21.00
∑*n*6	6.26	4.41
*n*6/*n*3	0.27	0.21
Unidentified	2.61	10.1

n.d.: below detection limit; Data are expressed as mean±SD of triplicate measurements.
Different letters (a,b) in the same row represent significant statistical differences ($p<0.05$).

tively. The results obtained in this study showed slightly similarity to the findings of the mentioned researchers. This difference may be due to geographical variation, seasonal conditions and different types of diet and feeding system. Fatty acid content is also influenced by species, maturity period, size and age of shrimp. The indices of PUFA/SFA and n-6/n-3 ratios were widely used to evaluate the nutritional value of fat for human consumption. According to some nutritional recommendations the PUFA/SFA ratio in human diets should be above 0.45 and, within the PUFA, the n-6/n-3 ratio should not exceed 4.0 (ALFAIA *et al.*, 2010. In the present study the PUFA/SFA and n-6/n-3 ratios of *A. foliacea* (for both female and male shrimps) were within the range reported for human diets. It could be demonstrated that the giant red shrimp (*A. foliacea)* is a desirable item in human diet when the levels of n3/n6 and PUFA/SFA ratios were considered.

Comparison of fatty acid composition between two sexes

The fatty acid compositions of female shrimp species found to be 43.69% saturated (SFAs), 29.33% polyunsaturated acids (PUFAs) and 24.37% monounsaturated (MUFAs) whereas the fatty acid compositions of male shrimp consist of 47.15% saturated (SFAs), 25.41% polyunsaturated acids (PUFAs) and 17.34% monounsaturated (MUFAs). Among these the highest concentrations of SFAs (47.15%) were detected in male shrimp species while the highest concentrations of PUFAs (29.33%) and MUFAs (24.37%) were detected in female shrimp. There is a significant difference between the SFA, PUFA and MUFA profiles in both sexes ($p<0.05$). Similar results were reported for female and male species of *Metapenaeus affinis* by DINCER and AYDIN (2014) and by ESKANDARI *et al.* (2014) for female and male species of *M. affinis*. Based on results, the amount of palmitic acid (C16:0) for female shrimp (27.59%) was almost the same as in male shrimp species (27.29%) ($p>0.05$), while the amount of oleic acid (C18:1) (21.68%) was higher than those in male shrimp (15.68%) ($p<0.05$). The present study also showed that the amount of docosahexaenoic acid (C22:6, DHA) of female shrimp (9.60%) are almost the same as in male shrimp species (9.53%) ($p>0.05$) whereas the levels of eicosapentaenoic acid (C20:5, EPA) and linoleic acid (C18:2) were higher than those in male shrimp ($p<0.05$). In a study, DINCER and AYDIN (2014) reported that the EPA content of male *Metapenaeus affinis* was lower than female *M. affinis*. The ratio of PUFA to SFA (0.54) and *n*-6 to *n*-3 (0.21) for the male shrimp was found to be lower than those in female shrimp. Although both shrimps were subjected to the same sea water and climate conditions, there were naturally some differences between them, in terms of their size, sex and quantity of lipid.

In conclusion, from a nutritional point of view, both male and female giant red shrimps demonstrated acceptable quality; in particular, the female giant red shrimps had the highest levels of PUFAs, and the MUFAs content. Both sexes are low in fat and are considered to belong to a low fat class group. Further investigations are required to obtain more information about this species.

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PAPER

EVALUATION OF LIPOLYSIS AND VOLATILE COMPOUNDS PRODUCED BY THREE *PENICILLIUM ROQUEFORTI* COMMERCIAL CULTURES IN A BLUE-TYPE CHEESE MADE FROM OVINE MILK

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ABSTRACT

The aim of this work was to compare the effect of three different *Penicillium roqueforti* commercial cultures (named PS1, PS2 and PS3) on proteolysis, lipolysis and volatile flavour profile of a blue cheese made from ovine milk and lamb paste rennet. Proteolytic parameters were not significantly affected by the *Penicillium roqueforti* culture, while cheeses manufactured using PS2 and PS3 cultures showed the higher amount of free fatty acids (FFA) and volatile FFA when compared with PS1 culture after 30 days of ripening. This study can provide important information for obtaining the desired extent of lipolysis in this type of blue cheese.

- Keywords: lipolysis, ovine blue cheese, *Penicillium roqueforti*, volatile compounds -

INTRODUCTION

Blue cheeses represent a cheese variety characterised by the presence of blue or blue-green veins, caused by induced growth of the mould *Penicillium roqueforti* within the cheese matrix. This category includes, among others, PDO (protected designation of Origin) or PGI (protected geographical indication) cheeses made from bovine (Gorgonzola, Italy; Danablu, Denmark; Stilton, United Kingdom), and ovine (Roquefort, France) milk. The manufacture process of blue mould cheeses has been well described previously (ARDÖ, 2011), but it can vary depending on country or region where cheese is produced. In particular in Sardinia island (Italy), a small production of ovine blue cheese is manufactured on industrial scale. This cheese is characterized by the use of lamb paste rennet for its production, differently from most of blue cheeses, where the milk coagulation is usually induced by the action of liquid rennet. Cheeses produced with paste rennet are characterized, at late ripening stages, by high amounts of free fatty acids due to the presence of lipolytic enzymes (lipases) in the rennet extract (ADDIS *et al.*, 2005; VIRTO *et al.*, 2003).

The ovine blue cheese is made following the process described herein. Thermised whole ovine milk is inoculated with a *Penicillium roqueforti* culture and a mesophilic starter at 36°C. Milk is coagulated using a water solution of lamb paste rennet, and the coagulum is cut into small granules (about 4 mm in size), drained, moved into moulds, dry salted and ripened for 30 days at 10°C and 85% of relative humidity. Cheeses are pierced using a stainless steel needle 7 days after production. At the end of ripening (30 days) cheeses are cylindrical in shape (height and diameter around 100 and 200 mm, respectively) and weigh between 2.5 and 3.0 kg.

The growth of *Penicillium roqueforti* within the cheese matrix results in a high production of its extracellular enzymes, and consequently in an extensive secondary proteolysis and lipolysis of blue cheeses during ripening (CALZADA *et al.*, 2013; CONTARINI and TOPPINO, 1995; PRIETO *et al.*, 1999; 2000).

Furthermore, blue cheeses are characterised by an high level of flavour compounds produced by lipid, lactose and protein catabolism (ARDÖ, 2011); in particular a large amount of methyl ketones is produced by the β-oxidation of free fatty acids followed by a decarboxylation reaction (QIAN *et al.*, 2002; VOIGT *et al.*, 2010).

The aim of this work was to compare the effect of three different *Penicillium roqueforti* commercial cultures on proteolysis, lipolysis and volatile flavour profile of Sardinian ovine blue cheese after 30 days of ripening, in order to provide useful information to cheese makers about the biochemical effects produced by each culture during ripening of this cheese.

MATERIALS AND METHODS

Mould cultures

Three commercially available *P. roqueforti* cultures, named PS1 (PRB 6 HYP 5 D, Danisco Deutschland GmbH, Niebull, Germany), PS2 (PR4, Chr. Hansen, Hørsholm, Denmark) and PS3 (PV LYO 10 D, Danisco Deutschland GmbH, Niebull, Germany), were separately used to produce blue-type cheeses. More details about the specific properties of each mould culture can be found in the respective product description documents provided by the Supplier.

Mould cultures were dissolved in water and added to 50 L of milk before renneting at a final concentration of 5.0E+6 CFU per L of milk.

Small-scale cheese-making

Cheese production was performed at the dairy technology laboratories of Agris Sardegna (Olmedo, Italy). Whole ovine milk was placed in a staining steel cheese vat, batch-heated at 65°C in 10 min, and quickly cooled down to 36°C (in 5 min). After cooling, a mould culture and a milk starter culture, prepared using a freeze-dried mixed culture (Bulk Set HM M4 LYO, Danisco, Deutschland GmbH, Niebull, Germany; 1 L·100 L^{-1} of milk), were added to milk. The composition of the milk starter culture was: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis biovar. diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*. Milk was coagulated using a water solution (30 g·100 L^{-1} of milk) of lamb paste rennet (Caglificio Manca, Thiesi, Italy). About 45 min after the addition of rennet, the coagulum was cut into small granules (about 4 mm in size), and the drained curd was moved into moulds and kept at 20-25°C under saturated humidity conditions for 18 h. Cheeses were then kept at 10°C for 24 h and 90-95% of relative humidity, dry salted and finally ripened for 30 days at 10°C and 85% of relative humidity. Cheeses were pierced using a stainless steel needle 7 days after production. Three replicates were carried out for each treatment level, for a total of nine cheese-making trials.

Cheese composition and nitrogen fractions

Samples were taken for analysis 1 day after production and after 30 days of ripening. Cheeses were analysed for pH (pH meter 420 A, Orion, Boston, USA), total solids (TS) (ISO, 2004), fat (Soxhlet method), total nitrogen (TN) (IDF, 1993), soluble nitrogen at pH 4.6 (SN), soluble nitrogen in 12% trichloroacetic acid (TCA-SN), and soluble nitrogen in 10% phosphotungstic acid (PTA-SN) (GRIPON *et al.*, 1975).

Free fatty acids analysis

The free fatty acids content was determined as previously described by ADDIS *et al.* (2005) on cheeses 1 day after production and after 30 days of ripening.

Volatile flavour profile analysis

Volatile compounds were determined by SPME (solid-phase microextraction) -GC-FID/MS on cheeses after 30 days of ripening. 0.5 g of freshly grated cheese was placed in a 10 mL vial, hermetically sealed with a seal and thin Viton septa. The vials were held at 37°C in a thermostated autosampler (8200 CX Varian, Walnut Creek, CA, USA) for 5 min to reach equilibrium between sample and above headspace prior to SPME headspace sampling. A divinylbenzene (DVB)/carboxen (CAR)/polydimethylsiloxane (PDMS) 50/30 μm fiber (Supelco Inc., Bellefonte, PA, USA) was exposed to headspace under constant stirring for 7 min in samples after 30 days of ripening. During headspace sampling, samples were maintained at 37°C, and volatile compounds adsorbed on the fiber were immediately thermally desorbed in the injector port of a 3800CX Varian GC (Walnut Creek, CA, USA) equipped with a 1077 split/splitless injector (250°C), coupled with a flame ionization detector (FID; 250°C), and a Saturn 2000 ion trap mass spectrometer system (MS detector) (Walnut Creek, Varian, CA, USA). Volatile compounds were injected in splitless mode in two identical capillary columns (DB-WAX 30 m, 0.32 mm i.d., 0.25 μm film thickness; J. & W. Scientific, Folsom, CA, USA) connected one to FID and the other to mass spectrometer. The column was operated with Helium (1 $mL \cdot min^{-1}$, constant flow), and the column temperature was held at 40°C for 3 min, then increased to 200°C at a rate of $4°C \cdot min^{-1}$, and finally held at 200°C for 5 min. MS detector was programmed in Electron Ionization (EI) mode at an ionization voltage of 70 eV in the acquisition range between 20-300 m/z, and at a scan rate of 1 scan/sec. The trap, manifold and transfer line temperature were set to 200°, 80° and 200°C respectively. Volatile compounds were identified by comparison of their mass spectral data with those of the NIST 98 library (NIST, USA), by their linear retention indexes (VAN DEN DOOL and KRATZ, 1963) and by comparison with authentic standard compounds (when available).

Statistical analysis

Statistical treatment of data was performed using the SPSS statistical package, release 11.5 (SPSS, Chicago, IL, USA). Data of chemical composition, nitrogen fractions and free fatty acids were examined using a bifactorial ANOVA model with "*P. roqueforti* culture factor" (PC) and "ripening stage factor" (R) as fixed effects, while LSD test (least significant difference test, $P < 0.05$) for multiple comparisons was used to separate treatment means. The results of volatile compounds were examined using a monofactorial ANOVA model with "*P. roqueforti* culture factor" (PC) as fixed effect.

RESULTS AND DISCUSSION

Chemical composition and nitrogen fractions

The chemical composition of cheeses 1 day after production and after 30 days of ripening is reported in Table 1. Gross composition was not significantly affected by the *Penicillium roqueforti* culture at 1 day and after 30 days, whereas it changed significantly ($P < 0.05$) during the ripening (with the exception of protein content). The values of moisture, fat and protein to total solids ratio after 30 days of ripening were in agreement with data reported by LAWLOR *et al.* (2003) for other blue-type cheeses. pH values increased of around 1.4 units for all treatments from day 1 to day 30, probably as consequence of the consumption of lactate and the oxidative formation of NH_3 from amino acids operated by moulds during ripening (CANTOR *et al.*, 2004). It has been seen, for example, that the pH may increase of around 2 units in Danablu during the first 5 weeks of ripening (ARDÖ, 2011).

The data reported in Table 1 indicate that all proteolytic parameters increased significantly throughout ripening ($P < 0.05$). The level of pH 4.6-SN (expressed as a percentage of total nitrogen) ranged from 31.03 to 33.97% after 30 days of ripening (Table 1), in some agreement with results published for a number of different blue-type cheeses with longer ripening times (from 34% up to 72% of pH 4.6-SN; FERNÁNDEZ-SALGUERO *et al.*, 1989; LAWLOR *et al.*, 2003; VOIGT *et al.*, 2010). The level of secondary proteolysis is higher in blue cheeses compared to other cheese varieties (CANTOR *et al.*, 2004); therefore, the raised values at 30 days reported here for TCA-SN and PTA-SN (both expressed as a percentage of total nitrogen; Table 1), compared with the values of pH 4.6-SN, highlighted that the most of the soluble fraction included non-protein substances (FERNANDEZ-SALGUERO *et al.*, 1989).

Free fatty acids analysis

Table 2 summarises the extent of lipolysis (expressed as mmol of free fatty acids per kg of cheese) of cheeses made with each of the three *Penicillium roqueforti* cultures both 1 day and 30 days after production.

The type of *Penicillium roqueforti* culture significantly influenced ($P < 0.05$) the amount of short-chain (C4:0-C8:0), medium-chain (C10:0-C14:0), and long-chain (C16:0-C18:3) FFA. Overall, all individual FFA increased significantly ($P <$

Table 1 - Composition and nitrogen fractions of cheeses (PS1, PS2, PS3) 1 day after production and after 30 days of ripening.

	1 day			30 days			SEM[e]	F test[f]	
	PS1	PS2	PS3	PS1	PS2	PS3		PC[g]	R[h]
pH	4.85	4.82	4.83	6.35	6.38	6.05	0.18	NS	*
Moisture (g.100g^{-1})	49.26	49.63	50.04	42.28	42.97	42.30	0.89	NS	*
Fata	55.02	54.93	54.88	56.15	56.63	55.60	0.26	NS	*
Protein[a]	38.25	38.02	38.19	37.50	37.67	37.42	0.16	NS	NS
pH 4.6-SN[b]	8.92	8.84	9.25	31.03	33.97	32.10	2.89	NS	*
TCA-SN[c]	5.40	5.60	5.65	28.28	30.86	29.42	2.93	NS	*
PTA-SN[d]	1.11	1.23	1.49	12.92	13.05	11.85	1.42	NS	*

[a]Expressed as a percentage of total solids (% w/w).
[b,c,d]Expressed as a percentage of total nitrogen (% w/w); [b]Soluble nitrogen at pH 4.6; [c]Soluble nitrogen in 12% trichloroacetic acid (TCA); [d]soluble nitrogen in 10% phosphotungstic acid (PTA).
[e]Standard error mean.
[f]Significant differences: * $P < 0.05$; NS, no significant differences.
[g]*P. roqueforti* culture factor.
[h]Ripening stage factor.

Table 2 - Free fatty acids content (mmol.kg^{-1} of cheese) in cheeses (PS1, PS2, PS3) 1 day after production and after 30 days of ripening.

	1 day			30 days			SEM[b]	F test[c]	
	PS1	PS2	PS3	PS1	PS2	PS3		PC[d]	R[e]
C4:0a	0.47	0.57	0.74	16.07	28.28	31.65	3.37	*	*
C6:0	0.11	0.14	0.17	3.89	9.46	11.55	1.19	*	*
C8:0	0.20	0.20	0.20	2.74	5.50	6.85	0.68	*	*
C10:0	0.15	0.17	0.21	5.83	10.93	13.81	1.39	*	*
C12:0	0.11	0.11	0.13	2.56	4.64	5.96	0.59	*	*
C14:0	0.21	0.22	0.23	6.53	9.11	12.68	1.25	*	*
C16:0	0.46	0.45	0.47	11.88	15.20	22.90	2.24	*	*
C18:0	0.12	0.11	0.11	2.71	3.51	4.98	0.49	*	*
C18:1	0.34	0.32	0.31	17.09	27.38	32.14	3.31	*	*
C18:2	0.07	0.05	0.02	2.62	3.97	4.51	0.47	*	*
C18:3	0.02	0.02	0.02	2.08	2.85	3.88	0.39	*	*
TFFAs	2.26	2.41	2.61	74.00	120.83	150.90	15.16	*	*

[a]C4:0, butyric acid; C6:0, caproic acid; C8:0, caprylic acid; C10:0, capric acid; C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; TFFAs, total free fatty acids.
[b]Standard error mean.
[c]Significant differences: * $P < 0.05$.
[d]*P. roqueforti* culture factor.
[e]Ripening stage factor.

0.05) during ripening, and cheeses manufactured with the cultures named PS2 and PS3 showed the higher amount of each FFA after 30 days of ripening when compared with PS1 culture. Three free fatty acids (C4:0, C16:0, C18:1) represented around 60% of total free fatty acids for all treatments both 1 day and 30 days after production. After 30 days of ripening, the content of C4:0, C16:0, and C18:1 ranged on average (depending on the culture tested) from 16.07 to 31.65 mmol.kg^{-1}, from 11.88 to 22.90 mmol.kg^{-1}, and from 17.09 to 32.14 mmol.kg^{-1} of cheese, respectively. In general, the action of *Penicillium roqueforti* lipases releases higher concentrations of long-chain FFA than short- and medium-chain FFA (ARDÖ, 2011). A recent study reported that palmitic and oleic acids reached the highest levels of long-chain FFA in a blue cheese (CALZADA *et al.*, 2013). On the contrary, the values of butyric acid presented in the present study were higher than those found by other authors in different blue cheese varieties (CALZADA *et al.*, 2013; WOO *et al.*, 1984). In particular, PS1, PS2 and PS3 cheeses after 30 days of ripening showed about 1.8-3.6 times higher values of C4:0 when compared with results of CALZADA *et al.* (2013), who reported that the content of butyric acid in a blue cheese after 90 days of ripening reached a value of 1.32 mg.g^{-1} (14.98 mmol.kg^{-1}) of cheese dry matter. The raised values of C4:0 and, in general, of short-chain FFA observed in the present study can be ascribed to the use of lamb paste

rennet for milk coagulation. Lamb paste rennet contains a pregastric lipase, which preferentially hydrolyzes short chain fatty acids (KIM HA and LINDSAY, 1993). Furthermore, it is important to point out that even the lipolytic system of *Penicillium roqueforti* can exhibit a selectivity similar to that of the pregastric lipase (KIM HA and LINDSAY, 1993).

SPME analysis

Cheeses (PS1, PS2 and PS3) after 30 days of ripening were subjected to volatile flavour profile analysis by SPME-GC-FID/MS (Table 3). A total of 34 volatile compounds were identified in cheese samples, and among them only some volatile FFA (butyric, pentanoic and hexanoic acids) and some alcohols (2-pentanol, 1-pentanol and phenyl ethyl alcohol) were significantly affected by *Penicillium roqueforti* culture ($P < 0.05$). Ketones and acids represented almost the totality of volatile fraction and resulted as more abundant in all samples (about 70 and 27%, respectively). Among ketones, 2-heptanone and 2-nonanone presented the highest values of FID Peak Area (Table 3). These results were in agreement with those reported in literature relating to concentration of ketones in this category of cheeses (ARDÖ, 2011; CANTOR *et al.*, 2004). The presence of ketones is correlated to the typical flavour of blue cheeses, and they are produced by the β-oxidation of free fatty acids followed by a decarboxylation reaction.

Table 3 - Volatile compounds (FID Peak Area) in cheeses (PS1, PS2, PS3) after 30 days of ripening.

LRI[a]		PS1	PS2	PS3	SEM[b]	PC[c]
839	2-propanone	275,452	196,359	179,488	70,535	NS
926	2-butanone	4,681	3,307	3,295	671	NS
1,004	2-pentanone	949,455	706,739	742,756	108,431	NS
1,107	2-hexanone	22,208	12,493	17,289	2,347	NS
1,211	2-heptanone	1,791,884	1,299,484	1,624,590	146,423	NS
1,315	2-octanone	28,394	19,954	30,148	4,275	NS
1,333	3-hydroxy 2-butanone	7,969	8,587	5,481	1,753	NS
1,422	2-nonanone	1,047,296	678,913	1,204,917	187,792	NS
1,481	8-nonen 2-one	78,175	55,547	87,753	12,625	NS
1,631	2-undecanone	13,624	3,499	11,418	3,510	NS
1,690	acetophenone	362	183	295	95	NS
	Ketones	4,219,501	2,985,068	3,907,431	493,937	NS
1,581	propanoic acid	1,805	2,052	2,763	435	NS
1,609	2-methyl propanoic acid	951	1,141	1,400	139	NS
1,671	butyric acid	407,334	1,137,832	957,216	136,292	*
1,712	3-methyl butyric acid	6,539	3,390	4,240	912	NS
1,787	pentanoic acid	2,476	8,945	6,831	1,142	*
1,889	hexanoic acid	314,878	673,581	540,975	63,090	*
1,990	heptanoic acid	1,313	2,965	2,445	363	NS
2,103	octanoic acid	62,326	102,518	81,564	8,519	NS
2,316	decanoic acid	8,299	17,473	11,960	1,935	NS
	Acids	805,921	1,949,897	1,609,395	208,018	*
910	ethyl acetate	293	176	125	36	NS
1,057	ethyl butyrate	5,024	6,800	3,500	1,703	NS
1,256	ethyl hexanoate	9,744	15,343	10,222	2,315	NS
1,459	ethyl octanoate	11,645	28,596	26,947	4,415	NS
	Esters	26,511	50,914	40,794	7,354	NS
945	2-propanol	10,390	2,791	7,434	2,096	NS
1,137	2-pentanol	75,497	24,527	45,705	9,397	*
1,270	1-pentanol	2,115	281	0	368	*
1,344	2-heptanol	90,554	34,925	52,055	14,579	NS
1,538	2-nonanol	8,074	2,898	8,414	2,048	NS
1,575	2,3-butanediol	1,402	2,296	2,664	264	NS
1,950	phenyl ethyl alcohol	236	0	0	30	*
2,047	phenol	323	322	341	31	NS
	Alcohols	188,591	68,368	117,699	26,439	NS
1,301	styrene	2,537	5,351	2,784	940	NS
	Hydrocarbons	2,537	5,351	2,784	940	NS
940	3-methylbutanal	229	403	245	66	NS
	Aldehydes	229	403	245	66	NS
	Totals	5,243,291	5,060,001	5,678,349	398,884	NS

[a]Linear Retention Indexes using a DB-WAX column.
[b]Standard error mean.
[c]*P. roqueforti* culture factor, significant differences: * $P < 0.05$; NS, no significant differences.

The reaction is catalysed by enzymes contained both in spores and mycelium of *Penicillium spp.* (QIAN *et al.*, 2002; VOIGT *et al.*, 2010).

Table 3 also highlights that PS1 presented significantly lower values of acids than other cheeses. This was consistent with what discussed above concerning the higher lipolytic activity of PS2 and PS3 compared to PS1 (Table 2), and was also probably due to the greater aptitude of PS1 in converting FFA to 2-alkanones.

Among volatile FFA, butyric acid was the most abundant (50, 58 and 59% of total FFA in PS1, PS2 and PS3, respectively) followed by hexanoic (39, 35 and 34% of total FFA in PS1, PS2 and PS3, respectively) and octanoic (8, 5 and 5% of total FFA in PS1, PS2 and PS3, respectively) acids. The origin of raised values of butyric acid has been discussed in the previous section, while hexanoic and octanoic acids are important flavor compounds of blue cheeses (ARDÖ, 2011).

Esters are produced by free fatty acids esterification with primary alcohols, and may attenuate the typical pungent flavour of blue cheeses due to the methyl ketones (MOIO *et al.*, 2000). They represented only 1% on average of totals volatile compounds (Table 3); PS2 and PS3 showed higher FID Peak Area (but without any statistical significance) of esters as a consequence of their higher content of FFA compared to PS1.

The strong reducing environment present in ripened cheese favoured the production of 2-alkanols from corresponding 2-alkanones. PS1 showed significantly higher values of 1- and 2-pentanol when compared with other samples ($P < 0.05$), and tended to have the highest levels both of 2-alkanones and 2-alkanols. The parallel evolution of these volatile compounds was previously observed in other blue cheeses (GONZÁLES DE LLANO *et al.*, 1990).

CONCLUSIONS

The results indicated that the ovine blue cheese made in Sardinia was more subjected to lipolysis and presented higher amounts of short chain fatty acids when compared to the most known blue cheese varieties. This evolution of lipolysis in the product was also due to the use of lamb paste rennet. Two cultures (PS2 and PS3) were characterised by the highest values of total free fatty acids. In contrast, proteolytic parameters and the most volatile compounds did not vary significantly depending on the culture tested.

In conclusion, this study may provide valid information about the use of the appropriate culture for managing the ripening process (with particular regard to lipolysis) also in blue cheeses different from that studied here.

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PAPER

EFFECT OF PARTIAL REPLACEMENT OF PORK MEAT WITH OLIVE OIL ON THE SENSORY QUALITY OF DRY-RIPENED VENISON SAUSAGE

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ABSTRACT

Six assays of low-fat venison salchichon were produced using varying proportions of olive oil to replace the traditional pork meat added. The control contained 75% lean venison and 25% pork meat; in the other assays, 15, 25, 35, 45 and 55% of the pork meat was replaced by olive oil. Samples were evaluated by quantitative descriptive sensory analysis and consumer testing. Descriptive sensory analysis revealed significant differences for most of the attributes studied. The replacement of 35% or more of pork meat by olive oil, prompted a decrease in odour intensity, spicy odour, hardness and an increase of fat mouthfeel, together with the olive oil perception. By contrast, the replacement of 25% of pork meat by olive oil yielded a salchichon not greatly different to the control. Consumers accepted all assays, but preferred those in which no more than 25% of the pork meat was replaced by olive oil. From a sensory standpoint, therefore, it is recommended that the replacement of pork meat by olive oil in this product should not exceed 25%.

- Keywords: dry-ripened venison sausage, olive oil, acceptance test, preference test, quantitative descriptive sensory analysis -

INTRODUCTION

Though the production of venison in Spain is high, its economic value is relatively low because it is considered to be simply a by-product of hunting, oriented to obtain flashy awards. The autonomous community of Castilla-La Mancha is also the main venison exporter in Spain, accounting the 80% of the total exportation, being Germany is primary destination. Despite the large venison production, its consumption in the region – an indeed in Spain generally – is fairly limited; venison is mainly consumed in certain rural areas and some restaurants.

Cynegetic venison is a highly nutritive meat, characterized by a high protein and heme iron content and a low presence of subcutaneous and intramuscular fat (ZOMBORSZKY *et al.,* 1996; HOFFMAN and WIKLUND, 2006). In addition, this meat has distinctive organoleptic properties, differing from those of other meats, such as its intense and attractive red colour, tenderness and variety of flavours, reflecting the fact that deers are raised in the wild and feed on natural pastures.

A wide range of ripened products is obtained from hunted deer, including cecina (dry-ripened meat), and dry fermented sausages, as chorizo and salchichon. These are generally labelled "gourmet products" in the international market. In the production of venison chorizo and salchichon, a certain amount of pork meat has to be added to lean venison in order to ensure gradual drying, acceptable tenderness and the development of their distinctive flavour. However, consumer interests are based on low-fat foods rich in unsaturated fatty acids to get a healthy diet.

On the other hand, olive oil is a staple of the Mediterranean diet, and its main source of fat. It is remarkable for its characteristic fatty acid composition, and particularly for its high oleic acid content, ranging between 55 and 83% (Codex Stan 33-1981). Virgin olive oil is a natural juice that can be consumed unrefined, thus retaining its original composition; this makes it a prime source of mainly-antioxidant micronutrients, including phenol compounds, vitamin E, carotenes and squalene (OWEN *et al.,* 2000).

Several investigations have been carried out on the partial replacement of pork meat by olive oil in pork and/or beef dry sausages (BLOUKAS *et al.,* 1997; MUGUERZA *et al.,* 2001, 2002, 2003; SEVERINI *et al.,* 2003; KAYAARDI and GÖK, 2003; DEL NOBILE *et al.,* 2009; BERIAIN *et al.,* 2011); however, there is not studies to address the use of olive oil in making venison salchichon.

The aim of this study was to elaborate cynegetic venison salchichon with the highest percentage of replacement of pork meat by olive oil, that allow to maintain sensory characteristic of the traditional salchichon sausage. It is also hoped to increase the venison products consumption and subsequently to raise the economic value of cynegetic venison.

MATERIALS AND METHODS

Raw materials

Lean venison was obtained from hind legs of male deer (*Cervus elaphus*) obtained during the 2009-2010 hunting season on two neighbouring reserves in Ciudad Real (central Spain). Vegetation in the two reserves was very similar, comprising pine forests, woodlands and scrub. A total of 67.5 kg of venison was used. Pork meat was obtained from castrated male pigs (progeny of a Pietrain male x Dalain female cross) raised intensively and slaughtered at the age of seven months. A total of 16 kg was used. Extra virgin olive oil was produced at an oil-mill in Ciudad Real from Cornicabra olives harvested in 2008-2009. A total of 3.5 l were used. The soy protein concentrate used, Arcon™S, is practically tasteless and guarantees high protein solubility. Its chemical composition was: ≤ 6% moisture, ≥ 72% protein, ≤ 3% fat and 20% fibre. Finally, a commercial salchichon formula (Salchichón Casero 933, Manufacturas Ceylan S.L., Valencia, Spain) was used, comprising salt, spices, lactose, saccharose, polyphosphates (E-450i, ii), sodium ascorbate (E-301) and potassium nitrate (E-252).

Venison salchichon production

Six assays of venison salchichon were made taking into account the findings of a previous study aimed at reducing the pork meat content of this product (UTRILLA *et al.,* 2014). All assays contained 75% lean venison. The original 25% pork meat was partially replaced by 0% (control), 15, 25, 35, 45 and 55% extra virgin olive oil, in Assays 1 to 6, respectively. Olive oil was added to the salchichon in the form of an organogel obtained by emulsifying olive oil with soy protein concentrate (Arcon™S) and mineral water, at a ratio of 10:1:8, respectively (Table 1).

Venison and pork meat were minced separately in an Unger W-98 mincer (Andher, Cam-

Table 1 - Relative percentages of lean venison, pork meat and organogel (olive oil + water + soy protein) in different assays of venison salchichon.

Assays	Lean venison (%)	Pork meat (%)	Organogel (%)
1	75	25.00	0.00
2	75	21.25	3.75
3	75	18.75	6.25
4	75	16.25	8.75
5	75	13.75	11.25
6	75	11.25	13.75

po de Criptana, Spain) with an 8 mm plate. Venison was then mixed with on the appropriate proportion of pork meat and the olive oil as organogel (to join a total of 15 kg), and the commercial salchichon mixture (Salchichón Casero 933, Manufacturas Ceylan S.L., Valencia, Spain). An amount of 33 g mixture/kg, previously dissolved in 1 l of cold mineral water, was used. All ingredients were minced in an AV-80 vacuum mixer (Andher, Campo de Criptana, Spain). The mixture was covered with a cotton cloth and left to settle for 20 h at 0° C, in order to the whole mass could involve the spices and additives. It was then fed through an H52 PAS hydraulic piston-based sausage stuffer connected to a VAE-10 vacuum system (Andher, Campo de Criptana, Spain), into synthetic collagen casings, with a 38-40 mm diameter. Horseshoe-shaped salchichon sausages were then tied off at 60 cm intervals. Salchichon sausages were maintained at 20°-22°C and a relative humidity of 60% for 2 h, and finally cured at 11°-12°C and a relative humidity of 75% for 28 days, in a Zanotti curing chamber (Grupo Momplet, Valencia, Spain). After ripening, salchichon sausages were vacuum-packed and stored at 8°-10°C for 45 days until its evaluation. Two replicates of the six assays of salchichon sausages were done.

Samples

To determine physicochemical characteristics (moisture, fat and protein content) in salchichon, the casing was removed and the entire content was ground in a domestic blender. All analyses were carried out in duplicate in two sausages of each replicate.

The samples for sensory analysis were presented to the panellists and consumers in 3 mm thick slices without skin, at 20°-22° C (room temperature) and tagged (number-letter-number). Samples were presented randomized at each tasting session in order to minimise any effects due to order of presentation. Unsalted crackers and mineral water were provided to the panellist and consumers to cleanse the mouth between samples. The trained panel evaluated three samples per session, however consumers tasted the six assays of sausages in the same session. The trained panel evaluated each sample in duplicated.

Physicochemical composition

Moisture content was measured in accordance with the standard ISO R-1442 (1973). Total fat was extracted with petroleum ether (40°-60°) following the standard ISO R-1443 (1973). Total nitrogen was measured using the Kjeldahl method (method 16245, AOAC 1980). Protein nitrogen content was obtained by multiplying total nitrogen by a factor of 6.25.

Quantitative descriptive sensory analysis

The quantitative descriptive sensory analysis was carried out in a tasting room equipped in accordance with UNE-EN ISO 8589:2010, by a 9 member sensory panel (6 women, 3 men, ages 25-52 years) with previous experience in fermented sausages. Three training sessions were held, employing three different commercial venison salchichon sausages, elaborated with cinegetic venison and pork meat. The qualification of the panel members was based on reproducibility verification and concordance between the tasters. Attributes intensities were rate on non-structured scales of 10 cm and in accordance with UNE-ISO 4121:2006. All the scales were anchored at the extremes with the terms "weak" and "very intense," except for the colour intensity scales in which the colour was indicated at the extremes. The visual attributes evaluated were: amount of fat (fat particles), fat colour (0=white; 10=yellow) and lean colour (0=pink; 10=black). The odour attributes studied were: black pepper, spices, cured and olive oil odour as well as odour intensity. The attributes that defined the texture profile of the samples were: hardness (strength to breakdown the product), juiciness (amount of juice released during chewing), chewiness (attribute related to the cohesiveness of the product) and fat mouthfeel (attribute related to the perception of the fat quantity of the product). Finally, the taste attributes (including retronasal perceptions) evaluated were the following: intensity of the taste, salty, pungent (nasal and oral mucosa irritation), pepper and olive oil taste and intensity of the aftertaste.

Consumer tests

The consumer tests were carried out in a tasting room equipped in accordance with UNE-EN ISO 8589:2010. An untrained group of 44 habitual consumers of pork salchichon participated in the study, 15 men aged between of 22 and 45 (mean age 31) and 29 women aged between of 21 and 52 (mean age 30). Consumers were recruited from students, staff and faculty of the Food Science and Technology Area of the University of Castilla-La Mancha. Consumers were instructed to express their evaluation for overall acceptability considering the external appearance, odour, taste and texture of the slices. In the same session they evaluated their acceptance and preference of the six assays of samples.

Acceptance test

To grade the acceptability of each sample, consumers used a non-structured or linear hedonic scale of 10 cm, anchored at either end by the phrases "strongly like" (left end) and "strongly dislike" (right end), enabling consumers to mark the point which best represented their satisfaction with the sample.

Preference test

A hedonic ranking test was used (UNE-ISO 8587:2010), whereby each consumer was presented with a sample from each assay and asked to order the samples by degree of preference, giving 1 point to the least preferred and 6 to the most preferred.

Statistical Analysis

One-way ANOVA was performed to study the influence of the olive oil amount in physicochemical parameters, and attributes evaluated by the quantitative descriptive sensory analysis and by the acceptance test. When the interaction was significant, the means were compared using the Student-Newman-Keuls test. The Friedman test was performed to check the significance of differences between consumer preferences. When significant differences were found, the Fisher's Least Significant Difference (LSD) test was calculated following standard UNE-ISO 8587:2010, to compare the means. All statistical procedures were carried out using SPSS 19.0 statistical software for Windows XP (SPSS, Inc., Chicago, Il, USA).

RESULTS AND DISCUSSION

Physicochemical composition

Results (mean±standard deviations) for moisture, fat and protein content of six salchichon assays at the end of ripening are shown in Table 2. Moisture values at the end of ripening were between 30.4-32.1 g/100 g, similar to those reported in other dry sausages varieties containing olive oil (BLOUKAS *et al.,* 1997; MUGUERZA *et al.,* 2002; BERIAIN *et al.,* 2011). Significant differences in fat content were found between assays, directly reflected the varying proportion of olive oil, whose fat content is higher than that of pork meat. Values at the end of ripening (21.1-40.2 g/100 g DM) were lower than those reported in pork and or beef sausages made with a partial replacement of pork backfat with olive oil (41.4-59.0 g/100 g DM) (BLOUKAS *et al.,* 1997; MUGUERZA *et al.,* 2001; MUGUERZA *et al.,* 2002; BERIAIN *et al.,* 2011), probably because the lean venison and pork meat used contains less fat than the raw materials used in those studies. On the other hand, significant inter assay differences in protein content were recorded at end of ripening process. The higher the olive-oil content, the lower the protein nitrogen content, as it was expected a significant inverse correlation being recorded between the two parameters (r=-0.948; $P<0.001$). Protein nitrogen values at the end of ripening (43.6-59.6 g/100 g DM) were higher than those reported by other authors (25.8-44.7 g/100 g DM) (BLOUKAS *et al.,* 1997; MUGUERZA *et al.,* 2001; MUGUERZA *et al.,* 2002; BERIAIN *et al.,* 2011), since venison salchichon had a lower fat content than those made with pork and/or beef.

Quantitative descriptive sensory analysis

Significant inter-assay differences were found for all the sensory attributes studied, except for fat colour, salty taste and taste intensity. Therefore, in all assays, the fat were white, presented scores ranged between 0.89 and 1.63 (0=white; 10=yellow), the salty taste was considered medium, approached 5.0, while taste intensity was judged to be moderate-to-high, scoring between 6.86 and 7.60.

Mean scores (± standard deviation) assigned by the tasting panel for each of the visual attributes studied are shown in Table 3. Assays

Table 2 - Physicochemical parameters (means±standard deviations) of venison salchichon with different percentage replacement of pork meat by olive oil.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Moisture (g/100 g)	31.69±2.29	32.07±1.64	31.15±0.46	30.79±0.73	32.02±0.26	30.40±0.09
Fat (g/100 g DM)	19.04^{a}±2.14	21.11^{a}±0.06	29.91^{b}±0.67	36.29^{c}±1.09	$37.88^{c,d}$±0.52	40.24^{d}±1.58
Protein (g/100 g DM)	59.57^{d}±1.54	$56.58^{c,d}$±1.97	54.19^{c}±0.62	49.76^{b}±1.39	48.06^{b}±0.82	43.62^{a}±0.55

Different superscripts (a,b,c,d) in the same row denote significant differences (P<0.05). Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement). DM: dry matter.

Table 3 - Visual attributes (means±standard deviations) of venison salchichon with different percentage replacement of pork meat by olive oil.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Amount of fat	6.07^{c}±0.93	6.00^{c}±0.61	5.91^{c}±0.75	4.65^{b}±0.82	4.52^{b}±0.88	3.28^{a}±0.74
Fat colour	0.89±0.64	1.16±0.46	1.31±0.52	1.27±0.67	1.63±0.90	1.46±0.68
Lean colour	7.44^{b}±0.50	6.81^{b}±0.95	6.56^{b}±0.70	7.41^{b}±1.06	7.16^{b}±0.89	5.84^{a}±0.90

Different superscripts (a,b,c) in the same row denote significant differences (P<0.05).
Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

Table 4 - Odour attributes (means±standard deviations) of venison salchichon with different percentage replacement of pork meat by olive oil.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Odour intensity	7.95^{b}±0.64	8.04^{b}±0.58	7.83^{b}±0.74	7.05^{a}±0.87	6.58^{a}±0.74	6.85^{a}±0.62
Black pepper odour	5.15^{c}±0.63	5.09^{c}±0.62	3.74^{b}±0.75	3.64^{b}±0.63	3.30^{b}±0.75	2.33^{a}±0.74
Spicy odour	4.99^{d}±0.77	4.35^{c,d}±0.58	4.45^{c,d}±0.78	3.96^{c}±0.99	3.30^{b}±0.86	2.44^{a}±0.88
Cured odour	6.08^{c}±0.87	6.79^{c}±0.81	5.97^{c}±0.54	6.13^{c}±0.65	4.91^{b}±1.06	4.16^{a}±0.88
Olive oil odour	0.00^{a}±0.00	0.00^{a}±0.00	2.56^{b}±0.78	3.33^{c}±0.81	4.04^{d}±1.20	6.01^{e}±1.01

Different superscripts (a,b,c,d,e) in the same row denote significant differences (P<0.05). Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

Table 5 - Texture attributes (means±standard deviations) of venison salchichon with different percentage replacement of pork meat by olive oil.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Hardness	6.05^{d}±0.66	5.74^{d}±0.80	4.91^{c}±0.55	4.03^{b}±0.79	3.12^{a}±0.54	2.79^{a}±0.91
Juiciness	4.63^{b}±0.61	5.18^{b}±0.79	6.05^{a}±0.77	6.80^{a}±0.83	6.68^{a}±0.57	6.03^{a}±1.15
Chewiness	6.17^{b}±0.40	4.95^{a}±0.42	5.20^{a}±0.53	5.00^{a}±0.00	5.00^{a}±0.00	5.07^{a}±0.27
Fat mouthfeel	4.86^{a}±0.75	4.88^{a}±0.70	6.06^{b}±0.86	6.64^{b,c}±0.88	7.02^{c}±0.98	7.34^{c}±0.98

Different superscripts (a,b,c,d) in the same row denote significant differences (P<0.05). Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

made without or with smaller amounts of olive oil (Assays 1, 2 and 3) displayed larger amounts of visible fat; as the proportion of olive oil increased, the amount of visible fat declined. Lean venison was dark brown in all except Assay 6, where it was pinker. Similar findings were reported by MUGUERZA *et al.* (2001) in a study of Pamplona-style chorizo made with lean pork (75%) and pork meat (25%) partially replaced by olive oil (0, 10, 15, 20, 25 and 30%) and in a later study (MUGUERZA *et al.*, 2002) of salchichon made with lean pork, lean beef and varying proportions of pork backfat (10, 20 and 30%) partially replaced by olive oil (0 and 20%). These authors recorded significant inter-assay differences in colour, noting that colour intensity decreased as the amount of olive oil increased. By contrast, BERIAIN *et al.* (2011), in another study of Pamplona-style chorizo in which pork backfat was partially replaced by olive oil emulsified with alginate, found no significant difference between appearance profiles. However, in that study 71.4% of the tasting panel expressed a preference for chorizo in which 50% of pork backfat had been replaced by olive oil, on the grounds that its appearance was more appealing than that of the control, due to its effective imitation of the rice-grain effect typical of this type of chorizo (BOE, 1980).

Mean scores (± standard deviation) assigned by the tasting panel for odour attributes are shown in Table 4. Assays containing the lowest amounts of olive oil (Assays 1, 2 and 3) were judged to display the greatest odour intensity (7.83-8.04). The black pepper odour characteristic of salchichon weakened as the proportion of olive oil increased, being most intense in Assays 1 and 2 (5.09-5.15). Spicy and cured odour also decreased with increasing proportions of olive oil; the lowest intensity for both attributes (2.44 and 4.16, respectively) was recorded in Assay 6 (55% replacement). Finally, olive oil odour was identified from Assay 3 onwards, becoming more intense as the proportion of olive oil increased. MUGUERZA *et al.* (2002) also reported greater oil odour intensity with rising proportions of olive oil in place of pork backfat.

Scores for the attributes defining the texture profile are shown in Table 5. The replacement of 35% or a high amount of pork meat by olive oil (Assays 4, 5 and 6) was considered to give rise to excessive softness (hardness scores below 5). The control scored highest for chewiness (6.17), whereas the assays containing varying proportions of olive oil received scores of around 5. Assays 1 and 2 (0 and 15% substitution) scored lowest for juiciness (4.63-5.18), the remainder received scores of between 6 and 6.8 points. Finally, fat mouthfeel increased with higher proportions of olive oil, scores rising from 4.86 (Assay 1) to 7.34 (Assay 6); the panellist judged the mouthfeel of Assays 5 and 6 (7.02 and 7.34, respectively) to be over-fatty. So, addition of olive oil to venison salchichon prompted an increase in juiciness and fat mouthfeel and a decrease in both hardness and chewiness. Similar findings were reported by MUGUERZA *et al.* (2001) who noted that types containing larger amounts of olive oil were too soft, although they recorded no difference in juiciness among types. BLOUKAS *et al.* (1997), in a study of salchichon made with lean pork, lean beef and pork backfat partially replaced by olive oil, with or without added soy protein,

found significant differences as a function of the way oil was added: salchichon to which oil was added in liquid form was judged to be too soft, and obtained lower scores for odour and flavour intensity, whereas scores for salchichon made with olive oil combined with soy protein were similar to those of controls.

Finally, scores for taste attributes are shown in Table 6. Significant inter-assay differences were recorded for most taste-related attributes. Olive oil taste was identified from Assay 3 (25%) onwards, increasing as a function of the proportion of added olive oil, to a maximum score of 6.96 for Assay 6 (55% substitution). Spicy taste decreased with rising proportions of olive oil, which masked the black pepper taste characteristic of salchichon. By contrast, BERIAIN *et al.* (2011) found no significant difference in taste between Pamplona-style chorizo types made with and without olive oil.

To summarise, variation in the percentage replacement of pork meat by olive oil had a marked influence on the results of descriptive sensory analysis. The replacement of 35% or more, of pork meat by olive oil, resulted in a reduction of the fat particles visibility. It also prompted a decrease in odour intensity, spicy odour, hardness and an increase of fat mouthfeel, together with the olive oil perception (odour and taste). By contrast, the replacement of 25% of pork meat by olive oil yielded a salchichon not greatly different in appearance, texture, odour and taste to the control.

Consumer tests

Acceptance test

The scores awarded by the consumers for different assays of venison salchichon sausage with different percentage of olive oil added are shown in Table 7. From these results, it can be concluded that all the samples were accepted because the average score was above 5.0 (satisfaction threshold). Significant inter-assay differences were recorded for appearance and odour, but not for the rest of the attributes studied. Assays 1, 2 and 3 received the highest scores for odour (7.10-7.60). Therefore, the replacement of up to 25% of pork meat by olive oil yields a product as acceptable to the consumer, in terms of all sensory parameters, as controls containing no olive oil.

Preference test

Consumers ordered samples by degree of preference, giving 1 point to the least preferred and 6 to the most preferred (Table 8). The preference order was Assay 3 > Assay 2 > Assay 1 > Assay 4 > Assay 5 > Assay 6. The Friedman test showed significant differences ($P<0.05$) between assays. After having applied the Fischer method to calculate the Least Significant Difference (LSD), it can be affirmed that the samples from Assay 6 were significantly different from the rest as the least preferred. On the other hand, the samples from Assays 4 and 5 were not significantly different from each

Table 6 - Taste attributes (means±standard deviations) of venison salchichon with different percentage replacement of pork meat by olive oil.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Taste intensity	7.26±0.78	7.06±0.78	7.50±0.84	7.50±0.78	7.60±0.93	6.86±0.91
Salty taste	5.07±0.27	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00	5.04±0.13
Pungent taste	2.86^{c}±0.80	$2.69^{b,c}$±0.78	1.93^{b}±0.90	2.00^{b}±0.81	2.11^{b}±0.79	0.93^{a}±0.48
Pepper taste	4.06^{c}±0.67	3.15^{b}±0.90	2.66^{b}±0.78	2.71^{b}±0.72	2.58^{b}±0.70	1.53^{a}±0.74
Olive oil taste	0.00^{a}±0.00	0.00^{a}±0.00	3.04^{b}±0.78	3.51^{b}±0.90	4.63^{c}±0.82	6.96^{d}±1.25
Aftertaste intensity	7.42^{b}±0.76	7.20^{b}±0.73	8.02^{b}±0.59	7.49^{b}±0.85	7.59^{b}±0.79	6.49^{a}±0.94

Different superscripts (a,b,c,d) in the same row denote significant differences (P<0.05). Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

Table 7 - Means and standard deviations of the scores obtained for different assays of venison salchichon with different percentage replacement of pork meat by olive oil in the consumers acceptance test.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Appearance	6.86^{b}±1.96	7.01^{b}±1.47	7.25^{b}±1.71	$6.63^{a,b}$±1.83	$6.47^{a,b}$±1.67	5.84^{a}±2.27
Odour	7.60^{b}±1.40	7.20^{b}±1.46	7.10^{b}±1.87	6.18^{a}±2.39	6.06^{a}±1.99	5.63^{a}±2.43
Taste	6.42±1.78	6.33±1.67	6.29±2.02	5.88±2.19	5.58±2.13	5.27±2.43
Texture	5.91±2.09	6.30±2.06	6.39±2.19	5.93±2.26	5.83±2.40	5.31±2.43
Overall acceptance	6.34±1.84	6.47±1.93	6.58±1.86	5.91±2.26	5.75±2.30	5.42±2.34

Different superscripts (a,b) in any row denote significant differences ($P<0.05$). Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

Table 8 - Total scores obtained for different assays of venison salchichon with different percentage replacement of pork meat by olive oil in the consumers preference test.

	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
Total scores	169	172	183	148	144	108

Assay 1 (0% replacement); Assay 2 (15% replacement); Assay 3 (25% replacement); Assay 4 (35% replacement); Assay 5 (45% replacement); Assay 6 (55% replacement).

other. The samples from Assays 1, 2 and 3 were not significantly different from each other as the most preferred by consumers. The samples from Assays 1, 2 and 3 were the most preferred mainly for six reasons (good flavour, proper texture, pleasant odour, good aspect and attractive colour). Among the reasons for preferring the sausage from Assay 6 the least, 36.4% of consumers thought the texture were not right and 31.8% highlighted the bad flavour. They also stressed the bad aspect, disagreeable odour and unattractive colour.

CONCLUSIONS

In sensory terms, low-fat venison salchichon in which equal or more than 35% of pork meat had been replaced by olive oil presented a lower acceptation than products containing less olive oil. Its appearance was deemed less favourable due to poorer visibility of fat particles, the texture was regarded as over-soft, and the mouthfeel was considered excessively fatty. There was also a decrease in odour quality and intensity, as well as in cured and spicy odour, together with an unfavourable taste and odour of olive oil. The trained panel found that replacement of 25% of pork meat by olive oil yielded a salchichon not greatly different to the traditional product. Consumers also preferred salchichon in which no more than 25% of the pork meat had been replaced by olive oil, largely because of its good taste and acceptable texture.

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PAPER

BIOLOGICAL ACTIVITY OF EGG-YOLK PROTEIN BY-PRODUCT HYDROLYSATES OBTAINED WITH THE USE OF NON-COMMERCIAL PLANT PROTEASE

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ABSTRACT

Enzymatic hydrolysis leads to improved functional and biological properties of protein by-products, which can be further used as nutraceuticals and protein ingredients for food applications.

The present study evaluated ACE-inhibitory, antioxidant and immunostimulating activities in hydrolysates of egg-yolk protein by-product (YP), generated during industrial process of delipidation of yolk. The protein substrate was hydrolyzed using non-commercial protease from Asian pumpkin (*Cucurbita ficifolia*). The reaction was conducted in 0.1 M Tris-HCl buffer (pH 8.0) at temperature of 37°C for 4 hours using different enzyme doses (100-1000 U/mg of substrate). The protein degradation was monitored by the determination of the degree of hydrolysis (DH), release of free amino groups (FAG) and by RP-HPLC. In the obtained hydrolysates we also evaluated biological activities. It was shown that the highest DH of substrate (46.6%) was obtained after 4h of reaction at the highest amount of enzyme. This hydrolysate exhibited antioxidant activity, including ferric ion reducing (FRAP) (56.41 μg Fe^{2+}/mg), ferric ion chelating (695.76 μg Fe^{2+}/mg) and DPPH free radical scavenging (0.89 μmol $trolox_{eq}$/mg) as well as ACE-inhibitory (IC_{50}=837.75 μg/mL) activities. The research showed improved biological properties of enzymatically modified YP by-product.

- Keywords: egg yolk proteins, *Cucurbita ficifolia* protease, hydrolysis, antioxidant, ACE-inhibitory activity, immunostimulating activity -

INTRODUCTION

Nowadays, the identification of bioactive food components, which can provide health benefits is one of the objectives of scientific research worldwide. Special attention is given to bioactive peptides due to their role in the prevention of numerous diseases (SHARMA and RANA, 2011). These peptides, released via the enzymatic hydrolysis of food proteins reveal numerous biological activities: antioxidant, antihypertensive, antimicrobial, antidiabetic, opioid and immunostimulating. These may have positive effects the cardiovascular, nervous, immune or digestive systems of the body (MINE and KOVAKS-NOLAN, 2006; CHAY PAK TING *et al.*, 2011; YU *et al.*, 2011; POKORA *et al.*, 2014).

The egg is recognized as a very valuable source of proteins for human nutrition, as well as proteins, which may be precursors of peptides with biological activity (MINE and KOVAKS-NOLAN, 2006; YU *et al.*, 2011; ZHIPENG *et al.*, 2011). ACE-inhibitory peptides are one of the best characterized peptides derived from eggs. The hydrolysis of ovalbumin, the main protein of egg white, conducted by gastrointestinal enzymes, results in the release of several ACE-inhibitory peptides (MIGUEL *et al.*, 2004; MIGUEL *et al.*, 2007). The effectiveness of these peptides was validated in tests *in vivo* conducted on spontaneously hypertensive rats (MIGUEL *et al.*, 2007). Antihypertensive activity was also demonstrated by some peptides released from egg-white protein treated by alcalase (LIU *et al.*, 2010; ZHIPENG *et al.*, 2011). As a result of peptide purification from those hydrolysates, ACE-inhibitory peptides: RVPSL and QIGLF were obtained (LIU *et al.*, 2010; ZHIPENG *et al.,* 2011).

It was also shown that egg is a rich source of proteins in which sequence numerous antioxidant peptides are encrypted. Phosphopeptides derived from egg phosvitin having molecular masses 1-3 kDa exert a strong ability to inhibit the oxidation of linoleic acid, to scavenge DPPH free radicals and to chelate iron ions (II) (XU *et al.*, 2007). Egg-yolk hydrolysates composed of peptides with a molecular weight lower than 1 kDa obtained with the use of proteinase from Bacillus ssp., also exhibited antioxidant capacities. Superoxide-scavenging activity and suppression of discoloration by β-carotene have also been observed (SAKANAKA and TACHIBANA, 2006). Egg yolk peptides obtained during alkalase and protease N digestion of delipidated egg yolk proteins were found to boost the systemic antioxidant status in the blood by increasing the GSH concentration in red blood cells (Young, Fan and Mine, 2010). It has been demonstrated that the consumption of egg yolk protein hydrolysates with antioxidative properties leads to the inhibition of tumor cell proliferation in the colon (ISHIKAWA *et al.*, 2009).

Some peptides derived from egg proteins can act as immune modulators and may be used as nutraceuticals for the prevention or treatment of lifestyle dependent diseases. Immunomodulatory peptides may exhibit anti-inflammatory activity by decreasing the production of pro-inflammatory cytokines (MATTSBY-BALTZER *et al.*, 1996; CROSS and GILL, 2000; MINE and KOVACS NOLAN, 2006). Egg yolk peptides significantly reduce pro-inflammatory cytokine, IL-8, in the Caco-2 cell line (YOUNG and MINE, 2010). Furthermore, immunostimulatory activity, assayed as the ability to enhance the capacity of phagocytic cells in mice, was present in ovalbumin hydrolysates prepared by gastrointestinal enzymes (BIZIULEVIČIUS *et al.*, 2005).

Bioactive peptides can be also released from protein by-products generated during isolation of biologically active substances naturally occurring in egg. One such protein waste is a by-product of lysozyme and cystatin extraction from hen egg white by ethanol method (SOKOŁOWSKA *et al.*, 2007). Our previous studies showed that this by-product, which itself exhibits poor functional properties, can be a rich source of ACE-inhibitory and antioxidative peptides (POKORA *et al.*, 2013; 2014; ZAMBROWICZ *et al.*, 2013). Attention is also drawn to egg yolk as a source of substances, which may find wide application in the prevention and treatment of various medical conditions. Egg yolk is mainly used for the extraction of valuable phospholipids such as lecithin, which is more valuable than plant-derived lecithin due to the specific chemical composition. The main by-products of this process are partially denatured and defatted egg yolk proteins in the form of insoluble granule fractions (SIEPKA *et al.*, 2010).

The preparation of bioactive peptides by enzymatic hydrolysis of proteinaceous by-products could become an interesting method of waste disposal if the process was cost- effective. Therefore cheap and effective enzymes for this process are preferred. Plant serine protease isolated from *Cucurbita ficifolia* pulp used in this study exhibits strong proteolytic properties and is a relatively cheap proteolytic enzyme (ILLANES *et al.*, 1985; CUROTTO *et al.*, 1988).

The aim of this study was the enzymatic hydrolysis of a by-product of egg yolk phospholipid isolation, in order to obtain hydrolysates with antioxidant, ACE-inhibitory and immunostimulatory activities.

MATERIALS AND METHODS

Substrate

Eggs from 40-45 weeks old Lohman brown laying hens (housed in a bedding system) were stored at 4°C for 1 week. The eggs were automatically broken and their macroscopic parts were separated on an industrial scale. Phospholipids were extracted from the egg yolks (Siepka *et al.*,

2010). Defatted granules, a by-product of phospholipid extraction from the egg yolk, were lyophilized and stored frozen until used.

Enzyme

Non-commercially available protease from *C. ficifolia* was isolated according to the procedure described by DRYJAŃSKI and WILUSZ (1990). Serine protease was obtained by extraction of the homogenized pumpkin pulp separated from the solids by centrifugation (5000 G, 20 min, 4°C). To the supernatant, ammonium sulfate was added to 50% saturation, and allowed to stand for 24 hours, and then centrifuged (9600 rev/min, 30 min). The resulting precipitate (the enzyme preparation) was desalted by dialysis for 12 hours using distilled water (4°C), and then 0.02 M of phosphate buffer at pH 6.0.

Determination of proteolytic activity of protease from *C. ficifolia*

Proteolytic activity was determined by reaction with 1% casein as a substrate (BDH, Ltd., England) at pH 8.3 (KUNITZ, 1945). The substrate with the enzyme was incubated for 10 min at 37°C. The reaction was stopped by the addition of 5% trichloroacetic acid (TCA). The samples were then centrifuged, and the absorbance of supernatants were measured at λ=280 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme giving an increase in absorbance of 0.1 at 280 nm under reaction conditions.

Determination of protein content

Total protein content (N x 6.25) in insoluble substrate was determined using the Kjeldahl method. Protein content in hydrolysates and peptide fractions was determined by the method of LOWRY *et al.* (1951).

Enzymatic hydrolysis

YP hydrolysis was carried out according to a modified method of Zambrowicz *et al.* (2013a). 1% substrate suspension in 0.1 M Tris-HCl buffer (pH 8.0) was hydrolyzed at 37° C for 4 hours using *C. ficifolia* protease at doses of 100, 200, 400 and 1000 U of active enzyme applied on 1 mg of YP substrate. The reaction was ended by heating the mixture at 100°C for 15 min. The hydrolysates were cooled, centrifuged (5500 G, 10 min, 10°C), then the supernatants were lyophilized and stored at 4°C until used.

The degree of hydrolysis

The degree of hydrolysis (DH %) was determined as the percentage ratio of protein soluble in 10% trichloroacetic acid (TCA) to total protein (SPELLMAN, 2003). TCA was added to the hydrolysates (1:1) and after 1 h of incubation at 4°C the samples were centrifuged (4500 G, 15 min, 20°C). The concentration of the trichloroacetic acid-soluble product in the supernatant was measured spectrophotometrically and calculated from the following equation:

$$DH\ (\%) = (\text{mg soluble protein after hydrolysis / mL} \div \text{mg soluble protein before hydrolysis/mL}) \times 100\%$$

The content of free amino acid groups

The content of free amino acid groups (FAG) (μmol/g) was determined by using trinitrobenzene sulfonic acid (TNBS, Sigma) according to a modified method by Kuchroo *et al.* (1983).

Reversed-phase high-performance liquid chromatography

Peptide profiles of hydrolysates were monitored by reversed-phase high-performance liquid chromatography (RP-HPLC). Separation was performed using a Zorbax XDB-C 18 Agilent column (1.8 mm × 50 mm). The operation conditions were as follows:
injection volume: 50 μL; mobile phase A – 0.1% TFA in water; mobile phase B – 0.1% TFA in acetonitrile, column temperature: 30°C. Flow rate: 1mL/ min. Analysis time and gradient conditions can be found in drawings. The absorbance of eluent was monitored at λ=230 nm.

Determination of ACE-inhibitory activity

ACE (EC 3.4.15.1) inhibitory activity was measured spectrophotometrically according to the method described by MIGUEL *et al.* (2004) with some modifications. A hydrolysate solution (40 μL) mixed with a Hippuryl-His-Leu (HHL) substrate solution (5 mmol/L in 100 mmol/L potassium phosphate containing 300 mmol/L sodium chloride, pH 8.3) was preincubated at 37°C for 5 min, and the reaction was initiated by adding 20 μL (2 mU) of ACE solution, and then incubated for 30 min at the same temperature. The enzymatic reaction was terminated by the addition of 150 μL of 1 M HCl. The liberated hippuric acid was extracted using 1 mL of ethyl acetate and vigorously shaking, 750 μL of the upper layer was transferred into a test tube and evaporated under vacuum. The hippuric acid left in the tubes was re-dissolved in 800 μl of distilled water. The content of hippuric acid was determined spectrophotometrically at λ=228 nm.

All samples were tested in 3 replications. Inhibition activity was calculated using the following equation:

$$\text{Inhibitory activity (\%)} = \frac{(Ac - As)}{(Ac - Ab)} \times 100$$

where Ac is the absorbance of the buffer (control), As is the absorbance of the reaction mixture (sample), Ab is the absorbance when the stop solution was added before the reaction occurred (blank).

The IC_{50} value was defined as the concentration of peptides in μg/mL required to reduce 50% of ACE activity, which was determined by analysis of ACE inhibition (%) versus peptide concentration.

Determination of antioxidant activity as the ability to scavenge of DPPH free radicals

Antioxidant activity was determined by a modified method of Yen and Chen (1995) as the ability to scavenge of DPPH (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl) free radicals in an aqueous solution of peptides. Absorbance measurements were made at λ=517 nm after 30 min incubation. The antioxidant activity of the analyzed peptides was determined on the basis of the standard curve prepared for trolox equivalent.

Determination of antioxidant activity by FRAP method

Antioxidant activity was determined as the ability to reduce the oxidation of iron Fe(III) to Fe(II) ions in a reaction with TPTZ (2,3,5-triphenyltetrazoliumchloride). The absorbance was measured at λ=593 nm. The concentration of Fe^{2+} ions was determined on the basis of the standard curve for known $FeSO_4$ solutions (BENZIE and STRAIN, 1996).

Determination of iron Fe(II) ion chelation

Chelation of iron ions was determined by colorimetric measurement of the quantity of Fe(II) not bound to the peptides in a reaction mixture with ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate) (Xu *et al.*, 2007). Absorbance measurement was made at λ=562 nm. The ability to chelate iron ions was determined on the basis of the standard curve for a $FeCl_2$ solution.

Determination of immunostimulatory activity

Immunostimulatory activity of the cytokine secretion in human whole blood was determined at the Department of Immunochemistry, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław (Poland). Cytokine secretion was induced according to the procedure described by INGLOT *et al.* (1996). Blood samples from at least 10 donors were collected in syringes containing sodium heparin. Within 1 h after collection, the blood was diluted 10-times with RPMI 1640 medium supplemented with penicillin/streptomycin, L-glutamine and 2% fetal bovine serum. 1 ml portions of the cell suspension were distributed in two 24-well flat-bottomed tissue culture plates. To the cell suspension of whole human blood (1 mL sample) the hydrolysates were added at 1.0, 10 and 100 μg. As a reference, the positive lipopolysaccharide inducer of *E. coli* at a concentration of 4 mg/mL was used. Control wells containing non-treated blood cell samples were used to measure the spontaneous production of cytokines (negative control). The plates were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. After incubation, the plates were centrifuged at 200 G for 15 min at room temperature. The supernatants were collected and used for determination of the cytokines. IL-6 and IL-10 were determined by microplate enzyme-linked immunosorbent assay using commercially available sets from Becton Dickinson (Franklin Lakes, NJ, USA) according to the procedure recommended by the manufacturer.

Statistical analysis

All experiments were carried out in triplicates. The data obtained were subjected to multi-factor variance analysis (ANOVA), followed by the Duncan's multiple range test to determine the significant difference between sample at $p<0.05$ level using the Statistica v.9.0.

The results of immunostimulating activity were considered significant by a non-parametric Wilcoxon test at $p\leq0.05$ (*) and $0.05\leq p\leq 0.1$ (**) versus control (untreated cells).

RESULTS AND DISCUSSION

Enzymatic hydrolysis

Egg yolk protein preparation (YP), as a by-product of lecithin extraction, was treated by a non-commercial serine protease isolated from *C. ficifolia* in order to evaluate antioxidant, ACE-inhibitory and immunostimulatory properties.

The progress of hydrolysis was monitored by determining the degree of hydrolysis (DH) (%) (Fig. 1), the increase in the concentration of free amino groups (FAG) (Fig. 2), and by RP-HPLC peptide profile analysis (Fig. 3). DH depended on the enzyme dose and reaction time. DH increased slowly in the first 0.5 h, followed by a faster rate of increase up to 4 h, indicating that the maximum cleavage of proteins occurred in the last hour of hydrolysis. Dissimilar kinetics of protein substrate degradation with various proteolytic enzymes has been observed by other authors (OTTE *et al.*, 1998; ZAMBROWICZ *et al.*, 2012). Typically, enzymatic hydrolysis is most extensive during the first 30 minutes and then slows down, indicating a maximum of protein degradation in the first hour of hydrolysis.

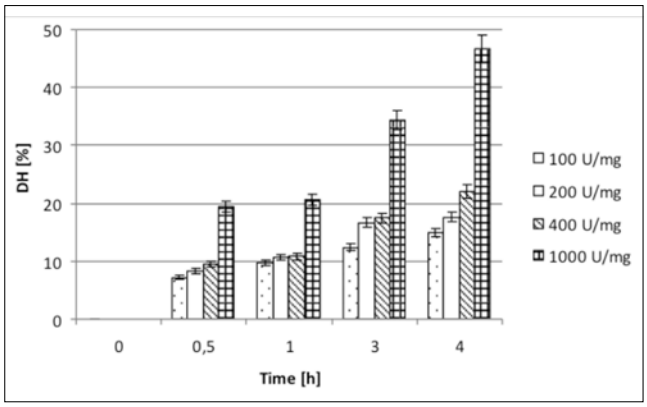


Fig. 1 - Effect of time on the degree of hydrolysis (DH) during hydrolysis of YP by protease from *C. ficifolia*. a-c means followed by the same letter do not differ significantly ($p \leq 0.05$).

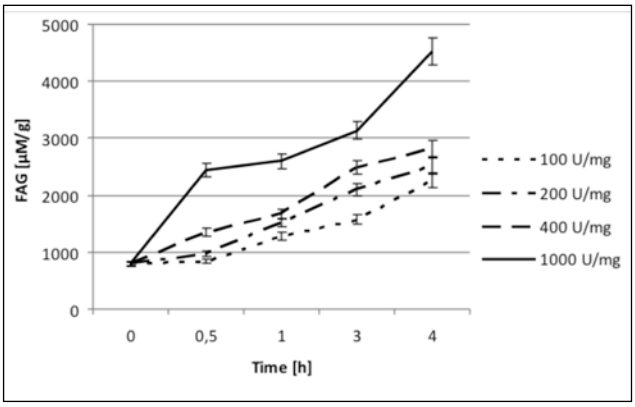


Fig. 2 - Effect of time on the free amino groups contents (FAG) during hydrolysis of YP by protease from *C. ficifolia*.

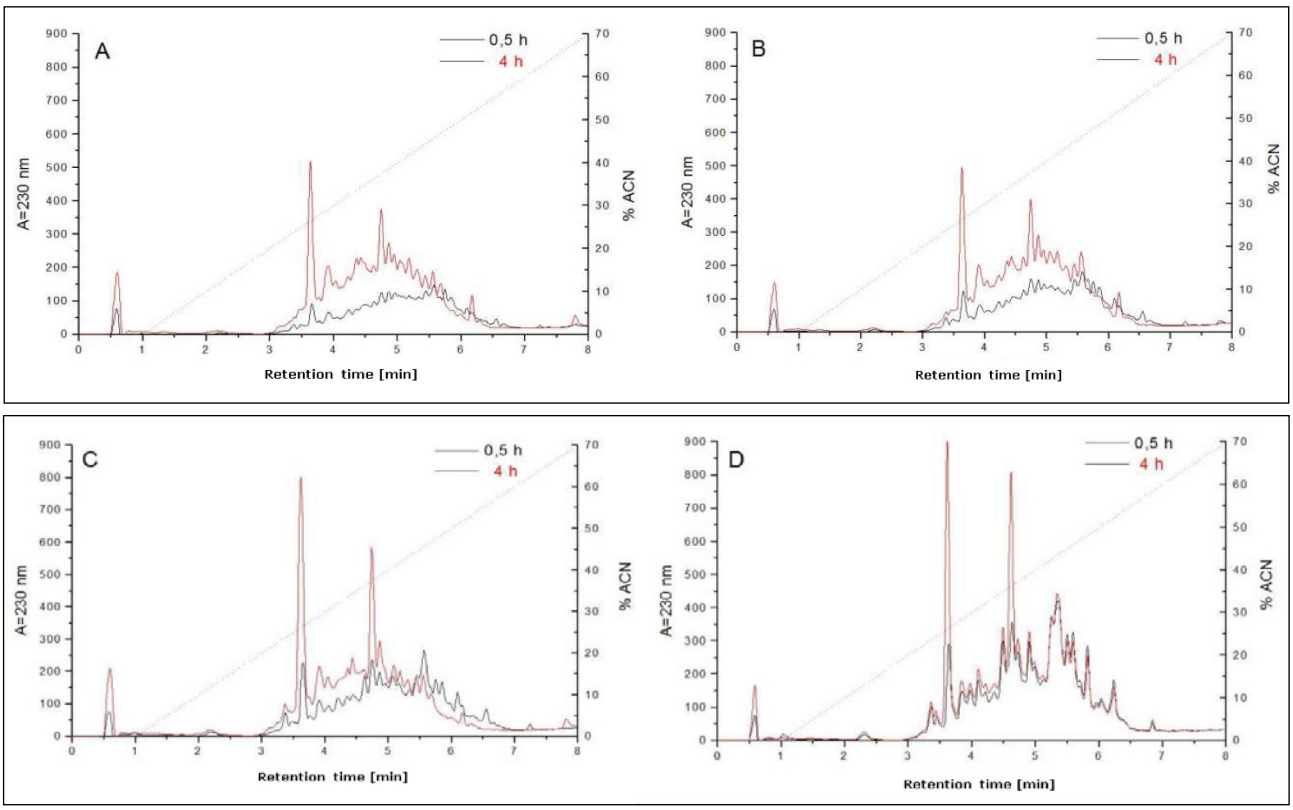


Fig. 3 - Peptide profiles (RP-HPLC) of YP protein hydrolysates obtained after using: A. 100 U/mg, B. 200 U/mg, C. 400 U/mg and D. 1000 U/mg of protease from *C. ficifolia*.

Probably, the hydrolysis process with the use of protease from *C. ficifolia* proceeded according to the "one-by-one" mechanism. During the initial stage of the reaction (determining the overall level of hydrolysis) it is necessary to partially unfold native protein molecules. As a result, the protein loses its stability, more peptide bonds are exposed on the outside of the molecule (intermediate products) and the enzyme has access to the hydrolyzed peptide bond. In a further step, intermediate products are very rapidly degraded to small peptides (KUNST, 2003).

The use of the lowest dose of enzyme (100 U/mg) resulted in a nearly 15% DH of YP. The increase of the dose of *C. ficifolia* proteinase to 400 U/mg did not exert any significant impact on DH rate, whereas the addition of the enzyme at 1000 U/mg resulted in a DH value of more than 46% after 4 hours of digestion. Analysis of the FAG concentration of the obtained hydrolysates confirmed the above results. The greatest increase in concentration was observed during the long time of hydrolysis. The application of enzyme at doses from 100 to 400 U/mg resulted in similar levels of increases of FAG from 2255.20 to 2325.74 µM Gly/g. The most intensive increase of FAG (4525.1 µM Gly/g) occurred during the 4-hour reaction using 1000 U/mg of protease.

Protein-peptide profiles obtained by RP-HPLC technique demonstrate the extent of YP hydrolysis (Fig. 3). They were identified with both a longer retention time (3-7 min) specific for the more hydrophobic peptides, and peaks with a short retention time (0.5-1 min) typical for hydrophilic peptides. The wide distribution of degradation products indicates that each of the hydrolysates is composed of peptides with different hydrophobic properties, which may impact the biological activity of the obtained hydrolysates. The results described above indicate that serine protease from *C. ficifolia* is an effective enzyme in hydrolyzing egg yolk protein by-product. Previously, the proteolytic properties of this enzyme had been tested on casein, a protein from corn gluten, and egg white proteins (ILLANES *et al.*, 1985; CUROTTO *et al.*, 1989; POKORA *et al.*, 2014).

The biological activities of YP enzymatic hydrolysates

The antioxidant activity of YP hydrolysates was studied in terms of the scavenging effect on DPPH radicals, ferric reducing power (FRAP), and iron chelating activity (Table 1).

The enzymatic treatment of YP leads to an increase in DPPH free radical scavenging activity in the hydrolysates. ELIAS *et al.* (2008) explained that the antioxidant activity of the hydrolysates/peptides is the result of the proteolytic action of the enzyme. The specific amino-acid sequence of peptides and their changed physical properties allow exposing the amino acid residues and their action as electron donors. As a result of these reactions, the peptides combine with radicals and form stable complexes, which inhibit oxidation processes. The various YP hydrolysates showed different potencies in scavenging DPPH radicals. The results indicated no direct relationship between DH and the values of DPPH free radical scavenging activity. The highest DPPH scavenging potency was shown by the hydrolysate obtained after 4 hours degradation with an enzyme dose of 1000 U/mg (0.89 μmol $trolox_{eq}$/mg) (Table 1). The significant level of DPPH free radical scavenging activity (0.63 μmol $trolox_{eq}$/mg) was also observed for the hydrolysate obtained with the use of 200 U/mg protease after 0.5 hour digestion. In previous works, YP protein by-product was treated with pepsin and neutrase leading to final hydrolysates with DH values: 45.3% and 27.6%, respectively (ZAMBROWICZ *et al.*, 2014; POKORA *et al.*, 2013). Peptic hydrolysate and hydrolysate obtained by neutrase showed DPPH free radical scavenging activity values: 0.5 troloxeq/mg and 0.44 μM $trolox_{eq}$/mg, respectively (ZAMBROWICZ *et al.*, 2014; POKORA *et al.*, 2013). It may be explained that the potency of YP-hydrolysates to scavenge DPPH free radical depends more on the specificity of the enzyme than on the degree of hydrolysis (DH). Our results also indicate that YP is a better source for peptides exhibiting DPPH scavenging activity than other protein waste, such as by-products of lysozyme and cystatin isolation from egg white. Egg white protein by-product hydrolysates obtained with trypsin and neutrase exhibited free radical scavenging activity up to 0.21 and 0.17 μmol $trolox_{eq}$/mg, respectively (ZAMBROWICZ *et al.*, 2013).

Previously, serine protease *C. ficifolia* was used by DĄBROWSKA *et al.* (2013) to evaluate the antioxidant activity of bovine casein. Casein hydrolysates possessed a different ability to scavenge of DPPH radicals (from 0.06 to 2.21 μmol $trolox_{eq}$/mg), depending on enzyme dose and reaction time. However, the DPPH scavenging potency of many of them was at the same level as the YP hydrolysates. In most of the hydrolysates obtained with different doses of protease, the ferric reducing ability was increasing gradually with the time of hydrolysis. The only exception was the 0.5 h hydrolysate obtained with the enzyme dose of 200 U/mg, which possessed significantly higher ferric reducing activity than the products obtained during long time of degradation (more than 30 minutes). A maximum value of this activity reached 56.41 μg Fe^{2+}/mg, for the 4 h hydrolysate obtained with the participation of 1000 U/mg of the enzyme (Table 1). The ferric reducing activity of the 4h hydrolysate increased with the increased doses of the enzyme. The application of protease at 100, 400 and 1000 U/mg resulted in hydrolysates 3.44, 5.0 and 5.47 times more potent than YP, respectively. On the other hand, hydrolysates exerted more than 3 times lower ferric reducing activity in comparison to the YP hydrolysate prepared with neutrase (177.35 μg Fe^{2+} /mg) (Pokora *et al.*, 2013). This results gives an indication that unconventional protease from *C. ficifolia* is characterized by a lesser ability to release peptides with ferric reducing activity from YP than commercially available neutrase. An increase in chelating activity was also observed as a result of progress in hydrolysis. The highest chelating activity was obtained in hydrolysates with DH above 35 %. YP degraded with participation of 1000 U/mg protease during 3 and 4 hour reactions exhibited ferrous ion chelating activity at 692.49 and 695.76 μg Fe^{2+}/mg, respectively (Table 1). Significant ferric chelating power was also shown by hydrolysates in which DH ranged from 15% to 20%. Similar results were obtained by Torres-Fuentes *et al.* (2011), who analyzed the antioxidant properties of plant protein hydrolysates in terms of their ability to complex iron ions.

Numerous antihypertensive peptides (eg. ovokinin, ovokinin 2-7, RADHP, YPI, DLIN) derived from egg white proteins by enzymatic hydrolysis have been characterized (MIGUEL *et al.*, 2004; LIU *et al.*, 2010). Interest has been aroused in ACE-inhibitory peptides generated from egg yolk proteins, because they have not been described as a

source of peptide inhibitors of ACE as much. The hydrolysates obtained in this work exhibited various abilities to inhibit the ACE enzyme (Table 1). The hydrolysates of DH lower than 10% did not exert any ACE inhibitory activity. The most active inhibitor of ACE (IC_{50}=467.5 μg/mL) was the hydrolysate obtained by an enzyme dose of 1000 U/mg after 4-hours digestion. Whole egg yolk in native form as a potential source of ACE-inhibitory peptides was tested by YOU and WU (2011). The level of this activity (IC_{50}) for hydrolysates prepared with the use of gastrointenstinal (pepsin, pancreatin) and microbial (thermolysin, alkalase) proteases ranged from 133.4 μg/mL to 210.2 μg/mL (YOU and WU, 2011). Such significant differences in the level of ACE-inhibitory activity may result from the fact that in the present study we used a by-product, denatured protein of the yolk granular fraction. Denaturation has a significant impact on the physico-chemical and biological properties of proteins.

The results indicate that protein by-product obtained from the isolation of phospholipids from hen egg yolk may be a better source of ACE-inhibitory peptides than other protein by-product from the isolation of cystatin and lysozyme from egg white. The peptic hydrolysate (DH: 38.3%) of this protein preparation exhibited an activity of IC_{50}=643.1 μg/mL (ZAMBROWICZ *et al.*, 2013). Recently, we indicated that serine protease from *C. ficifolia* may be effective in the conversion of this protein by-product to a value added product with ACE-inhibitory activity (POKORA *et al.*, 2014). The 50% inhibition of ACE was obtained with the presence of 9071.7 μg/mL of the hydrolysate.

Studies have shown a high linear correlation between DPPH free radical scavenging activity and immune activity with a positive correlation coefficient of 0.96 (HE *et al.*, 2014). Therefore hydrolysates with the highest DPPH free radical scavenging potency were also evaluated in terms of their immunostimulatory properties. It was assessed as the results of cytokines IL-10 and IL-6 induction by hydrolysates in whole human blood cell cultures (*ex vivo*) (Fig. 4). 4-hour hydrolysate obtained with the use of 1000 U/mg of *C. ficifolia* protease appeared to be slight inducer. The use of 100 μg/ mL of protease resulted in a low increase the concentration of IL-6, which reached the value: 3.05 ng/ mL (Fig. 4 A). However, the results were not statistically significant compared to the positive control (LP). Extremely different cytokine inducing activity was exerted by the yolkin, naturally occurring in egg yolk. Yolkin is a mixture consisting of several peptides of an apparent molecular weight of 1 to 35 kDa, produced as a result of vitellogenin II hydrolysis by cathepsins during the formation of an egg. Its constituent peptides were found to be efficient inducers of IL-1β, IL-6 and IL-10 secretion. A complex at a concentration of 100 μg/mL showed almost the same activity as the LPS-treated control in stimulating cytokine production. (POLANOWSKI *et al.*, 2013). The biological activity of enzymatic hydrolysates

Table 1 - Biological activities of YP hydrolysates obtained with using serine protease from *C. ficifolia*. All data were expressed as mean values (mean±SD, n=3). Values sharing the same letter at the same enzyme dose and test group are not significantly different at p<0.05.

Enzyme dose [U/mg]	Time of hydrolysis	DPPH scavenging activity [μM $Trolox_{eq}$/mg]	Ferric reducing ability (FRAP) [μg Fe^{2+}/mg]	Ferrous ion-chelating activity [μg Fe^{2+}/mg]	ACE inhibitory activity [IC_{50}] [μg/ml]
0	substrate	0.15±0.01^{a}	10.3±0.16^{a}	376.2±18.80^{a}	nd^{a}
100	0.5	0.28±0.03^{a}	33.26±1.38^{a}	497.10±8.22^{b}	nd^{c}
	1	0.32±0.03^{b}	36.44±1.62^{b}	466.26±3.89^{c}	nd^{c}
	3	0.18±0.02^{c}	48.78±1.20^{c}	474.25±4.01^{d}	968.5±17.25^{b}
	4	0.42±0.02^{b}	35.40±0.67^{d}	574.38±13.67^{a}	837.75±15.25^{a}
200	0.5	0.63±0.02^{a}	46.14±1.33^{a}	460.72±11.74^{b}	nd^{c}
	1	0.20±0.03^{c}	35.53±1.21^{a}	507.59±2.88^{d}	nd^{c}
	3	0.28±0.01^{d}	37.11±0.73^{b}	553.75±13.79^{c}	890.75±11.25^{b}
	4	0.27±0.01^{b}	44.68±1.66^{b}	626.16±6.56^{a}	777.0±14.25^{a}
400	0.5	0.20±0.03^{ab}	38.91±1.41^{a}	468.70±12.07^{d}	nd^{c}
	1	0.21±0.03^{a}	36.77±1.20^{a}	513.73±3.81^{c}	nd^{c}
	3	0.20±0.04^{a}	35.44±1.30^{ab}	573.56±3.77^{b}	803.12±10.25^{b}
	4	0.26±0.02^{a}	51.46±0.78^{ab}	625.86±7.11^{a}	718.75±4.5^{a}
1000	0.5	0.22±0.02^{a}	21.71±1.37^{d}	642.32±2.83^{d}	657.75±4.5^{a}
	1	0.25±0.02^{c}	27.78±1.75^{c}	654.79±5.78^{b}	650.0±4.5^{a}
	3	0.43±0.03^{b}	43.33±1.06^{b}	692.49±1.86^{c}	554.75±7.75^{a}
	4	0.89±0.03^{a}	56.41±1.13^{a}	695.76±14.91^{a}	467.5±6.0^{a}

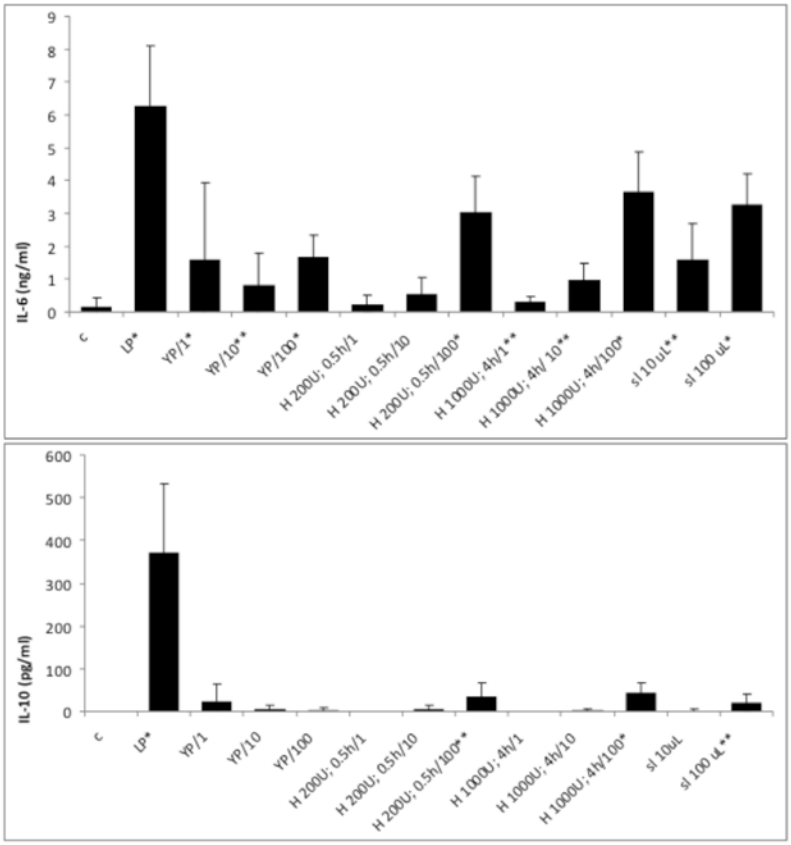


Fig. 4 - Induction of the cytokines A - interleukin-6 and B - interleukin-10 by YP and YP hydrolysates in whole blood cells cultures. C- control (untreated cells), LP -lipopolicascharide from *Escherichia coli* was used as positive control, sl- reagents used in enzymatic hydrolysis. YP and YP hydrolysates were applied at doses 1, 10 and 100 μg/mL. Data are presented as the median ± standard deviation. The results were considered significant by the non-parametric Wilcoxon test when $p \leq 0.05$ (*) and $0.05 \leq p \leq 0.1$ (**) versus control (untreated cells).

is determined by the protein sequence (type and location of amino acid residues) as well as by the specifity of the enzyme (CLEMENTE, 2000; PARK *et al.*, 2001). These two crucial factors are responsible for the disparate level of immunostimulatory activity of YP-hydrolysates.

CONCLUSIONS

The effect of enzymatic modification of an egg yolk protein preparation (YP), obtained as a by-product of phospholipid extraction, on its biological properties were evaluated. The hydrolysis process of YP was performed with the use of noncommercial serine protease from *C. ficifolia*. The most effective degradation of YP was noticed under the conditions: enzyme dose 1000 U/ mg and duration 4 h, when a significant degree of hydrolysis (46.6%) was obtained. Enzymatic hydrolysis of YP provided hydrolysates/ peptides exhibiting antioxidant and ACE-inhibitory activities. The YP hydrolysates showed significant antioxidant and degree of hydrolysis-dependent ACE-inhibitory activity. The 4-hour hydrolysate obtained with the highest amount of enzyme (1000 U/mg) showed the highest biological activity among the tested hydrolysates. It exhibited ferric ion reducing potential (FRAP) (56.41 μg Fe^{2+}/mg), ferric ion chelating activity (695.76 μg Fe^{2+}/mg), DPPH free radical scavenging activity (0.89 μmol $trolox_{eq}$/mg) and ACE- inhibitory (467.5 μg/mL) activity. Novel biological effects of egg-yolk protein by-product hydrolysates was shown.

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PAPER

DETERMINATION OF DRYING CHARACTERISTICS AND QUALITY PROPERTIES OF EGGPLANT IN DIFFERENT DRYING CONDITIONS

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ABSTRACT

Drying is the most traditional process used for preserving eggplant a long time. The aim of this study was to determining drying characteristics and quality properties of eggplant dried by sun drying, hot air convective drying and infrared assisted convective drying. Convective drying and infrared assisted convective were carried out in a convective dryer at three different temperatures (40°, 50°, 60°C) and air velocity at 5 m/s.

The increasing of temperatures during the drying of eggplant led to a significant reduction of the drying time. However loss of nutrition was observed in eggplant samples dried at higher temperature. The biggest change in colour parameters was observed in samples dried with sun drying. So it was thought that sun drying had a negative effect on quality properties of eggplant samples.

- Keywords: eggplant, air drying, infrared -

INTRODUCTION

Eggplant (*Solanum melongena* L.) is a common annual vegetable crop grown in the subtropics and tropics (CONCELLON, 2012). Eggplant is an important market vegetable of Asian and Mediterranean countries and has a very limited shelf life for freshness (WU *et al.*, 2007; BOULEKBACHE-MAKHLOUF *et al.*, 2013). Its shelf-life at temperature of 10–15°C is about 10 days (HU *et al.* 2010). The limited shelf-life constitutes a heavy drawback for commercial purpose (BRASIELLO *et al.*, 2013).

Drying which is the process of removal of most of the moisture present in the food is the oldest preservation method applied since ancient times (AYHAN and ALİBAŞ, 2005; ER and AKBULUT, 2011; ALİBAŞ, 2012). The removal of moisture from the food materials prevents the growth and reproduction of spoilage microorganisms, slows down the action of enzymes and minimizes many of the physical and chemical reactions (CEYLAN *et al.*, 2006; WU *et al.*, 2007; GUINE *et al.*, 2012a). Nowadays drying process of product is carried out by various methods such as sun drying, contact, convective, radiation, dielectric, vacuum, freeze drying and osmotic drying (KARABAYIR, 2006). Natural sun drying is practiced widely in the World and also Turkey, but has some problems related to the contamination by dirt and dust and infestation by insects, rodents and other animals (KOCABIYIK and DEMIRTÜRK, 2008). Therefore, the convective drying process carried out in closed equipments is preferred (ERTEKIN and YALDIZ, 2004).

Convective drying is the most traditional dehydration method used to preserve foods; it mainly consists of forcing air through the product to be dried. The surface area of the product to be dried, the drying time, drying temperature, air velocity, moisture content of air and atmospheric pressure determine drying efficiency (CEMEROĞLU, 2004). Convective drying processing effectively extends the shelf life of agricultural products, however this drying process involves chemical, physical, structural and nutritional changes, linked to the water loss and the high temperatures applied, which affect the product quality (GARCIA-PEREZ *et al.*, 2012). Loss of sensory and nutritive qualities is considered inevitable during traditional drying process due to the undesirable textural and biochemical changes (WU *et al.*, 2007). The expansion of dehydrated food market demands high quality products that maintain at a very high level the nutritional and sensorial properties of the initial fresh product (RUSSO *et al.*, 2013). Infrared radiation has significant advantages over conventional drying. These advantages are higher drying rate, energy saving, and uniform temperature distribution giving a better quality product. At present, many driers use infrared radiator to improve drying efficiency, save space and provide clean working environment, etc. Therefore infrared drying can become popular as an energy saving drying method (WANG and SHENG, 2006).

Drying times of carrot pomace dried at the infrared power levels of 83, 125, 167 and 209 W were studied. According to the results, it was determined that drying rate increased and drying time decreased with increasing infrared power level (DOYMAZ, 2013).

The effect of harvest time and drying techniques on the quality characteristics, which are specifically important for maize crop were investigated. Energy expenses of the drying techniques were calculated for all harvest periods and it was found out that the expenses to reduce the moisture level from 15 to 13% with hot air drying are higher than the expenses to reduce moisture level from 29 to 13% with infrared-hot air drying combination (YILMAZ and TUNÇEL, 2008).

ABDELMOTALEB *et al.* (2009) investigated thin layer drying of garlic slices under convection and combined infrared–convection heating modes and observed increases in drying rate, thermal efficiency, rehydration ratio, flavor strength and colour difference and decreases in drying time and specific energy consumption for the combined (infrared–convection) heating mode in comparison with convection only.

Effects of infrared power, air temperature and air velocity on drying rate and quality of onion slices dried in infrared-convective dryer were studied. It was found that drying time of onion slices increased with increasing air velocity, and decreased with increasing temperature and infrared power (SHARMA *et al.*, 2005).

The objectives of this study were to investigate the drying characteristics of the eggplant samples, to examine the effect of drying conditions on the drying process, and to choose optimum drying method for quality of dried eggplant samples.

MATERIALS AND METHODS

Sample preparation

Fresh eggplants were obtained from Öcal Agricultural Product Limited Company (Turgutlu, Manisa, Turkey). Vegetables were washed and sliced (30 mm diameter and 6 mm thickness). Eggplant slices were placed over a metal grating in a convective oven operating at constant temperature (RUSSO *et al.*, 2013).

Drying experiments

Eggplant slices were subjected to drying with three methods. These drying methods were:

sun drying, hot air convective drying and infrared assisted convective drying. Convective drying and infrared assisted convective drying of eggplants were carried out in a drying system consisting of solar collector, carbon fiber infrared heaters, drying chamber, condenser, heat exchanger, hot water tank, and PLC (Programmable logic controller) panel at three different temperatures (40°, 50°, 60°C) and air velocity of 5 m/s.

Drying kinetics

In our study drying time was defined as the time passing from initial moisture content of the samples until final moisture content of samples. Drying rate was described as the amount of water removed from the sample per unit of time. Effect of temperature on drying time and drying rate of eggplant samples was determined (NASIROĞLU and KOCABIYIK, 2007).

Specific energy consumption

Specific energy consumption is amount of energy required for removing unit amount of water from samples during drying of samples. Specific energy consumption of eggplant samples dried in different conditions was calculated as follows Eq. (1):

$$E_s = E_T / W_R \quad (1)$$

E_s : Specific energy consumption (MJ/kg),
E_T : Total energy (MJ),
W_R : Amount of water removed during drying (kg) (SHARMA and PRASAD, 2006).

Shrinkage

Shrinkage, which occurred during drying as a result of water evaporation, was evaluated by determination of the relative volume of dried material. The relative volume was the ratio of eggplant slices volume after drying to that before drying as follows Eq. (2):

$$V_s = V/V_0 \quad (2)$$

V_s = Shrinkage
V = Volume of dried samples
V_0 = Volume of fresh samples (FIGIEL, 2010).

Rehydration

Rehydration kinetics study was carried out for dried eggplant slices. The samples were placed in water at 45°C and waited for 5 h. The rehydrated samples were spread on absorbent paper for the removal of free water on the surface of vegetable. The change in weight was recorded after a regular interval of time. The rehydration capacity was calculated from the ratio of sample weight after and before the rehydration as follows Eq. (3):

$$R_R = M_R/M_D \quad (3)$$

R_R = Rehydration ratio
M_R = Weight of rehydrated samples
M_D = Weight of dried samples (RUSSO *et al.*, 2013).

Colour parameters

Colour of dried and fresh samples was evaluated by means of a Minolta Chroma Meter CR-300 (Minolta Co. Ltd., Osaka, Japan). Instrumental colour data were expressed as CIE L*, a*, b* coordinates, which define the colour in a three-dimensional space: L* (dark–light), a* (redness–green) and b* (yellowness–blueness). Total colour difference (ΔE), chroma (C), hue angle (h) and R (a/b) values were calculated by using L*, a*, b* values in Eqs. (4)-(7). Eggplant slices were placed in container without space. Colour measurements were performed twice (DEMIR and AKBULUT 2010; NASIROĞLU and KOCABIYIK, 2007).

$$\Delta E^* = \sqrt{(L^*_0 - L^*)^2 + (a^*_0 - a^*)^2 + (b^*_0 - b^*)^2} \quad (4)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (5)$$

$$h = \tan^{-1}\frac{b^*}{a^*} \quad (6)$$

$$R = \frac{a^*}{b^*} \quad (7)$$

Textural properties

For determining the textural properties of fresh and rehydrated eggplant slices, texture profile analysis (TPA) was performed using a Texture Analyser (model TA.XT.Plus). The texture profile analysis was carried out by two compression cycles between parallel plates performed on cylindrical samples (diameter 10 mm, height 3 mm) using a flat 35 mm diameter plunger, with a 5 s of time between cycles. The parameters that have been used were the following: 50 kg force load cell and 0.5 mm s^{-1} test speed (NAYAK *et al.*, 2007; GUİNE and BARROCA, 2012b; RUSSO *et al.*, 2013).

The textural properties: hardness, springiness, cohesiveness, gumminess and chewiness were calculated after Eqs. (8)-(12):

Hardness, H=F1 (8)
Springiness, S=ΔT2/ΔT1 (9)
Cohesiveness, C=A2/A1 (10)
Gumminess, G=H x C (11)
Chewiness=H x S x C (12)

Total Dry Matter

Dry matter of dried and fresh samples was determined by drying the samples cut into small pieces at 105°C to constant weight. Total dry matter content of the samples was calculated from the difference in mass before and after the drying process (CEMEROĞLU, 2007; ÖZTÜRK and ÇAPUR, 2010).

Water activity

Water activity measurement set was used for determination of water activity values of all the samples. In this system, the product to measured water activity was cut into small pieces, placed in a hermetic steel chamber. When humidity of air inside the container reached equilibrium with the product, equilibrium moisture content of samples was measured by probe in the container (HASTÜRK-ŞAHİN and ÜLGER, 2010).

Ascorbic acid (Vitamin C)

A spectrophotometric method was used to determine the total amount of vitamin C in the eggplant slices. The absorbance value of samples was measured by means of a Uv-Visible spectrophotometer (Shimadzu Corp., Kyoto, Japan) with wavelength at 518 nm. Ascorbic acid of samples were calculated from standard curve showed absorbance values to concentrations of ascorbic acid and expressed as microgram of ascorbic acid per 100 gram of sample (HIŞIL, 2010).

Statistical analysis

In our study drying methods in different conditions were designed as applications and a completely randomized design was used for statistical analysis. These applications were sun drying, hot air convective drying (40°, 50°, 60°C) and infrared assisted convective drying (40, 50, 60°C). Effect of different drying methods and drying conditions on drying characteristics, chemical, physical and textural properties of eggplant samples was determined. Number of replication was two. In order to determine the differences between applications, analysis of variance (ANOVA) was carried out using Statistical Analysis Software (SAS, 2001). Data found important in result of ANOVA were evaluated with PROC MIXED procedure. For every data LSMEANS values were determined and least significant differences (LSD) between data were calculated.

RESULTS AND DISCUSSION

Drying kinetics of eggplant samples

In our project it was observed that drying method affected drying time of eggplant slices. Drying time of infrared assisted convective drying and convective drying was shorter than drying time of sun drying. Infrared application decreased drying time of eggplant samples during convective drying at air temperature of 50° and 60°C. Also UMESH-HEBBAR *et al.* (2004) reported that the combined infrared and hot air dryer reduced the processing time dramatically (48%), in addition to consuming less energy (63%) for water evaporation compared to hot air drying.

In convective drying, drying time of the eggplant samples showed reduction with increasing temperature. However drying rate of eggplant samples increased at higher temperature (Fig. 1). Similarly the effect of temperature on the drying kinetics and quality attributes of apple (var. Granny Smith) slices during drying was investigated and the experimental results of study showed that dehydration were faster

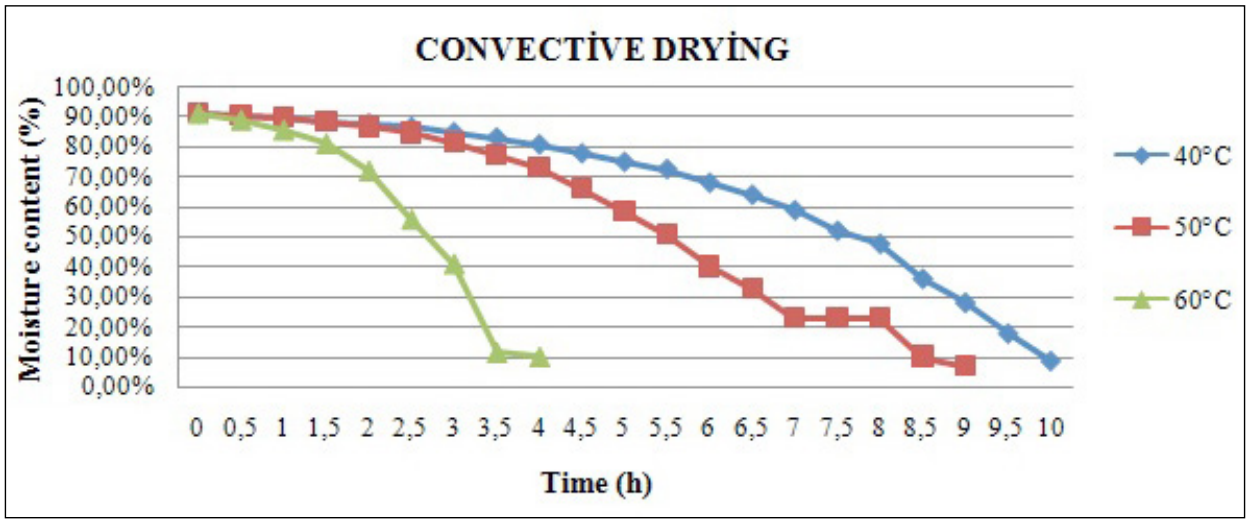


Fig. 1 - Drying curves of eggplant samples dried with convective drying at three different temperatures.

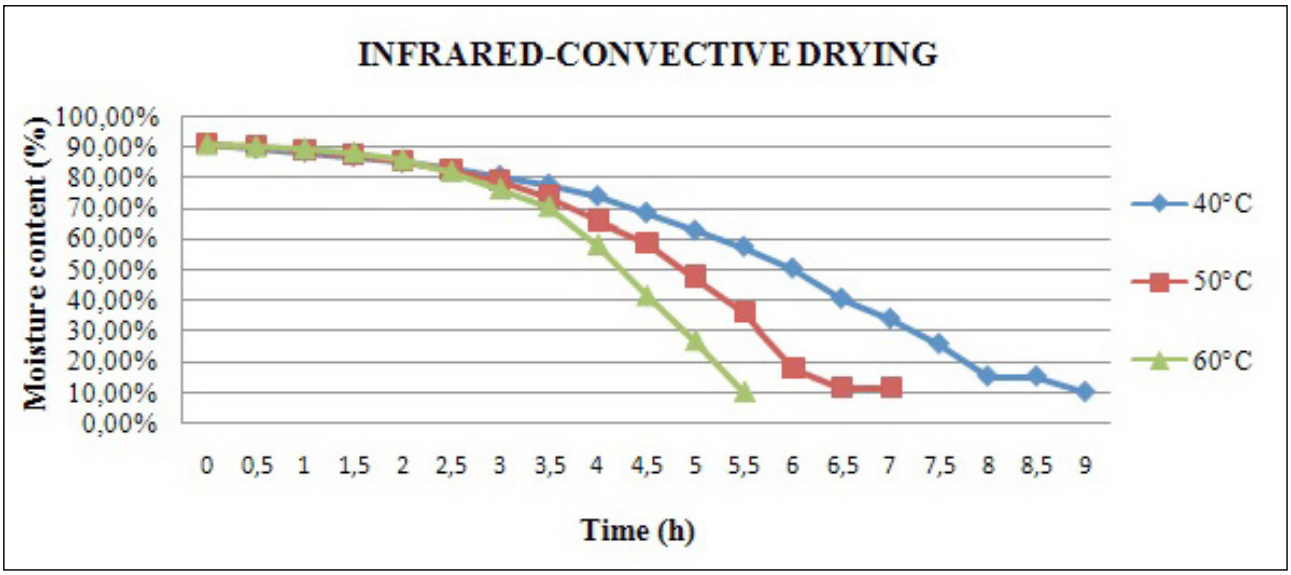


Fig. 2 - Drying curves of eggplant samples dried with infrared-convective drying at three different temperatures.

when air temperature increased (VEGA-GALVEZ *et al.*, 2012). Effect of air temperature on drying time of cornelian cherry fruits dried in convective dryer was investigated, it was observed that increasing air temperature reduced drying time by 34% (KAYA and AYDIN, 2008). Similar result were described by ERTEKIN and YALDIZ (2004) working about drying characteristics of eggplants dried using heated ambient at air temperatures from 30° to 70°C and it was stated that drying time decreased with increasing drying air temperature.

During the infrared assisted convective drying, drying time of the eggplant samples showed reduction with increasing temperature. However drying rate of eggplant samples increased at higher temperature (Fig. 2). TOĞRUL *et al.* (2005) studied drying characteristics of banana slices dried in infrared dryer at drying temperature ranging from 50° to 80°C and it was found that drying rate increased with increasing drying temperature.

When eggplant samples were dried with sun drying, it was determined that sun drying took a longer time than convective drying and infrared assisted convective drying (Fig. 3).

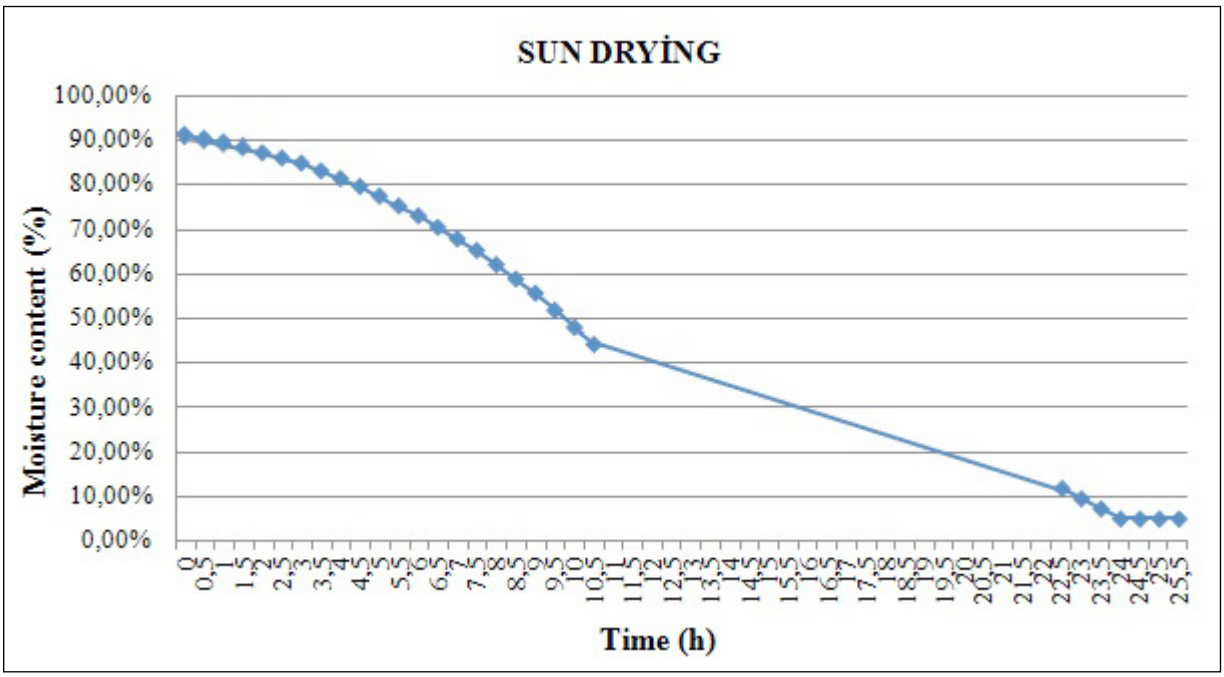


Fig. 3 - Drying curves of eggplant samples dried with sun drying.

Specific energy consumption of dried eggplant samples

In our study amount of energy required for drying of samples was calculated. Specific energy consumptions of dried eggplant samples are showed in Table 1. As a result of statistical analysis, it was determined that there were not important differences between specific energy consumption values of dried eggplant samples at different drying methods ($p>0.05$). The lowest specific energy consumption of eggplant slices was measured during infrared assisted convective drying at air temperature of 40°C. According to results obtained from previous study, specific energy for drying of mushroom slices in a hot air flow-infrared combination dryer increased with increasing temperature, while the specific energy for mushroom drying in the convection dryer decreased with increasing temperature (MİNAEİ *et al.*, 2011).

Table 1 - Specific energy consumption values of dried eggplant samples (kj/kg).

Drying methods	Eggplant
CD (40°C)	0,0023±0,001
CD (50°C)	0,0040±0,004
CD (60°C)	0,0033±0,000
ICD (40°C)	0,0018±0,001
ICD (50°C)	0,0036±0,001
ICD (60°C)	0,0038±0,0001
SD	0±0,000
	p=0.3071

CD: Convective drying, ICD: Infrared assisted convective drying, SD: Sun drying.

Table 2 - Rehydration ratios of dried eggplant samples.

Drying methods	Eggplant
CD (40°C)	$5,27±0,23^{c}$
CD (50°C)	$5,71±0,26^{cb}$
CD (60°C)	$5,81±0,35^{ab}$
ICD (40°C)	$5,42±0,07^{cb}$
ICD (50°C)	$5,92±0,10^{ab}$
ICD (60°C)	$6,33±0,08^{a}$
SD	$5,50±0,31^{cb}$
	p=0.0283
	LSD=0.539

Rehydration ratio of dried eggplant samples

In our project rehydration ratio values of eggplant samples dried with different drying methods were determined. Data obtained as a result of analysis were given in Table 2. Increasing air temperature increased rehydration ratio values of eggplant samples. Similarly RUSSO *et al.* (2013) did scientific study about dried and rehydrated eggplant and state that samples dried at higher temperature showed faster water uptake during rehydration because of wrinkled structure.

It was determined that drying methods had a significant effect on rehydration ratio values of dried eggplant samples ($p<0.05$). Especially it was observed that there was important difference between the rehydration ratio values of eggplant samples dried with convective drying at 40°C and the rehydration ratio values of eggplant samples dried with infrared assisted convective drying at 60°C. Change in rehydration ratio of dried eggplant samples was given in Fig. 4. Rehydration ratio of dried eggplant samples reached to maximum value in five hour. Drying method did not affect rehydration time of dried eggplant samples.

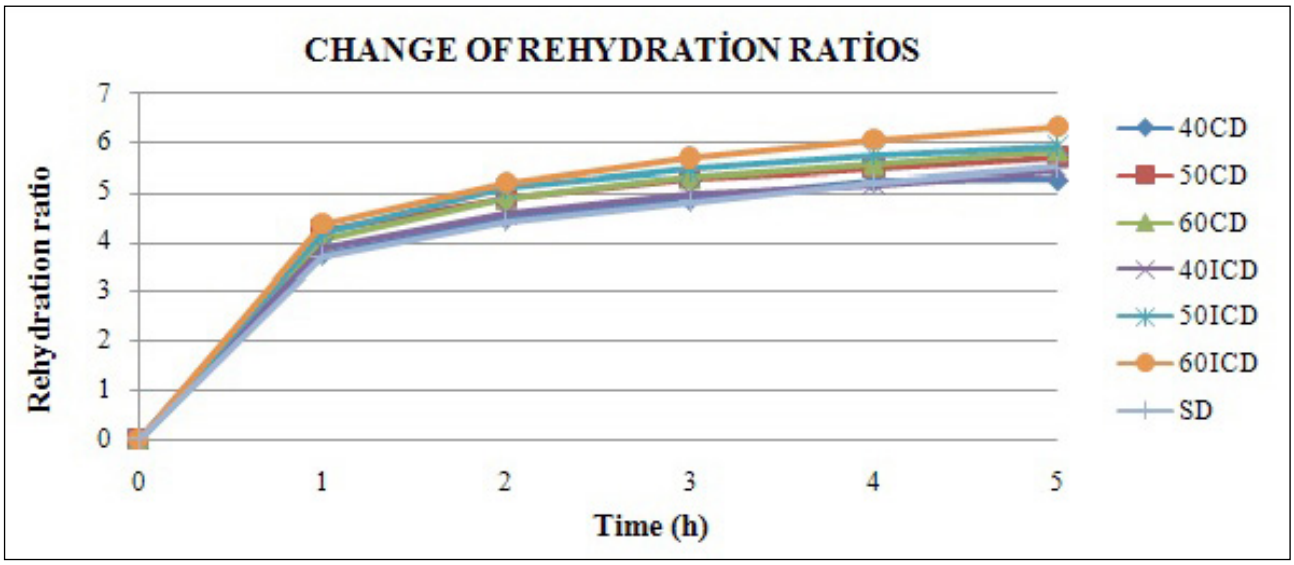


Fig. 4 - Change of rehydration for eggplant samples dried at different conditions.

Shrinkage of dried eggplant samples

Shrinkage values of dried eggplant were determined with analysis and showed in Table 3. Increasing of air temperature in convective dryer caused increasing of shrinkage values of eggplant slice. As a result of statistical analysis it was found that drying conditions did not have an important effect on shrinkage values of dried eggplant samples ($p>0.05$). However LEWICKI and JAKUBCZYK (2004) investigated mechanical properties of apples dried at drying temperature ranging from 50° to 80°C in a laboratory convection dryer and found that the increasing drying temperature caused the gradual decrease of shrinkage values.

Table 3 - Shrinkage of dried eggplant samples.

Drying methods	Eggplant
CD (40°C)	0,225±0,05
CD (50°C)	0,239±0,02
CD (60°C)	0,278±0,024
ICD (40°C)	0,217±0,032
ICD (50°C)	0,254±0,076
ICD (60°C)	0,271±0,012
SD	0,230±0,034
	p=0.6868

Chemical properties of eggplant samples

In our project total dry matter, water activity, ascorbic acid and loss of ascorbic acid of eggplant samples were detected. These data were showed in Table 4. Eggplant samples were dried to 90% dry matter content. The highest ascorbic acid loss in dried samples was determined in eggplant samples dried with infrared assisted convective drying at 60°C. The lowest ascorbic acid loss was observed in samples dried with sun drying.

Effect of different drying methods on chemical properties of eggplant samples was examined as statistical, it was determined that drying methods had a significant effect on total dry matter, water activity, ascorbic acid and loss of ascorbic acid of eggplant samples ($p<0.05$).

Textural properties of eggplant samples

In our thesis project hardness, springiness, cohesiveness, gumminess and chewiness values of fresh and rehydrated samples were measured for determining textural properties of eggplant samples. Textural properties of eggplant samples were given in Table 5.

As a result of statistical examination of textural properties of rehydrated samples, rehydration process was found important in terms of hard-

Table 4 - Chemical properties of eggplant samples.

Samples	Total Dry Matter	Water Activity	Ascorbic Acid (mg/100g)	Loss of Ascorbic Acid (%)
Fresh	8,81±0,516^{a}	0,991±0,010^{a}	16,22±0,08^{a}	-
CD (40°C)	90,73±2,520^{b}	0,594±0,013^{b}	12,36±0,625^{bc}	23,76±4,20^{cd}
CD (50°C)	92,12±0,177^{b}	0,501±0,026^{d}	11,44±0,577^{c}	29,46±3,24^{bc}
CD (60°C)	90,25±1,582^{b}	0,555±0,033^{bcd}	9,74±0,481^{d}	39,92±3,25^{ab}
ICD (40°C)	90,27±1,117^{b}	0,568±0,002^{bc}	12,53±0,577^{bc}	22,73±3,21^{cd}
ICD (50°C)	91,43±4,582^{b}	0,584±0,040^{bc}	11,37±0,866^{c}	29,86±5,03^{bc}
ICD (60°C)	89,21±4,509^{b}	0,591±0,050^{b}	9,54±0,962^{d}	41,18±5,65^{a}
SD	87,75±0,080^{b}	0,522±0,005^{cd}	13,76±0,962^{b}	15,18±5,57^{d}
	p<0.0001	p<0.0001	p=0.0002	p=0.0053
	LSD=5.864	LSD=0.064	LSD=1.6135	LSD=10.465

Table 5 - Textural properties of fresh and rehydrated eggplant samples.

Samples	Hardness (N)	Springiness	Cohesiveness	Gumminess (N)	Chewiness (N)
Fresh	148,8±2,73^{a}	0,670±0,026	0,577±0,005^{e}	85,90±2,28^{a}	57,17±0,160^{a}
RCD (40°C)	50,78±8,23^{b}	1,877±1,428	0,708±0,023^{dc}	36,00±4,29^{b}	32,12±2,56^{b}
RCD (50°C)	47,27±3,88^{b}	0,816±0,003	0,693±0,019^{d}	32,81±3,47^{b}	27,49±3,56^{b}
RCD (60°C)	44,65±7,71^{b}	1,895±1,465	0,705±0,017^{dc}	31,48±6,02^{b}	27,19±4,28^{b}
RICD (40°C)	52,59±3,67^{b}	1,331±0,792	0,754±0,043^{ab}	39,22±0,814^{b}	32,39±2,13^{b}
RICD (50°C)	47,77±9,51^{b}	0,878±0,084	0,686±0,010^{d}	32,55±5,77^{b}	28,77±7,84^{b}
RICD (60°C)	42,08±8,68^{b}	0,889±0,013	0,747±0,016^{abc}	31,21±7,27^{b}	27,69±5,71^{b}
RSD	41,63±7,04^{b}	0,881±0,033	0,774±0,009^{a}	32,26±5,10^{b}	28,48±3,56^{b}
	P<0.0001	p=0.6138	p=0.0003	p<0.0001	p=0.0015
	LSD=15.879	LSD=0.048	LSD=11.07	LSD=9.9053	

RCD: Rehydrated Convective drying.

Table 6 - Colour parameters of eggplant samples.

Samples	L*	a*	b*	Chroma	Hue angle	R (a/b)	ΔE
Fresh	61,64±1,23^{a}	3,74±0,02	19,55±0,648^{a}	19,91±0,636^{a}	79,27±0,197^{a}	0,190±0,004^{b}	-
CD (40°C)	50,10±0,108^{b}	6,97±1,72	17,96±0,616^{ab}	19,91±0,308^{a}	69,69±4,98^{b}	0,378±0,107^{a}	12,53±2,12
CD (50°C)	47,52±2,52^{bc}	7,20±0,101	16,98±0,062^{bc}	18,45±0,015^{ab}	66,95±0,372^{b}	0,426±0,008^{a}	14,84±1,37
CD (60°C)	47,85±2,28^{bc}	6,09±0,318	15,45±0,847^{c}	16,61±0,670^{c}	68,48±2,16^{b}	0,395±0,044^{a}	14,72±1,34
ICD (40°C)	47,81±0,986^{bc}	5,67±1,02	15,31±0,554^{c}	16,34±0,872^{c}	69,77±2,62^{b}	0,369±0,052^{a}	14,26±0,522
ICD (50°C)	47,91±1,64^{bc}	6,48±0,621	17,87±1,37^{ab}	19,01±1,50^{ab}	70,05±0,325^{b}	0,363±0,006^{a}	13,93±2,40
ICD (60°C)	50,17±1,00^{b}	5,30±0,477	17,04±1,25^{bc}	17,86±1,05^{bc}	72,58±2,66^{b}	0,315±0,051^{a}	12,10±0,055
SD	44,77±4,01^{c}	5,85±1,03	16,79±0,268^{bc}	17,80±0,082^{bc}	70,79±3,41^{b}	0,349±0,067^{a}	17,27±5,16
	p=0.001	p=0.0591	p=0.0126	p=0.0121	p=0.0343	p=0.0491	p=0.496
	LSD=4.7391		LSD=1.8826	LSD=1.832	LSD=6.0647	LSD=0.1246	

ness, cohesiveness, gumminess and chewiness values of samples ($p<0.05$). However it was determined that there was not an important difference between the springiness values of eggplant samples ($p>0.05$).

Results obtained from research indicated that hardness, gumminess and chewiness values of rehydrated samples were smaller than those of fresh samples. VEGA-GALVEZ *et al.* (2008) studied with red pepper samples (*Capsicum annuum* L.) dried at four air inlet temperatures from 50° to 80°C and rehydrated in water at 30°C and found that firmness was significantly affected by the temperature used during drying.

Colour parameters of eggplant samples

In our project L*, a*, b* values of fresh and dried eggplant samples were measured during determining colour parameters of samples. Chroma, hue angle, R(a/b) and total colour difference (ΔE) values were calculated by using L*, a*, b* values of samples. Colour parameters of samples were showed in Table 6.

As a result of statistical examination, it found that drying method did not affect to a* and ΔE values of eggplant samples ($p>0.05$), however it was determined that there were significant difference between L*, b*, chroma, hue angle, R(a/b) values of samples ($p<0.05$). So it was concluded that drying process had a significant effect on colour parameters of fresh samples. ERTEKIN and YALDIZ (2004) also investigated the effect of drying air temperature on colour parameters of the eggplant samples and suggested that increasing drying air temperature decreased the colour lightness and raised the saturation.

From the results of the present work it was concluded that drying process increased a*, R(a/b) values of eggplant samples, while decreased L*, b*, chroma and hue angle values of eggplant samples. The biggest change in colour parameters was observed in samples dried with sun drying during the examination of colour parameters of fresh and dried eggplant samples. Therefore it was possible to determined that sun drying had a negative effect on quality properties of eggplant samples.

CONCLUSIONS

In our thesis project, the drying characteristics of the eggplant slices dried by sun drying, hot air convective drying (40°, 50°, 60°C) and infrared assisted convective drying (40°, 50°, 60°C) were studied. Air temperature in a convective dryer affected drying time of eggplant slices. Increasing drying air temperature decreased drying time and increased drying rate. Ascorbic acid value of eggplant samples dried by sun drying was quite high. However quality losses were observed in eggplant samples dried by sun drying. Convective drying at low temperature should be applied to maintain ascorbic acid content and quality of eggplant slices.

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PAPER

DEVELOPMENT OF AN ACTIVE BIODEGRADABLE FILM CONTAINING TOCOPHEROL AND AVOCADO PEEL EXTRACT

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ABSTRACT

Thermoplastic starch (TPS) films and poly(butylene adipate co-terephthalate) (PBAT) (60/40 m/m) containing TOCO-70 (tocopherol/soybean oil 70/30 m/m) and avocado peel extract (ExA) were produced using blown film extrusion. The formulations of the 5 films (FC/F1/F2/F3 and F4) were established through mixture design with constraints maintaining constant PBAT and TPS proportion, and varying the antioxidant concentrations. Adding antioxidants reduced the water vapour permeability (K^w) of the films, with formulation F2 presenting higher decrease in relation to FC, 77.8%. The presence of ExA improved the mechanical properties of the films. The production of the films was determined to be viable after they presented good processability in a pilot extruder, as well as mechanical properties appropriate to production and utilization in industry. The presence of ExA and TOCO 70 provided the films with antioxidant activity; their application as active packaging requires further studies.

- Keywords: active packaging, antioxidant, biodegradable polymer, poly (butylene adipate co-terephthalate), thermoplastic starch -

1. INTRODUCTION

The fundamental function of food packaging is to delay the deterioration of the product in all physical, chemical, microbiological, sensory and nutritional aspects, ensuring the quality of the product until it is consumed (RESTUCCIA *et al.*, 2010). The oxidation of food sensitive substances form hydroperoxides that decompose into aldehydes, ketones, alcohols, acids, esters or hydrocarbons, altering the color, texture, smell and taste, as well as reducing the shelf life (MCCLEMENTS and DECKER, 2000). Active packaging interacts with the food to increase its shelf life, and materials containing antioxidants are a promising alternatives used to develop new active packaging (BYUN *et al.*, 2010; PEREIRA DE ABREU *et al.*, 2012; PEREIRA *et al.*, 2013).

Tocopherol is a liposoluble antioxidant that is widely applied in food as a primary antioxidant. Tocopherol is highly stable when applied in films of polyethylene and polypropylene, even under severe extrusion conditions. (AL-MALAIKA *et al.*, 1999; AL-MALAIKA *et al*, 2001). Recent studies regarding films elaborated from biodegradable polymers with tocopherol have generated positive results concerning antioxidant migration when applied to food (BYUN *et al.*, 2010; GRACIANO-VERDUGO *et al.*, 2010; LÓPEZ *et al.*, 2011; HWANG *et al.*, 2013; MARTINS *et al.*, 2012).

Several active principles originating from plants have been applied as anti-oxidants (DI-CASTILHO *et al.*, 2011; PEREIRA DE ABREU *et al.*, 2012; RUBILAR *et al.*, 2013), such as the avocado; the avocado is an oleaginous fruit native of Tropical America (Mexico) that is rich in phenol derivatives. Only 69% of this fruit are edible, the remaining is constituted of stone and skin. The use of the skin to extract antioxidants may add value to the residue and minimize environmental issues.

(WANG *et al.*, 2010) studied the antioxidant capacity of peels and seeds in various avocado species and concluded that these residues also contain large quantities of phenol compounds, among other antioxidants. RODRÍGUEZ-CARPENA *et al.* (2011) used avocado peel and seed extracts to inhibit lipid oxidation in meat products.

Active packaging can be manufactured using ecofriendly materials, such as biodegradable polymers. Starch has been used as an alternative to produce biodegradable films, but the materials present a low mechanical strength and are hygroscopic (MALI *et al*, 2005; REN *et al.*, 2009; SCAPIM, 2009). The conversion of starch into thermoplastic starch (TPS) involves heating and shearing with a plasticizer agent, such as glycerol.

The materials produced from blends of thermoplastic starch and other biodegradable polymers may be used to develop low-cost biodegradable packaging with better mechanical and barrier properties (BRANDELERO *et al.*, 2012; OLIVATO *et al.*, 2011; REN *et al.*, 2009; SCAPIM, 2009). Poly(butylene adipate co-terephthalate) (PBAT) is a biodegradable polyester with mechanical properties similar to the those of polyethylene films with resistance to fat and humidity and temperature variations (BASF, 2013). Previous studies have studied biodegradable materials produced using blends of thermoplastic starch and PBAT (BRANDELERO *et al.*, 2012; OLIVATO *et al.*, 2011; REN *et al.*, 2009; SCAPIM, 2009). The objective of this study was to characterize and assess the mechanical and structural properties of biodegradable films composed of thermoplastic starch, PBAT and antioxidants (tocopherol and bark extract avocado) seeking better interaction among these compounds in order to suggest further studies on the application of these films in active packaging.

2. MATERIALS AND METHODS

2.1. Materials

The film production utilized native cassava starch (Indemil Com. Ind. Ltda, Brasil), glycerol (Synth P.A, Brasil), poly(butylene adipate co-terephthalate) (Ecoflex®, BASF Chemical Company, Brasil) and TOCO-70 (tocopherols solution/soybean oils 70/30 m/m) (Danisco, Brasil). The avocado fruits (Persea americana Mill) were utilized at an intermediate maturity stage as moderately tender and green peel derived from São Paulo state after being purchased in Maringá PR.

2.2. Production and characterization of avocado peel extract

The peels were separated from fruits, cut into 2-cm squares, dried in a greenhouse at 60°C for 24 h, ground in an analytical mil and sieved. The extracts were prepared according to SANTOS *et al.* (2011) with some modifications. The previously dried and ground peels were soaked in ethanol at 95% v/v (proportion 1/10 m/v) with stirring for 4 h. The solution was vacuum filtered and concentrated via rotary evaporation at 40°C. The dry extracts (ExA) were stored in amber flasks at -18°C, under nitrogen atmosphere (N_2) until the analyses.

The compounds in ExA were characterized and quantified using Ultra-High Performance Liquid Chromatography coupled to Electrospray Ionization Mass Spectrometry in negative ion mode (UPLC-ESI(-)-MS) (Acquity UPLC-TQD, Waters, EUA). Approximately 10.0 mg of dry ExA and 1.0 mg of standard in HPLC grade methanol were injected (3 μL) into UPLC-ESI(-)-MS equipped with an Acquity column UPLC BEH C^{18} (2,1 x 50 mm) with 1.7 μm particles at 30°C. A gradient elution at 0.20 mL/min with solvent A (formic acid at 0.1%, in Milli-Q water) and B (methanol) was used with the following

method: from 95% of A and 5% of B to 100% B in 8.00 min, and the system was stabilized at 10.00 min. The spectra were acquired under the following conditions: capillary and cone stress of -3000 V and -30 V, respectively, with a source temperature of 150°C and a temperature of desolvation of 350°C.

2.3. Preparation of the biodegradable films

We produced 5 different types of biodegradable films, where the film Control (FC) was used as a reference and was composed of starch/glycerol/PBAT the proportions 42/18/40 (w/w/w). In the other films concentrations TOCO-70 and ExA were determined using a mixture design with constraints (Table 1), were proportion of starch/glycerol/PBAT was maintained at 42/18/40 (m/m/m). The results were analyzed using the STATISTICA 7.0 software (Statsoft, EUA).

The films were processed in the Technology Laboratory of Food Science and Technology Department of State University of Londrina (UEL - Brazil). The antioxidant compounds were mixed with glycerol. Initially, the pellets were produced in a pilot twin screw extruder (BGM, model D-20, Brazil) equipped with screws measuring 20 mm in diameter and 680 mm in length. The speed of the screws was set at 100 rpm, and the temperature profile in all five heating zones was 90/120/120/120/120°C. The film was produced in a pilot single-screw extruder (BGM, model EL-25, Brazil) equipped with a screw measuring 250 mm in diameter. The speed was maintained at 35 rpm, and the temperature profile in all four heating zones and in the matrix for balloon formation was 90/120/120/130/130°C.

2.4. Biodegradable Films

2.4.1. Thickness (δ) *and density* (ρ)

The thickness (δ) of the films was determined using a digital micrometer (0.001 mm resolution, Mitutoyo, Japan). Twelve random points on each film formulation were assessed. For the density (ρ), the average masses of 10 square samples (25 mm x 25 mm) of film were calculated after being conditioned in desiccators with anhydrous calcium chloride for 20 days.

2.4.2. Water vapour permeability (K^W)

The films were conditioned at 64±2% RH and 25±2°C for 72 h. The film samples were fixed to a circular opening of a permeation cell (area of 60 mm2) with silicone grease. The interior of the cell was filled with magnesium chloride saturated solution (33% RH) and was stored at 25±2°C in a desiccator that contained sodium nitrate saturated solution (64% RH) to maintain a 31% RH gradient across the film. The sample were weighed every 3 h during 72 h of testing time. The changes in the weight of the cell or mass gain (m) were plotted as a function of time (t). The slope of each line was calculated using linear regression, and the water vapour permeation ratio (K^wR) was obtained using Equation (1):

$$K^wR = (m/t).(1/A)$$

where m/t is the angular coefficient of the curve and A is the sample permeation area.

The K^w was calculated as Equation (2):

$$K^w = K^wR.st/sp(RH_1-RH_2)$$

where st is the mean sample thickness (m), sp is the water vapour saturation pressure at the assay temperature (Pa), RH1 is the relative humidity of the desiccator and RH2 is the relative humidity in the interior of the permeation cell. The tests were conducted in triplicate.

Table 1 - Formulation of biodegradable films according to mixture design and the constraints.

Film formulation	Films component (m/m/m)		
	Starch + Glycerol + PBAT	ExA	TOCO-70
	x_1	x_2	x_3
FC	1.000	-	-
F1	0.994	0.006	-
F2	0.992	-	0.008
F3	0.986	0.006	0.008
F4	0.993	0.003	0.004

° $x_1 + x_2 + x_3 = 1$

2.4.3. Sorption isotherms

The sorption isotherms of the films were determined according to the methodology described by SCAPIM (2009) using the following relative humidities at 25°C: 11.3, 33, 43.2, 52.9, 64.5, 75.3, 84.3 and 90.2%.

The isotherms were modeled according to the Guggenheim-Anderson-de Boer (GAB) model (Equation 3) using the Quasi-Newton method in Statistica 7.0.

$$X_w = \left(\frac{m_0.C.K.a}{[(1-K.a_w).(1-K.a+C.K.a_w]}\right)$$

where X_w (g of water/g of dry matter) is the relative humidity of balance, m_0 is the water content in the monolayer (g of water/g of solids), a_w is the water activity, and C and K are constants from the GAB model that represent the sorption heat in the first layer and sorption heat of the multilayer.

2.4.4. Determination of solubility (β) and diffusion (D^w) coefficients

The method used to calculate the solubility coefficient (β) was proposed by LAROTONDA *et al.* (2005) using the first order derivative of the GAB. This model is correlated with the water activity and water vapor pressure (P_s) at 25°C according to Equation 4.

$$\beta = \frac{C k m_o}{p_s}\left[\frac{1}{(1-ka_w)(1-ka_w+Cka_w)} - \frac{a_w}{[(1-ka_w)(1-ka_w+Cka_w)]^2}[-k(1-ka_w+Cka_w)+(1-ka_w)(-k+Ck)]\right]$$

where β(g/g.Pa)is the solubility coefficient, C, K, and m_0 are the GAB model parameters and Ps (Pa) is the water vapor pressure at 25°C. The a_w was the average of the relative humidity gradient used in K^w(g/m.Pa.day) (item 2.4.2).

The coefficient of water vapor diffusion (D^w) was calculated according to Equation 5 using the values of β and K^w, as determined in section 2.4.2, where ρ is the film density:

$$D^w = K^w/\rho.\beta\,\frac{PVA}{\rho.\beta}$$

2.4.5. Mechanical properties

The mechanical properties (ultimate tensile strength and elongation at rupture) were assessed according to ASTM D 882-91 (ASTM, 1996) using a texturometer (Stable Micro Systems, model TA.TX2i, England) with a 30-mm distance between the clutches and a traction speed of 500 mm/min. The samples were 80 mm long and 6 mm wide; they were conditioned under 64.5% RH at 25°C for seven days before the analyses. For each formulation, 10 repetitions were performed.

2.4.6 Microstructures analysis

The analyses were conducted using scanning electron microscope (Shimadzu, model SS-550N, Japan) based on the methodology descripted by SCAPIM (2009). The samples were immersed in liquid nitrogen and fractured with the aid of tweezers before being conditioned in a desiccator with calcium chloride to remove any humidity for three weeks. The samples were subsequently overlaid with two golden covers through a metallizer (Shimadzu IC-50 Ion Coater, Japan). After preparation, the samples were visualized through electronic scanning microscope to have its fracture area surface analyzed.

2.4.7. Antioxidant capacity of avocado peel extract and films

The analyses were carried out according to the method described by SERPEN *et al.*, (2012). Sample of 10.0 mg of avocado peel extract and each film formulation was reacted with 5 mL of 2.2-diphenyl-1- picrilhidrazil (DPPH•) in ethanol/water (50/50, v/v) over 1, 2, 3 and 4 hours under magnetic stirring and protection from light. Afterward, the blend was centrifuged for 1 min at 300-400 rpm (8 G) and the absorbance of the supernatant was measured through a spectrophotometer (ThermoScientific, model Genesys 10 UV, USA) at 525 nm. Methanolic solutions of (±)-6-hydroxy-2,5,7,8-tetramethylcromano-2-carboxylic acid (Trolox) in different concentrations were used to obtain the calibration curve for 5 mL (y = 29.448x + 5.4989, R^2 > 0.99) with the oxidant capacity expressed in μmol of Trolox equivalents (ET)/g for each film formulation.

3. RESULTS AND DISCUSSION

3.1. Antioxidant compounds and antioxidant capacity of avocado peel extract.

The following antioxidant compounds were identified and measured in the avocado peel extract: citric acid (133±10 μg/100 g extract), catechin hydrate (82±3 μg/100 g extract), malic acid (75±2 μg/100 g extract), epicatechin (62±3 μg/100 g extract) and tartaric acid (11.8±0.1 μg/100 g extract). Prior studies have revealed positive results for active packaging developed using plant extracts with compositions similar to ExA (DICASTILHO *et al.*, 2011; LÓPEZ *et al.*, 2011; PEREIRA DE ABREU *et al.*, 2012).

The antioxidant capacities of ExA and TOCO-70 were 188±8 mmol ET/g o and 162±8mmol ET/g, respectively. WANG *et al.* (2010) assessed different avocado species and concluded that both the seed and peel contain copious quantities of phenolic compounds and high antioxidant capacities. The authors found variations from 38 to 189.8 μmol of ET/g in peels from different species; the peel contributes 38% of the antioxidant capacity of the entire fruit. Therefore, the extraction concentrated the relevant compounds, presenting an antioxidant capacity more than a thousand times higher than that reported by (WANG *et al.*, 2010).

3.2. Films characterization

3.2.1. Sorption isotherms

The films all exhibited practically constant water sorption until 60% UR, and above 60%

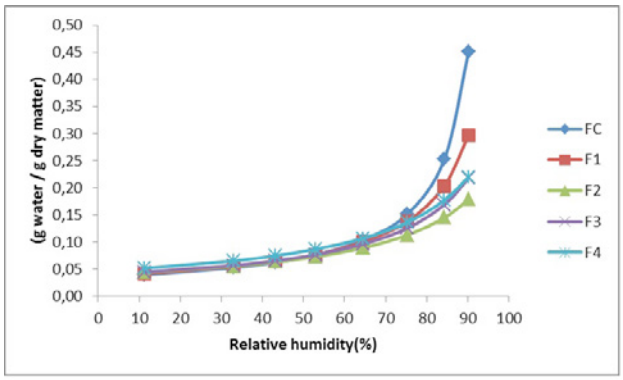


Fig. 1 - Water sorption isotherms of biodegradable films at 25 °C.

an increase in sorption occurred (Fig. 1) due to the hydrophilic nature of the starch. According to TALJA *et al.* (2008), for relative humidities above 60%, a replacement of starch-starch and starch-glycerol interactions with starch-water and water-glycerol interactions may occur, justifying the increase in water sorption from the humidity.

Adding TOCO-70 and ExA decreased the hydrophilicity of the films under UR above 75% while formulations F2, F3, F4 and F1 presented reductions of 34, 21, 11 and 10%, respectively, concerning water sorption in the control film (FC). The formulation containing only TOCO-70 (F2) presented the lowest water sorption in high relative humidities most likely due to the compatibility between the tocopherol and the soybean oil present in the compound with the starch and PBAT blend (BRANDELERO *et al.*, 2012).

Table 2 - Parameters for the GAB equation at 25°C for the biodegradable films.

Parameters for GAB model	Films formulations				
	FC	F1	F2	F3	F4
m_0	0.0348	0.0370	0.0394	0.0399	0.0465
C	168.52	159.57	150.49	163.361	139.76
K	1.02	0.97	0.86	0.91	0.87
R^2	0.97	0.99	0.89	0.94	0.96

The GAB model was satisfactorily adjusted to the experimental isothermal data, and the determination coefficients (R^2) varied from 0.89 to 0.99 (Table 2). The constant values of sorption for the multilayer (K) are correlated with the isothermal behavior in relative humidity above 65% (Fig. 1); specifically, the higher the water sorption for the films in that area are, the higher the K values are.

3.2.2. Water vapour permeability (K^W), the coefficient of solubility (β), the coefficient of diffusion (Dw), the thickness (δ) and the density (ρ)

The films containing antioxidants presented values for the water vapour permeability *(K^W)*, the coefficient of solubility (β) and the coefficient of diffusion (Dw) lower than for the control film (FC) (Table 3) due to the hydrophobic nature of ExA and TOCO-70. Film F2 contained only TOCO-70 and presented the lowest hydrophobicity, with a decrease of 77.8% in relation to the control formulation (CF). Considering that the hydrophilicity of the cassava starch is a major obstacle for its application in packages, the addition of antioxidants may also contribute to improve this characteristic (AVÉROUS and BOQUILLON, 2004; BRANDELERO *et al.*, 2011; OLIVATO *et al.*, 2011; REN *et al.*, 2009).

According to the model generated using the mixture design (R^2=0.83) that was correlated with the K^W in accordance with the film components (Fig. 2), the films containing only one type of antioxidant present a reduced K^W that has proven to be more effective than the blends. This behavior may be verified using the positive interaction model $+5.x_2.x_3$; specifically, when the film contains both antioxidants (x_2 = avocado extract; x_3 = TOCO-70), an increase in K^w occurs.

BRANDELERO *et al.* (2012) verified that the presence of soybean oil in the ATP/PBAT blends facilitated an interaction between the carboxyl group of the fatty acids and the PBAT, as well as film compaction promoted by the starch, which explains why formulation F2 presented the lowest β e D^w coefficients. Both ExA and TOCO-70 acted as compatibilizers, helping polymers interact and enabling the formation of a more compact structure that gives the molecules less

Table 3 - *Water vapour permeability (K^W)*, thickness (δ), density (ρ), coefficient of solubility (β) and coefficient of diffusion (D^w) for the biodegradable films.

Formulation	K^W (x10^{-6}) (g/m.Pa.day)	Δ (μm)	ρ (g/cm³)	β (x10^{-5}) (g/g.Pa)	D^w (x10^6) (m²/day)
FC	14.51±0.47	196±37	1.278	4.50	0.25
F1	6.75±0.46	149±19	1.475	4.12	0.11
F2	3.21±0.52	146±13	1.165	3.24	0.08
F3	6.03±0.81	130±17	1.279	3.69	0.13
F4	7.53±0.98	161±17	1.153	3.95	0.17

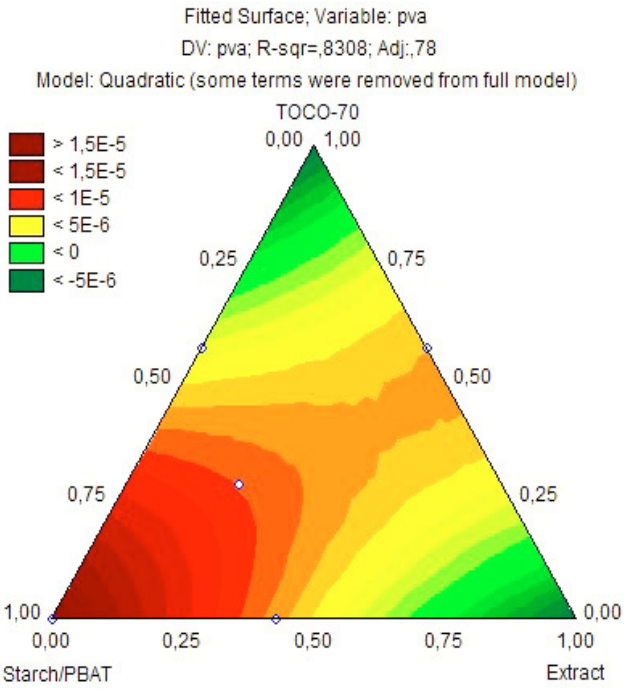


Fig. 2 - Permeability of water vapour of films according to starch/PBAT, ExA and TOCO-70 contents.

space to move, as shown by the coefficient of diffusion. These additives also provide lower hydrophilicity, as the coefficient of solubility indicates. Possibly, such improvement in the interaction of polymers promoted by the presence of antioxidants contributed to the lower thickness of the films since all of the formulations with an antioxidant presented reduced thickness compared with the control formulation, FC, with higher

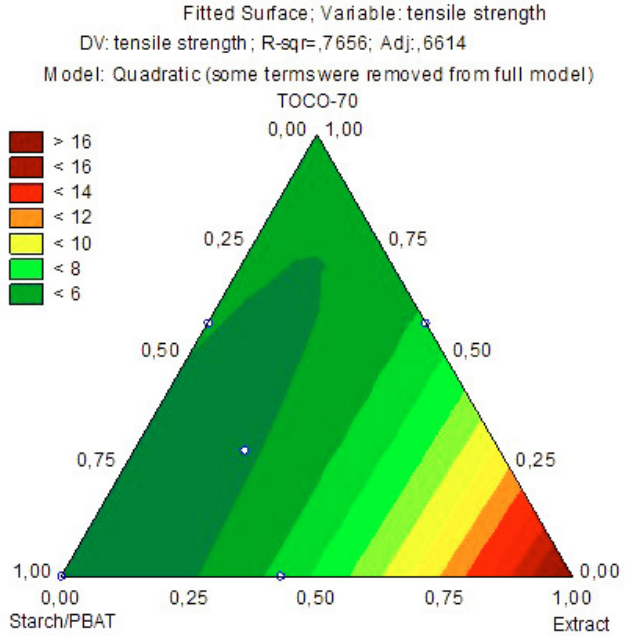


Fig. 3 - Tensile strength of the films according to Starch/PBAT, ExA and TOCO-70 contents.

mean value, 196±37μm, and F3 with the lower mean value, 130 ±17μm.

3.2.3. Mechanical properties

According to the model generated using the mixture design (R^2=0.77) to correlate the mechanical properties with the film components, the avocado extract (ExA) had a larger effect than TOCO-70 concerning the tensile strength (Fig. 3) and the elongation of the films (Fig. 4). According to OLIVATO *et al.* (2012), both citric and malic acids promote reticulation (*crosslinking*) between thermoplastic starch

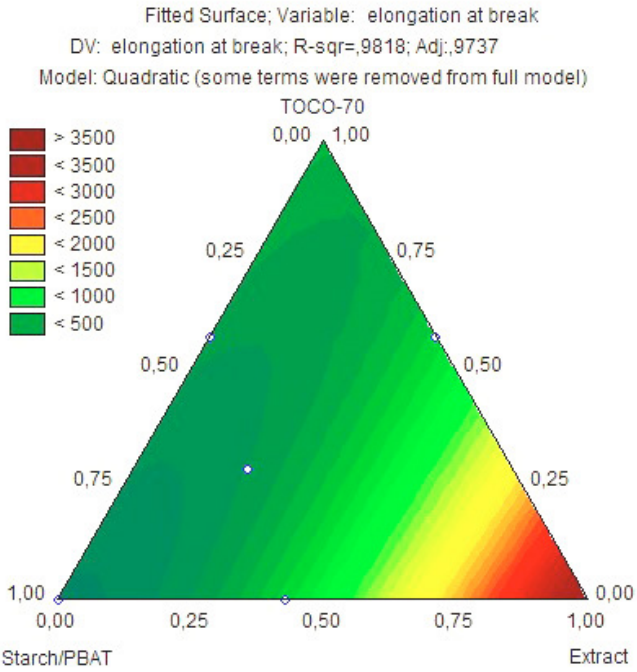


Fig. 4 - Film elongation at break according to Starch/PBAT, ExA and TOCO-70.

and PBAT, making the films resistant toward traction. ExA contains both acids in its composition, improving the mechanical properties of the films.

3.2.4. Antioxidant capacity of biodegradable films

According to the model generated while using the mixture design (R^2=0.98) and correlating the antioxidant capacity with the film components, the effect of the avocado peel extract (ExA) on the antioxidant capacity of the films was higher than that of tocopherol (TOCO-70) (Fig. 5).

The antioxidant capacity of the ExA extract and the TOCO-70 solution are similar, however, the films containing ExA presented a higher antioxidant capacity compared to the films containing TOCO-70, most likely due to the higher thermal stability of the ExA.

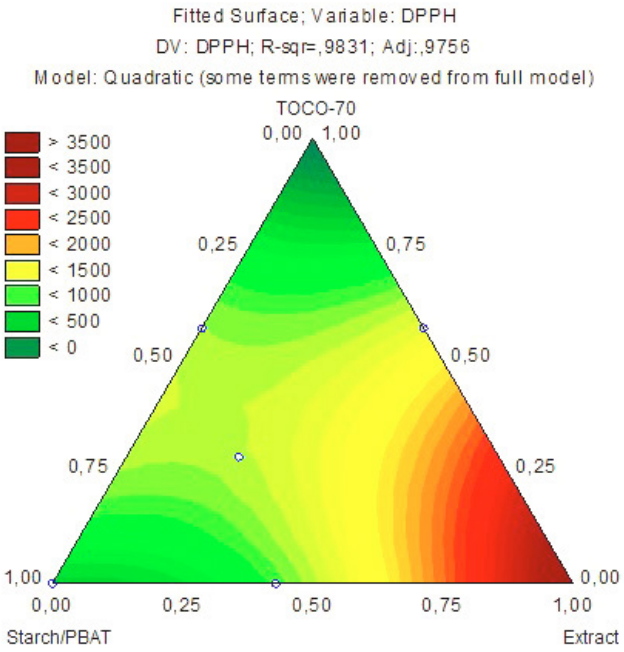


Fig. 5 - Antioxidant capacity of the films according to Starch/PBAT, ExA and TOCO-70 contents.

3.2.5. Desirability function

As observed in the analyses of K^w the presence of TOCO-70 provided better result when compared with formulation FC, with 77.8% decrease. However, by assessing the contour surfaces of the mechanical properties and the antioxidant capacity, it is possible to observe the occurrence of a tendency to have these characteristics improved when ExA is more present than TOCO-70. The desirability function indicated that the formulation that maximizes the ultimate tensile strength, elongation at rupture, and the antioxidant capacity while minimizes the permeability to water vapor (K^w) is a film whose composition should consist of 98.95% starch + PBAT + glycerol, 0.556% EXA, and 0.494% TOCO-70. After these results, further studies are going carry out with the same composition defined by Desirability function.

3.2.6 Microstructure

In the surface and film fracture micrographs neither pores, non-gelatinized granular neither starch nor phase separation were detected, thereby indicating good interactions between the components of the films for all formulations.

4. CONCLUSIONS

The production of biodegradable films composed of thermoplastic starch, PBAT and antioxidants (tocopherol and avocado peel extract) is viable since the films presented good processability in a pilot extruder, as well as the appropriate mechanical properties for industrial production and applications. The presence of ExA provided the films with antioxidant activity enabling their application as active packaging. It is required to conduct further studies to test the application of this blend of polymers and antioxidants in biodegradable and active packaging for products rich in lipids, such as hamburguers, nuggets, peanuts and soy, to verify the occurrence of antioxidant action of the package produced using such film on the product stored. Still, we suggest the development of a film with composition established through the desirability function to carry out the application tests.

5. ACKNOWLEDGMENTS

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PAPER

CHARACTERISTICS AND OXIDATIVE STABILITY OF BREAD FORTIFIED WITH ENCAPSULATED SHRIMP OIL

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ABSTRACT

Characteristics and oxidative stability of bread fortified with micro-encapsulated shrimp oil (MSO) were determined. The addition of MSO could improve the loaf volume of bread. Chewiness, gumminess and resilience of resulting bread were decreased. Bread crust and crumb showed higher redness and yellowness when MSO was incorporated ($P<0.05$). Microstructure study revealed that MSO remained intact with bread crumbs. The addition of MSO up to 3% had no adverse effect on bread quality and sensory acceptability. Oxidation took place in bread fortified with 5% MSO to a higher extent, compared with those with 1 or 3% MSO. Therefore, the bread could be fortified with MSO up to 3%.

- Keywords: shrimp oil, encapsulation, bread quality, lipid oxidation -

INTRODUCTION

Hepatopancreas, a byproduct generated from the manufacturing of hepatopancreas-free whole shrimp, is the excellent source of lipids with high polyunsaturated fatty acids (PUFA) (37.42 g/100g oil) and carotenoids (2.02 mg/g oil). Shrimp oil from hepatopancreas contained linoleic acid as the most abundant fatty acid, followed by oleic acid. Additionally, shrimp oil also contained PUFA including eicosapentaenoic acid (2.15 g/100 g oil) and docosahexaenoic acid (6.20 g/100 g oil) (TAKEUNGWONGTRAKUL *et al.*, 2012), (TAKEUNGWONGTRAKUL *et al.*, 2014a). The recommended daily intakes for linoleic acid, linolenic acid and long-chain n-3 PUFA are 4.4-20 g/d; 1.35-2.2 g/d and 0.16-1.6 g/d, respectively (MEYER *et al.*, 2003). Nevertheless, oil from hepatopancreas is very susceptible to oxidation, leading to undesirable off-odor (TAKEUNGWONGTRAKUL *et al.*, 2012). Rancidity is the major drawback for application of shrimp oil. Encapsulation of oil under the appropriate condition can be a promising means to extend its shelf-life. Encapsulation has appeared as a key technology in delaying or inhibiting oxidation and masking undesirable odor and flavor in the final product. The process involves the conversion of the oil into a free flowing powder, which can be easily handled and used for food fortification. Encapsulation can be defined as a process, in which tiny droplets, namely core, are surrounded by wall materials (GALLARDO *et al.*, 2013). Proteins and carbohydrates are frequently used as matrices for micro-encapsulation of lipophilic compounds by spray drying (GHARSALLAOUI *et al.*, 2007). TAKEUNGWONGTRAKUL *et al.* (2014b) reported that the use of whey protein and Na-caseinate in combination with glucose syrup as the wall materials could improve encapsulation efficiency of micro-encapsulated shrimp oil more effectively than protein alone and protein in combination with gum arabic or maltodextrin. Amongst several encapsulation techniques, spray-drying is the most common micro-encapsulation technology used in food industry due to its low cost, continuous production, ease of industrialization and available equipment (GHARSALLAOUI *et al.*, 2007).

The world's food market is currently focused on foods that provide nutritive values and health benefits to consumers. Functional foods are rapidly expanding and draw the great attention (EZHILARASI *et al.*, 2014). Fortification of highly nutritive ingredients such as polyunsaturated fatty acid rich oil, etc. is gaining the interest for food industry. The incorporation of micro-encapsulated oil into foods enables the development of new functional foods with minimal impact on the organoleptic properties of the food products (EZHILARASI *et al.*, 2014). Wall materials surrounding oil droplets can act as the shield, preventing the oil from oxidation. BORNEO *et al.* (2007) fortified micro-encapsulated n-3 fatty acids in cream-filled sandwich cookies without any adverse effect on sensory properties.

Bread has become popular, especially for the new generation (CLEARY *et al.*, 2007). The fortification of shrimp oil rich in PUFA and astaxanthin in the encapsulated form could increase the nutritive value of bread. As a consequence, the consumers can obtain the active nutrients with the health benefit from the bread. Nevertheless, no information regarding the fortification of micro-encapsulated shrimp oil in bread has been reported. The objective of this study was to investigate the effects of micro-encapsulated shrimp oil fortification on the characteristics and sensory property of bread.

MATERIALS AND METHODS

Chemicals

Ethylenediamine tetraacetic acid (EDTA) was obtained from Merck (Darmstadt, Germany). Tannic acid (99.5% purity) was purchased from Sigma (St. Louis. MO, USA). Essential oil (100% purity) from lemon was obtained from Botanicessence (Bangkok, Thailand). Sodium caseinate was procured from Vicchi enterprise Co., Ltd. (Bangkok, Thailand). Whey protein concentrate was obtained from I.P.S. International Co., Ltd. (Bangkok, Thailand). Glucose syrup (Dextrose equivalent; 40-43) was purchased from Charoenworrakit Co., Ltd. (Samut Prakan, Thailand). Wheat flour, sugar, salt, shortening, milk powder and yeast were procured from a local market in Hat Yai, Songkhla, Thailand.

Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during February and March, 2014. Pooled hepatopancreas (3-5 kg) was placed in a polyethylene bag. To maintain the quality of hepatopancreas during transportation, the bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18°C until use, but the storage time was no longer than 1 month. Prior to oil extraction, hepatopancreas was thawed using running water (25°C) and ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

Extraction of oils from hepatopancreas

Oil was extracted from hepatopancreas following the method of TAKEUNGWONGTRAKUL *et al.* (2014). The prepared hepatopancreas (20 g) was homogenized with 90 mL of cold solvent mixtures (isopropanol: hexane, 50: 50, v/v) (4°C) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4°C. The extract was filtered using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, England). The residue was extracted with cold solvent mixtures for another two times. The hexane fraction was pooled and repeatedly washed with an equal quantity of 1% NaCl in order to separate the phases and remove traces of polar solvents. Hexane fraction (approximately 135 mL) was then added with 2-5 g anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No. 4 filter paper. The solvent was evaporated at 40°C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. The obtained oil with the yield of 19.04% (w/w) was used for micro-encapsulation.

Preparation of shrimp oil-in-water emulsion

Aqueous stock mixed solution of whey protein concentrate, sodium caseinate and glucose syrup at a ratio of 1: 1: 2 (w/w/w) in deionized water was prepared. The mixture was stirred overnight using a magnetic stirrer at room temperature (28°-30°C) to obtain the homogenous wall material solution as per the method of TAKEUNGWONGTRAKUL and BENJAKUL (2014b). Shrimp oil was added into the solution at a core/wall material ratio of 1:4 (v/v). The mixtures were homogenized at a speed of 10,000 rpm for 3 min using a homogenizer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia). The obtained coarse emulsions were then passed through a Microfluidics homogeniser (Model HC-5000, Microfluidizer, Newton, MA, USA) at a pressure level of 4,000 psi for four passes. Emulsions were added with lemon essential oil + tannic acid + EDTA. Prior to the incorporation, lemon essential oil (200 ppm) was dissolved in shrimp oil, whereas EDTA (50 ppm) and tannic acid (100 ppm) were dissolved in aqueous stock solution.

Preparation of micro-encapsulated shrimp oil (MSO)

The prepared emulsion was subjected to drying using a laboratory scale spray-dryer (LabPlant Ltd., LabPlant SD-06A, Huddersfield, UK) equipped with a 0.5 mm diameter nozzle. The emulsion was fed into the main chamber (215 mm diameter × 500 mm long) through a peristaltic pump. Feed flow rate was 8.08 mL/min; drying air flow rate was 4.3 m/s and compressor air pressure was 40.61 psi. Air inlet temperature was 180°±2°C. The outlet temperature was controlled at 90°±2°C. The obtained powder referred to as micro-encapsulated shrimp oil (MSO) was collected in the amber bottle and capped tightly. MSO was determined for the total oil content using the mixture of chloroform and methanol as per the method of SHAHIDI and WANASUNDARA (1995). MSO contained 18±1.34% shrimp oil and had 1.06±0.05% moisture content.

Fortification of MSO in bread

Bread was prepared with the following formulation: wheat flour (500 g), sugar (20 g), salt (8 g), shortening (20 g), milk powder (25 g) and yeast (7 g). Flour and other ingredients were mixed and kneaded uniformly, in which water (300 mL) was added during kneading. Thereafter, MSO was directly added to dough at different levels (0, 1, 3 and 5%, w/w). The dough was kneaded for another 10 min. Dough (150 g) was then subjected to bulk fermentation for approximately 1 h at 30°C and 75% relative humidity, followed by scaling, intermediate proving, moulding and second proving (for about 1-1.25 h). Finally, baking was carried out at 220°C for 20 min in baking oven (YXD-20, Guandzhou Xinnanfang electro-thermal equipment Co., Ltd., Guandzhou, China). After baking, bread samples were removed from mold and allowed to cool at room temperature. Bread incorporated with 5% (w/w) spray dried empty capsules (without the addition of shrimp oil) was used as the control bread. The bread samples were subjected to analyses.

Characterization of bread fortified with MSO

Loaf volume

The volume of bread was determined by sesame seed displacement method after the loaves were cooled to room temperature for approximately 2 h (JIAMYANGYUEN *et al.*, 2005).

Texture profile analysis

Texture profile analysis (TPA) was performed using a TA-XT2 texture analyzer (Stable Micro Systems, Godalming, Surrey, UK) with a cylindrical aluminum probe (35 mm diameter). The samples were sliced (each 2.0 cm thickness) and placed on the instrument's base. The tests were performed with two compression cycles. Texture measurements were performed ten times for each sample and mean values were reported. Hardness, springiness,

cohesiveness, gumminess, chewiness and resilience were calculated from the force-time-curves generated for each sample (GÖKMEN *et al.*, 2011). Hardness is expressed as the maximum force for the first compression, which relates to the strength of the samples under penetration. Gumminess is defined as the force required to disintegrate a semi - solid food before it is ready for swallowing. Springiness is a measure of how much the samples structure is broken down by the initial penetration and is calculated as the ratio of the time from the start of the second area up to the second probe reversal over the time between the start of the first area and the first probe reversal. Cohesiveness is a measure of the degree of difficulty in breaking down the internal structure of sample. Resilience reflects the redeformation capacity of sample tissues after penetration (CHANG *et al.*, 2012). Chewiness is related to the time required for masticating a bread piece prior to swallow, and the low chewing value means easy break of the bread in the mouth (KRUPA-KOZAK *et al.*, 2012).

Color measurement

The color of crust and crumb samples were determined using a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system, including L^*, a^*, b^* and ΔE*, representing lightness, redness/greenness, yellowness/blueness and total difference of color respectively. ΔE* was also calculated using the following equation:

$$\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$$

where ΔL^*, Δa^* and Δb^* are the differences between the color parameter of the samples and the color parameters of the white standard (L^* = 92.85, a^* = - 1.20, b^* = 0.46).

Scanning electron microscopy (SEM)

Bread morphology was evaluated by scanning electron microscopy (SEM). Fresh bread was sliced by a razor blade and mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 15 kV with magnification of 2000×.

Sensory evaluation

Sensory evaluation was performed by 30 untrained panelists with ages ranging from 20 to 35 years, who were familiar with the consumption of bread. Panelists were asked to evaluate for crust color, crumb color, odor, texture, appearance and overall likeness of bread samples using a nine-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely, respectively. Panelists were asked to hand-feel the sample for texture. Freshly prepared bread was taken randomly for sensory evaluation at day 0 and 3. Each bread loaf was cut in half and slices were subsequently cut to a thickness of 2 cm. Bread was served in a closed odorless plastic container at room temperature. The samples were labeled with random three-digit codes. The order of presentation of the samples was randomized according to "balance order and carry-over effects design" (CARR *et al.*, 1999).

Changes in volatile compounds in bread during storage

Bread was placed in polyethylene bag and sealed. Packaged bread samples with different treatments were stored at room temperature (28°-30°C). The samples were taken at day 0, 1, 2 and 3 for analyses. Volatile compounds in bread was analysed by headspace GC-MS using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of GÖKMEN *et al.* (2011) with a slight modification.

Extraction of volatile compounds by SPME fiber

To extract volatile compounds, 1 gram of bread slice was placed in a headspace 20 mL -vial (Agilent Technologies, Palo Alto, CA, USA). The vial was tightly closed by means of a capper. A carboxen–polydimethylsiloxane solid phase micro-extraction fiber (50/30 μm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was used to adsorb the volatile lipid oxidation compounds released from the sample. The fiber was inserted into the vial and equilibrated at 40 °C for 30 min prior to GC-MS analysis.

GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography (GC) coupled with HP 5972 mass-selective detector equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on a HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m±0.25 mm ID, with film thickness of 0.25 μm). The GC oven temperature program was: 35°C for 3 min, followed by an increase of 3°C/min to 70°C, then an increase of 10°C/min to 200°C, and finally an increase of 15°C/min to a final temperature of 250°C and holding for 10 min. Helium was employed as a carrier gas with a constant

flow of 1 mL/min. The injector was operated in the splitless mode and its temperature was set at 270°C. Transfer line temperature was maintained at 260°C. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250°C. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 μA, and the electron multiplier voltage at 500 V.

Analyses of volatile compounds

Identification of the compounds was done by consulting ChemStation Library Search (Wiley 275.L). Identification of compounds was performed, based on the retention time and mass spectra in comparison with those of standards from ChemStation Library Search (Wiley 275.L). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

Statistical analysis

All experiments were run in triplicate. All analyses were conducted in five replications. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (STEEL and TORRIE, 1960).

RESULTS AND DISCUSSION

Characteristics of bread fortified with MSO

Loaf volume

Loaf volume of bread fortified with MSO at different levels is shown in Table 1. Incorporation of MSO (1-5%) resulted in the increase in loaf volume ($P< 0.05$). However, similar loaf volume was obtained, regardless of amount of MSO added ($P > 0.05$). Loaf volume of bread incorporated with spray dried empty capsule (control bread) was not different from that of bread containing MSO at a level of 1% (w/w) ($P> 0.05$). Nevertheless, bread fortified with MSO at the levels of 3 and 5% (w/w) had higher loaf volume than the control bread ($P< 0.05$). Spray dried empty capsule, including whey protein concentrate, sodium caseinate and glucose syrup, could increase loaf volume of bread to some degree. Those proteins as well as glucose syrup might strengthen the loaf structure via interaction with wheat gluten, in which the bread matrix could hold gas more efficiently. Whey proteins demonstrated the ability to increase the loaf volume of the bread (NUNES *et al.*, 2009). Gluten proteins of wheat flour create unique visco-elastic properties of dough, which allow dough to expand due to the formation of carbon dioxide during fermentation and retain most of this gas inside the dough texture (WEHRLE *et al.*, 1997). GÖKMEN *et al.* (2011) and EZHILARASI *et al.* (2014) reported that increasing amount of micro-encapsulated oil or active compounds decreased loaf volume of bread. Encapsulated substances could decrease the concentration of gluten in the formulation and lower the retention of gases during the baking process. Therefore, bread fortified with MSO with the range of 3-5% (w/w) had

Table 1 - Loaf volume and textural at properties of breads incorporated with MSO at different levels at day 0 and 3 of storage.

Storage time (day)	MSO (% w/w)	Loaf volume (mL)	Hardness (g)	Springiness (mm)	Cohesiveness (g.mm)	Gumminess	Chewiness	Resilience
0	Control	296.06±9.36bA	1362.22±80.23aB	0.90±0.02aA	0.59±0.02aA	759.25±50.08aB	739.70±38.73aB	0.22±0.01bA
	0 %	269.25±10.84cA	1020.99±54.21bB	0.91±0.03aA	0.61±0.02aA	670.14±57.86abB	712.87±51.31aB	0.24±0.01aA
	1 %	315.22±10.47abA	1022.73±46.70bB	0.91±0.01aA	0.59±0.02aA	622.09±46.49bB	554.13±38.71bB	0.22±0.01bA
	3 %	317.67±9.13aA	938.58±65.57bB	0.90±0.02aA	0.60±0.03aA	610.30±42.41bB	480.51±37.57bcB	0.22±0.01bA
	5 %	317.47±8.86aA	927.85±69.48bB	0.90±0.03aA	0.60±0.01aA	503.91±42.57cA	466.17±47.67cB	0.22±0.02bA
3	Control	290.83±8.96bA	2454.22±84.41aA	0.89±0.02aA	0.50±0.02aB	1146.05±54.24aA	1034.57±56.96aA	0.19±0.01aB
	0 %	267.22±11.34cA	2158.52±49.27bA	0.90±0.01aA	0.52±0.02aB	1097.85±39.01aA	979.92±70.76aA	0.19±0.02aB
	1 %	312.17±8.86aA	1532.61±58.13cA	0.90±0.02aA	0.52±0.03aB	838.77±44.37bA	759.42±64.20bA	0.19±0.01aB
	3 %	313.89±10.37aA	1257.53±77.38dA	0.90±0.01aA	0.53±0.01aB	720.33±40.92cA	622.02±59.62cA	0.19±0.01aB
	5 %	313.33±5.77aA	1225.42±34.58dA	0.90±0.01aA	0.52±0.02aB	672.64±54.40cA	617.68±60.45cA	0.17±0.01aB

Control = Added with 5% (w/w) spray dried empty capsule without the addition of shrimp oil.
Data are expressed as mean±SD (n=3).
Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).
Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

Table 2 - Color of breads incorporated with MSO at different levels at day 0 and 3 of storage.

Storage time (day)	MSO (% w/w)	Crust color				Crumb color			
		*L**	*a**	*b**	ΔE*	*L**	*a**	*b**	ΔE*
0	Control	56.16±3.89bA	15.20±2.37bA	36.30±1.36abA	54.28±4.32bA	71.08±1.12aA	0.13±0.02dA	11.67±0.89dA	25.17±1.47eA
	0 %	69.13±2.98aA	9.76±1.12cA	32.12±1.07cA	41.91±1.56cdA	67.66±1.22bB	0.12±0.02dA	11.89±0.60dA	28.35±1.19dA
	1 %	67.88±3.46aA	12.28±1.08bA	35.38±1.06bA	45.42±2.65cA	64.53±1.56cB	2.15±0.41cA	13.72±1.42cA	32.08±1.91cA
	3 %	64.95±2.55aA	13.75±1.17bA	35.73±0.43bA	47.77±1.77cA	63.90±2.07cdB	7.19±0.68bA	18.92±0.91bA	35.89±0.76bA
	5 %	47.27±1.99cA	20.08±0.52aA	38.67±1.28aA	63.47±1.56aA	60.77±1.48dB	11.45±0.51aB	22.59±0.61aB	41.46±0.83aA
3	Control	56.68±3.95bA	14.38±2.26bA	36.31±1.42aA	53.69±0.91bA	72.98±1.09aA	0.12±0.01dA	12.63±1.76dA	23.97±1.36dA
	0 %	67.58±2.16aA	9.67±0.99cA	32.69±0.92cA	42.77±1.88dA	71.99±1.33aA	0.11±0.04dA	12.20±0.76dA	24.61±1.12cdB
	1 %	66.72±2.20aA	11.59±2.17bcA	35.72±1.27bA	46.09±1.09cA	70.94±1.69aA	2.27±0.33cA	13.90±1.28cA	26.55±1.19cB
	3 %	64.10±3.29aA	13.19±2.04bA	35.96±1.10bA	48.28±1.37cA	66.17±2.02bA	7.58±0.36bA	20.13±0.86bA	34.80±1.39bA
	5 %	46.36±0.57cA	20.44±0.69aA	38.54±0.97aA	64.32±.041aA	65.11±1.54bA	13.34±0.50aA	26.12±0.99aA	40.90±0.69aA

Control = Added with 5% (w/w) spray dried empty capsule without the addition of shrimp oil.
Data are expressed as mean±SD (n=3).
Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).
Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

the higher loaf volume, in comparison with the control bread.

After storage of 3 days at room temperature, in which mold was not detected, no difference in loaf volume was noticeable in comparison with that found at day 0 (P> 0.05). Thus, bread structure was not collapsed within 3 days of storage. It was noted that the addition of MSO had no influence on the shelf-life of bread.

Textural properties

Textural properties of bread samples containing MSO at various levels are presented in Table 1. The addition of MSO generally had the effects on the texture profile of bread. However, MSO had no effect on hardness (P> 0.05), irrespective of amount used. It was noted that, the control bread had higher hardness value than others (P< 0.05). The proteins in spray dried empty capsules with smaller size might be distributed more uniformly and strengthened bread structure more efficiently. For gumminess, the addition of MSO decreased the value. The decrease was more pronounced as the level of MSO increased (P< 0.05). It was found that bread incorporated with 5% MSO showed the lowest gumminess, compared with others (P< 0.05). The addition of MSO or spray dried empty capsule had no impact on springiness and cohesiveness (P> 0.05). Chewiness of bread decreased as the amount of MSO in bread increased (P< 0.05). Control bread showed similar chewiness to that without MSO (P> 0.05). For resilience, bread without MSO (0%) showed the higher value than others (P< 0.05).

After the storage at room temperature for 3 days, hardness, gumminess and chewiness increased, whilst the cohesiveness and resilience decreased (P< 0.05). Nevertheless, no changes in springiness were observed after the storage (P> 0.05). These results indicated that bread staling took place upon storage, probably due to retrogradation (HENNA-LU and NORZIAH, 2011). Therefore, MSO addition had the direct impact on textural property to different degrees, depending on the amount of MSO incorporated.

Color

Color of bread crust was affected by the amount of MSO added as shown in Table 2. The photographs of bread crust are shown in Fig. 1 A. Bread crust had the decrease in *L**-value, but the increases in *a**-, *b**- and ΔE*- values as the level of MSO increased (P< 0.05). Amongst all samples, that added with 5% MSO showed the lowest *L**-value but highest *a**-, *b**- and ΔE*- values (P< 0.05). It was found that bread incorporated with spray dried empty capsule (control) had the lower *L**- value than others, except for that added with 5% MSO. The color of bread crust is mostly attributed to non-enzymatic chemical reactions such as Maillard and caramelization reaction that produce colored compounds (formation of the golden yellow color) during bread baking (GÖKMEN *et al.*, 2011). Proteins in spray dried empty capsule could serve as the reactant, especially for browning reaction, especially at crust region. GÖKMEN *et al.* (2011) reported that the particles in the crust region were partially destroyed due to more severe thermal conditions during baking. Furthermore, the wall materials also contained some sugars, which more likely underwent caramelization at high temperature. This could contribute to the brown color of bread crust. PURLIS and SALVADORI (2009) reported that bread had high browning reaction rates, particularly when caramelization oc-



Fig. 1 - The photographs of breads incorporated with MSO at different levels. A) Bread crust, B) Bread crumb.

curred. Therefore, browning reaction had a pronounced influence on bread color, particularly during bread baking.

For bread fortified with MSO, the increases in redness (a^*- value) were more likely due to the orange/red color of MSO. Shrimp oil contained a high amount of astaxanthin (TAKEUNGWONGTRAKUL *et al.*, 2014). As a result, the bread crust turned to be more orange in color, when MSO was added, especially at higher levels. Thus, the color of bread crust might depend on both non-enzymatic browning reactions and astaxanthin partially released from MSO. However, the changes in color of crust were more likely caused by the releases of astaxanthin from MSO as evidenced by the more a^*-value (redness) in color of resulting bread. When comparing L^*-, a^*-, b^*- and ΔE^*- values of all bread crust, all bread samples had no change in color after 3 days of storage ($P > 0.05$). The result suggested that the pigments in MSO were stable after 3 days of storage as evidenced by the unchanged color of bread crust. It was presumed that wall material might protect the oxidation of astaxanthin to some degree during the storage.

The color of bread crumb was determined (Table 2). The levels of MSO incorporated in bread were coincidental with the color. The decrease in L^*- value and increases in a^*-, b^*- and ΔE^*- values of bread crumb were found as the level of MSO increased ($P < 0.05$). For color of bread crumb, crumb does not undergo Maillard reaction, but is affected by the ingredients in the formula (CONFORTI and DAVIS, 2006). Oils from shrimp hepatopancreas were reddish orange in color due to the presence of astaxanthin (TAKEUNGWONGTRAKUL *et al.*, 2014). Additionally, surface oil and oil released to the surface of MSO during bread making could also contribute to color of bread crumb. When MSO at a level of 5% was incorporated, bread crumb had the lowest L^*- value but highest a^*-, b^*- and ΔE^*- values than others ($P < 0.05$). For control bread (with only spray dried empty capsule), a^*- and b^*- values of crumb were not different from those of bread without MSO ($P > 0.05$). Spray dried empty capsule was visually white in color without red or yellow color. After 3 days of storage, the control bread had no change in L^*- value ($P > 0.05$), whilst other bread had the increase in L^*- value ($P < 0.05$). For a^*- and b^*- values, all bread crumb had no change in a^*-, b^*- and ΔE^*- values ($P > 0.05$). Nevertheless, crumb of bread incorporated with 5% MSO had the increases in a^*- and b^*- values. It was noted that those with 0 and 1% MSO had the decrease in ΔE^*- value after 3 days of storage ($P < 0.05$).

The photographs of bread crumb are shown in Fig. 1B. During storage, the oil might be released from the wall to some degree. As a result, oil with high content of astaxanthin could contribute to the increase in a^*- and b^*- values to some extent. This was obvious for bread fortified with 5% MSO. Therefore, the addition of MSO directly affected the color of both crust and crumb of bread.

Microstructure

SEM microphotographs of all bread crumbs incorporated with the different levels of MSO are shown in Fig. 2. In general, MSO was embedded in the crumb of bread, which was constructed by gluten network. These results were consistent with GÖKMEN *et al.* (2011) who incorporated nano-encapsulated flax seed oil into bread. Powders added to dough remained intact in the bread crumb. For the control bread, the bead of spray dried empty capsule was observed throughout the crumb (Fig. 2A). How-

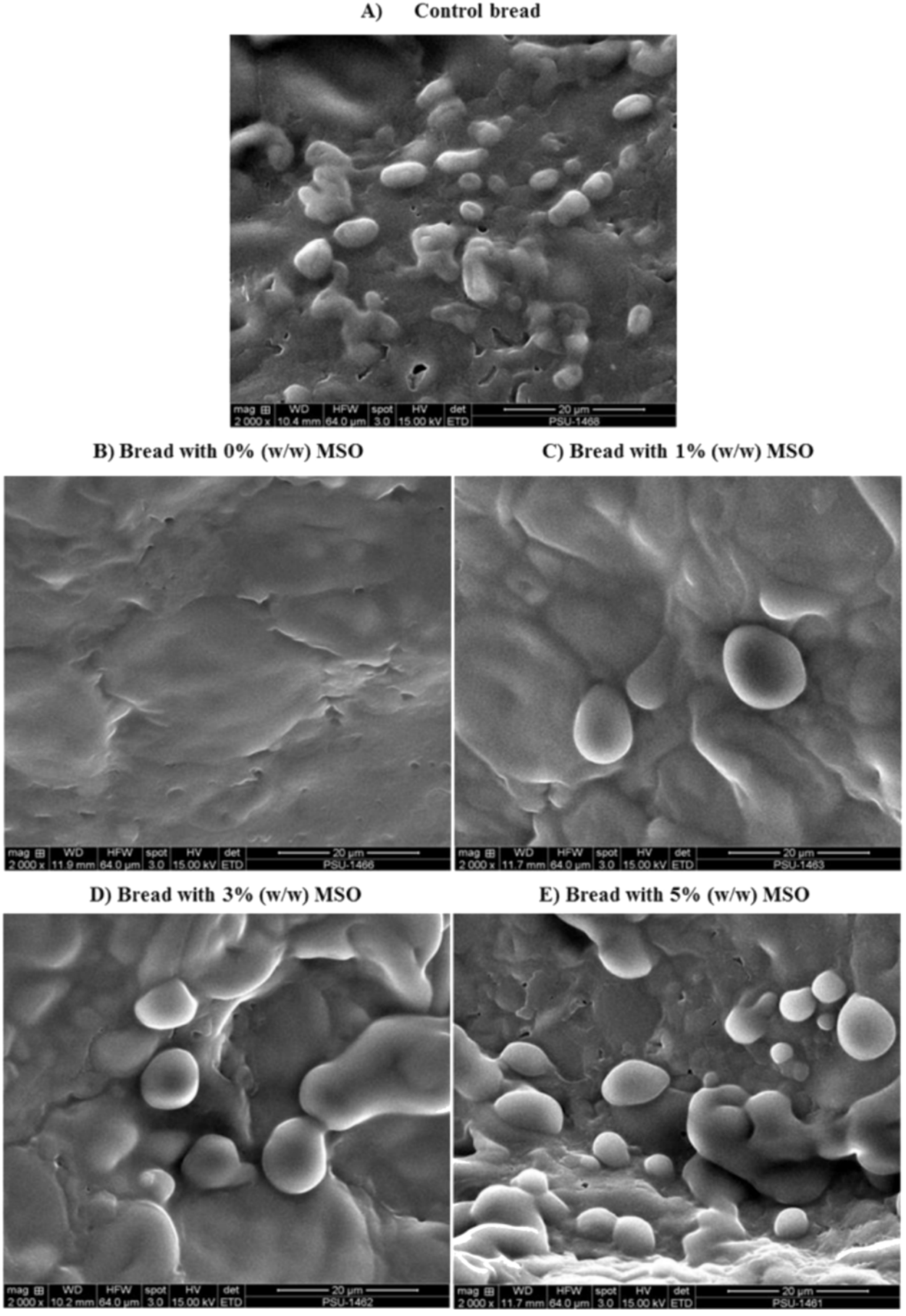


Fig. 2 - Surface morphology of breads incorporated with MSO at different levels. (Magnification: ×2000).

ever, there was no bead in the bread without MSO and spray dried empty capsule (Fig. 2B). It was clearly illustrated that the number of bead, representing MSO, increased as the level of MSO increased. In general, MSO were located uniformly in the crumb matrix. Those MSO could serve as the source of PUFA and astaxanthin rich shrimp oil.

Sensory property

Crust color, crumb color, texture, appearance, odor and overall likeness scores of all bread samples added with different amounts of MSO at day 0 and 3 of storage are shown in Table 3. There were no differences in all attributes amongst all bread samples (P> 0.05) at day 0 of storage, except for crumb color and overall likeness scores of bread incorporated with 5% MSO, which had the lower score (P< 0.05). The addition of 5% MSO to bread had negative effect on crumb color and overall likeness of bread. This was due to the marked increases in a^* and b^* values of bread crumb (Table 2). GÖKMEN *et al.* (2011) reported that the addition of micro-encapsulated n-3 fatty acids could increase functionality of bread. Shrimp oil is one of the important sources of n-3 fatty acids (TAKEUNGWONGTRAKUL *et al.*, 2012). Thus, MSO at 3% (w/w) could be added into bread to improve the nutritive values of bread without the negative effect on sensory property of bread.

After 3 days of storage, no differences in all attributes were observed amongst all bread samples (P> 0.05). Nevertheless, crumb color, odor and overall likeness of bread added with 5% MSO were lower than others (P< 0.05). Wall materials could protect the entrapped core by providing a physical barrier against environmental conditions (GALLARDO *et al.*, 2013). Food fortification is good way to induce the general population to consume components, such as n-3 fatty acids, and will add value to food product manufactured by the food industry (BORNEO *et al.*, 2007). However, the addition of 5% MSO might result in the increased free oil, especially at the surface of MSO. This led to more free oil, which was susceptible to oxidation. As a consequence, the lower score of odor likeness was found. Therefore, MSO must be incorporated at the appropriate level to avoid the undesirable attributes of bread.

Volatile compounds

Volatile compounds in bread samples added with MSO at different levels after 3 days of storage are displayed in Table 4. Volatile compounds in bread without MSO (days 0) were also determined. Lipid oxidation generates a number of products, including volatile compounds, which are the major contributors to the rancid off-flavors and off-odors in the food product (ROSS and SMITH, 2006).

In general, all compounds present in bread without MSO at day 0 were lower in abundance than those found after 3 days of storage. Nevertheless, 3-methyl-1-butanol, 2-pentyl-furan, heptenal and 2-octen-1-al were also lower in abundance after storage, plausibly due to the volatilization or decomposition. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of lipids (VARLET *et al.*, 2006). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used to successfully follow lipid oxidation in a number of foods (SHAHIDI and PEGG 1994). After the storage, the bread without MSO contained new volatile compounds including decanal. The highest amount of lipid oxidation products such as 3-methyl-1-butanol, benzeneethanol, benzaldehyde and 2-methyl-1-propanol

Table 3 - Likeness score of breads incorporated with MSO at different levels at day 0 and 3 of storage.

Storage time (day)	MSO (% w/w)	Crust color	Crumb color	Texture	Appearance	Odor	Overall likeness
0	Control	7.04±0.78aA	6.90±1.08aA	6.47±1.17aA	7.00±1.04aA	7.13±0.94aA	7.03±1.00aA
	0 %	6.92 ±0.65aA	6.75±1.16aA	6.70±0.76aA	6.79±0.92aA	7.00±0.94aA	6.90±0.82aA
	1 %	7.04±0.81aA	6.90±0.90aA	7.03±1.27aA	7.07±1.16aA	6.93±0.82aA	6.83±0.95aA
	3 %	7.31±0.89aA	7.10±0.90aA	7.00±0.98aA	6.93±1.00aA	6.97±0.88aA	6.80±0.77aA
	5 %	6.67±0.88aA	5.54±1.12bA	6.97±1.37aA	6.63±1.36aA	6.47±0.95aA	5.63±1.12bA
3	Control	6.94±0.89aA	6.90±1.24aA	6.04±0.91aA	7.00±0.89aA	6.52±1.23aA	6.44±0.93aA
	0 %	6.72±0.96aA	6.74±0.96aA	6.38±1.10aA	7.00±1.21aA	6.58±1.31aA	6.36±0.91aA
	1 %	6.83±1.33aA	6.87±1.41aA	6.74±0.90aA	7.06±1.21aA	6.56±1.01aA	6.28±0.96aA
	3 %	7.00±0.80aA	7.00±0.91aA	6.70±0.95aA	6.74±1.21aA	5.96±0.75aA	6.14±1.09aA
	5 %	6.66±1.14aA	5.43±1.07bA	6.60±1.22aA	6.39±1.36aA	5.16±0.94bA	5.36±0.99bA

Control = Added with 5% (w/w) spray dried empty capsule without the addition of shrimp oil.
Data are expressed as mean±SD (n=3).
Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).
Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

Table 4 - Volatile compounds in breads incorporated with MSO at different levels after storage of 3 days at 30°C.

Compounds	Peak area (Abundance) × 10^6				
	Control	0% MSO	1% MSO	3% MSO	5% MSO
2-methyl-1-propanol	ND	424 (309)	414	388	264
3-methyl- 1-butanol	1350	1383 (1406)	1293	1264	1255
2-pentyl-furan	ND	ND (128)	ND	ND	ND
3-hydroxy-2-butanone	180	ND	ND	ND	168
Heptenal	ND	ND (74)	ND	ND	118
1-hexanol	414	302 (253)	402	426	441
Nonanal	296	207 (122)	303	366	414
2-octen-1-al	ND	ND (33)	ND	ND	ND
1-octen-3-ol	102	89 (63)	133	176	226
Decanal	41	40 (ND)	26	78	83
Benzaldehyde	347	450 (354)	285	274	477
1-octanol	29	35 (33)	48	52	53
2-octen-1-ol	ND	ND	21	17	20
2-furanmethanol	ND	ND	ND	152	220
(E)-6-nonen-1-ol	89	ND	102	119	ND
(Z)- 3-decen-1-ol	58	ND	59	67	135
Benzenemethanol	22	ND	ND	ND	44
Benzeneethanol	795	716 (683)	728	856	1437
3-hydroxy-2-methyl-4H-pyran-4-one	ND	ND	ND	ND	50
Dihydro-5-pentyl-2(3H)-furanone	ND	19 (15)	23	30	20

ND: non-detectable
* Value in the parenthesis represents the abundance of compound in sample at 0 day.
Control: Added with 5% (w/w) spray dried empty capsule without the addition of shrimp oil.

was found in bread without MSO after 3 days. Among all the aldehydic compounds, benzaldehyde was found to be the major aldehyde in bread without MSO (0% MSO), followed by nonanal and decanal, respectively. Additionally, volatile ketones (dihydro-5-pentyl-2(3H)-furanone) and volatile alcohols (3-methyl-1-butanol, benzeneethanol, 2-methyl-1-propanol, 1-hexanol, 1-octen-3-ol and 1-octanol) were also found in bread without MSO. MAIRE *et al.* (2013) reported that flour appeared relatively rich in alcohols (3-methyl-1-butanol, 1-pentanol, 1-hexanol and 1-octen-3-ol). These compounds were also reported by HANSEN and HANSEN (1994) in flour with different millings, formed by either lipid oxidation or microorganism metabolism. 1-Octen-3-ol is a volatile generated from linoleic acid oxidation in the presence of singlet oxygen (LEE and MIN, 2010). This indicated that lipid oxidation took place in bread without MSO. MAIRE *et al.* (2013) reported that dough preparation seemed to be the crucial step toward lipid oxidation due to enzymes (lipoxygenase and lipase) as well as air inside the dough texture. Additionally, auto-oxidation could occur during baking by high temperatures, which promote an accelerated oxidation of ingredients in bread without MSO.

After storage, the formation of most volatile compounds in bread increased as the amount of MSO increased from none to 5%. Those compounds included 1-hexanol, nonanal, 1-octen-3-ol, 1-octanol, (Z)-3-decen-1-ol and benzeneethanol. However, 3-methyl-1-butanol decreased with increasing MSO. This could be due to volatilization. Benzaldehyde of sample added with 1 and 3% MSO showed the lower abundance than that without MSO. Benzaldehyde formed might bind with protein matrix of bread. Heptenal was also found in the sample added with 5% MSO. Higher abundance in nonanal and benzaldehyde was observed in the sample incorporated with 5% MSO, compared with others after 3 days of storage. Volatile compounds in bread added with 5% MSO were generally highest in abundance, compared with those added with others. TAKEUNGWONGTRAKUL *et al.* (2014) reported that surface oil content of MSO prepared using whey protein concentrate: sodium caseinate: glucose syrup (1: 1: 2, w/w/w) as wall materials was 2.48 % (w/w). Bread with higher level of MSO incorporated showed the higher amount of surface oil, which was more susceptible to oxidation. As a result, oxidation took place to a higher extent. Abundance of volatile compounds in all bread correlated well with the sensory property as shown in Table 3, in which bread added with 5% MSO had the lowest score of odor likeness. For bread added with 1% or 3% MSO and control bread, similar amount of volatile compounds was noticeable and no difference in sensory property of bread was observed ($P > 0.05$) (Table 3). Therefore, 3% MSO was the appropriate level to fortify in bread without negative effect on quality and acceptability.

CONCLUSION

MSO prepared using whey protein concentrate, sodium caseinate and glucose syrup (1: 1: 2, w/w/w) as wall materials could be fortified in bread product. Fortification of MSO had impact on the bread loaf volume, color and sensory properties. MSO up to 3% could be incorporated into bread to improve the nutritive value without affecting its sensorial properties. The fortified bread was quite stable up to 3 days of storage, in which no marked changes in color occurred and only slight increases in volatiles were obtained.

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PAPER

RELATIONSHIP BETWEEN SENSORY RESULTS AND COMPLIANCE SCORES IN GRATED PARMIGIANO-REGGIANO CHEESE

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ABSTRACT

The regulations for Protected Designation of Origin require a certification body to verify compliance with the provisions of the product specification. The grated Parmigiano-Reggiano is evaluated with a scorecard containing 21 quantitative descriptors and 4 qualitative evaluations of compliance with the regulations. To better understand the relationship between sensory compliance and quantitative descriptors we have tested 24 samples of grated Parmigiano-Reggiano. Correlations and Partial Least Squares gave us a better understanding of compliance evaluation. The work allowed us to define the most important descriptors in relationship with compliance and showed that it is possible to predict compliance values using descriptor values.

- Keywords: sensory analysis, Parmigiano-Reggiano, grated cheese, compliance score -

INTRODUCTION

Worldwide the generic term used to describe products attributed to a defined region is products with a Geographic Indication (GI) as for example Darjeeling tea in India, Tequila spirit in Mexico or Napa Valley wines in USA. Within the European Union (EU), Protected Geographical Indication (PGI) is applied to products whose characteristics originate mainly, but not exclusively, from a particular region (e.g. Scottish farmed salmon, Bayonne ham, Turron de Alicante), whereas Protected Designation of Origin (PDO) have proven characteristics resulting solely from the region of production (i.e. Parma Ham, Isigny butter, Valencia rice). Cheese is the most important PDO product.

The European regulations on quality schemes for agricultural products and foodstuffs number 2081 of 1992, number 506 of 2006 and the latest number 1151 of 2012 provide for product specifications to include organoleptic characteristics. In the EU database DOOR, the so called "single document" for each PGI and PDO product gives general information about the characteristics of the product and it's processing (EUROPEAN COMMISSION DOOR, 2013). These regulations require a certification body to verify compliance with the provisions of the product specification (OFFICIAL JOURNAL OF THE EUROPEAN UNION, 2012). Since an organoleptic specification is included in the product description, compliance indicates agreement with the registered specification.

Testing for compliance with the registered organoleptic specification is different from sensory quality control. The latter is less demanding since production conditions are tightly controlled within a food processing plant and consequently food attributes are relatively stable.

GI is always connected to the artisanal process with the product being produced in many small scale plants with different conditions of production i.e. source of raw material, climatic condition, production equipment, operation of this equipment, packaging of the product, management of the product prior to distribution, etc. Such heterogeneity makes for problems in defining the characteristic in the official production standard, hence the official sensory definitions tend to be general and thus not precise.

Sensory properties define the distinctiveness of the product at the moment of consumption. For GIs many different sensory characteristics have to be measured in order to allow both control of the process and certification.

Within Europe, there is no unanimity in how sensory properties should be evaluated; in most cases there is expert evaluation that is not easily translated into sensory analysis. When a sensory panel is employed there are many different approaches to panel composition, the scorecard and to the presentation of the results of the data analysis.

The compliance evaluation of a GI has something in common with quality control judgments made by product experts on the conformity with pre-defined sensory characteristics. Compliance is an assessment of how "typical" the product is of the GI. This is different from quality as perceived by consumers. A product can be compliant with the specification ('typical' of the GI) but might be considered poor quality by consumers who do not know and appreciate the qualities of this GI. On the other hand a product could be found to be of good quality by consumers but not compliant with the specification of the GI.

Sensory evaluation of cheeses is also employed to evaluate compliance with pre-established sensory specifications. Thus it is used both in quality control (in cheese producing companies) and in scoring compliance (for GIs).

The most common system of organoleptic evaluation by a control body is that using the traditional method of forming an overall quality score by summing up the scores on number of characteristics (BODYFELT, 1988). Another system of quality scoring of some attributes (appearance, consistency, odour/flavour) is detailed by the norm ISO 22935-3/IDF 99-3 "Milk and milk products – Sensory analysis" part 3 (INTERNATIONAL STANDARD ORGANISATION 2009, KRAGGERUD *et al.*, 2012). A further method with some differences from the above is used for the Spanish PDO cheese Idiazabal which uses a scorecard with 8 parameters (4 appearance, 1 texture, 1 odour, 1 taste and 1 aftertaste) each with a compliance score and hence a total compliance score (PÉREZ-ELORTONDO, 2007). For this cheese compliance is determined with a decision tree. Yet another system of quality scoring for compliance is used by the Italian Parmigiano-Reggiano PDO cheese. The scorecard (GARAVALDI *et al.*, 2010) contains attributes for compliance as well as Quantitative Descriptive Attributes (QDA). A compliance score is derived for each of 4 properties (appearance, odour, taste and texture). The Italian Asiago PDO cheese is evaluated with a 6-attributes (colour, "holes", sweet, salty, sour, bitter) QDA scorecard with quality ranges (ZANNONI and MARANGON, 2011), i.e. should the intensity of the perceived attribute be out of the range specified for any one of the characteristics then the product is not compliant.

Availability of funds, technical support, the interests of producers and market requirements are all factors, which affect the method chosen to score compliance. There is no general agreement on how to tackle this most important problem; the choice of approach is currently determined by the specific requirements of each GI.

The only nation where there is uniformity in

sensory evaluation of GIs is France. The Institute for the Designation of Origin (INAO, 2008) has given guidance notes for the organization and operation of sensory panels for all PDO/PGI products. In practice for French Appellation d'Origine Protegée (AOP) PDO cheeses a common system of evaluation is a total compliance score (CANTAL, 2011) (SALERS, 2011) (PICODON, 2008) (ABONDANCE, 2010) (FOURME D'AMBERT, 2008) derived from the sum of compliance scores of 3 parameters (appearance, texture and taste).

From those examples, it is evident that there are four types of methods to evaluate the compliance of GI with their specifications. The most common is to give a total score indicating the deviance from pre-established sensory specification (CANTAL, 2011) (SALERS, 2011) (PICODON, 2008) (ABONDANCE, 2010) (FOURME D'AMBERT, 2008) as indicated by the ISO norm 22935-3. Another method employs a quality score for every parameter (PÉREZ-ELORTONDO *et al.*, 2007).

A less common method (ZANNONI and MARANGON, 2011) employs a QDA scorecard with quality (or compliance) ranges for every attribute. A fourth model (GARAVALDI *et al.*, 2010) employs a compliance score for visual, odour, texture and taste together with a QDA with 24 descriptors.

Parmigiano-Reggiano is one of the most popular Italian cheeses, with Protected Designation of Origin (PDO) from 1954 (from 1996 in EU). The most common end use of this cheese is grated over pasta. The increasing success in foreign markets and customer demand for convenience has led to an increasing proportion of the cheese being grated before being packaged in a modified atmosphere prior to distribution.

The grated cheese comes from Parmigiano-Reggiano PDO wheels, which are cut into large pieces, grated by a grating machine and then transferred by a conveyor belt to a packing machine. The process of grating/packaging lasts only few minutes. The quality of the final products depends not only on the quality of the original cheese, but also on the operation of the grating process (Fig. 1).

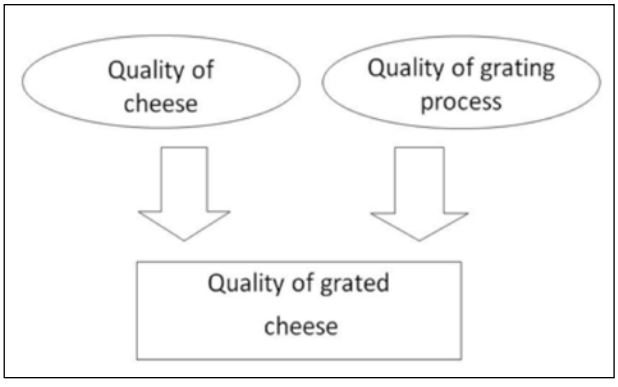


Fig. 1 - Determinants of grated Parmigiano-Reggiano quality.

For the product to be marketed as grated Parmigiano-Reggiano cheese, it must be certified by the official control body, Organismo Controllo Qualità Produzioni Regolamentate (OCQPR), for compliance with the Parmigiano-Reggiano regulations. These state that in the grated form the product must keep the characteristics of the original cheese (ZANNONI, 2007). The sensory analysis used by the certification body, for assessing the compliance of grated Parmigiano-Reggiano has been used since 2002. The scorecard for grated Parmigiano-Reggiano has been evaluated in a previous paper (ZANNONI and HUNTER, 2013).

Bearing in mind that there is no unanimity on how the compliance evaluation of GIs is carried out, this paper contributes to knowledge of the relationship between compliance and quantitative descriptors, using grated Parmigiano-Reggiano cheese as an example.

MATERIALS AND METHODS

Samples

The regulations for Parmigiano-Reggiano cheese state that the cheese can be grated only in a plant, located in the production area of the cheese, operating as prescribed by the regulations. Moreover the producer has to be authorized and regulated by the control body OCQPR. The minimum age of the product is 12 months but a maximum age is not defined.

Twenty four samples of grated Parmigiano-Reggiano, each from a different processor, were collected. Four hundred grams for each sample were collected in the production plant in four 100g bags under modified atmosphere.

Sample preparation

Each of 24 samples was divided in two parts (sub-samples of two 100g bags) for sensory analysis; the products were identified with letters A – X. Thus 48 sub-samples were evaluated by the panel. In each tasting session four sub-samples were evaluated. Order of presentation for each assessor was defined by sets of 4x4 Latin squares. Samples were refrigerated to between 2 and 8°C and their temperature was raised to 13°C temperature during the evening before tasting. The samples were served at room temperature raising the tasting temperature of the samples to approximately 16°C. Each panellist was served with 20 g sub-sub-samples on a plastic Petri dish.

Scorecard

For evaluating the sensory compliance of PDO

Table 1 - Correlations of each variable of each modality with the appropriate Compliance variable.

a) Variables of Colour modality correlated with Compliance - visual variable.

Variable	Mean	Corr	Prob
Colour Intensity	3.19	0.04	ns
Brown	0.07	-0.44	0.014
Lemon yellow	0.09	0.19	ns
Other colours	0.08	-0.45	0.013

b) Variables of Appearance modality correlated with Compliance - visual variable.

Variable	Mean	Corr	Prob
Particles size	3.29	-0.05	ns
Large grains	0.31	-0.84	<.001
Long threads	0.49	-0.47	0.009

c) Variables of Odour modality correlated with Compliance - odour variable.

Variable	Mean	Corr	Prob
Odour Intensity	3.56	0.06	ns
Rancid	0.53	-0.57	0.001
Rind	0.76	-0.39	0.028
Sour	0.23	-0.16	ns

d) Variables of Texture modality correlated with Compliance - texture variable.

Variable	Mean	Corr	Prob
Degree of Solubility	3.90	0.92	<.001
Dryness	0.83	-0.81	<.001
Rind particles	0.62	-0.72	<.001
Sandy	0.63	-0.61	0.001

e) Variables of Aroma/taste modality correlated with Compliance - aroma/taste variable.

Variable	Mean	Corr	Prob
Aroma/Taste Intensity	4.31	0.37	0.036
Salty	0.60	0.11	ns
Pungent	0.77	-0.04	ns
Sour	0.70	-0.07	ns
Rancid	0.89	-0.63	<.001
Rind	1.11	-0.66	<.001

Parmigiano-Reggiano grated cheese with the regulations, the designated control body Organismo Controllo Qualità Produzioni Regolamentate (OCQPR), uses a "mixed" scorecard, with both descriptive and compliance scores (ZANNONI and HUNTER, 2013). The quantitative descriptive part of the scorecard has 21 descriptors; a 1-7 scale for 5 attributes (colour intensity, odour intensity, aroma/taste intensity, particle size and degree of solubility). For the 16 descriptors connected to defects a 1-4 scale is used because it is more acceptable to the panellists. In addition the scorecard has an additional 4 qualitative evaluations of compliance for visual, odour, texture and aroma ("back of the nose" odour)/ taste using a 1-7 scale with 1 minimum and 7 maximum score.

Panel

The nine panellist were aged between 34 and 69 years and had from 5 to 18 years experience of sensory analysis of Parmigiano-Reggiano cheese.

Statistical data analysis

The univariate (one variable at a time) analysis of this data is fully described in Zannoni and Hunter (2013). The starting point for the analysis described in this paper is the 24 Sample means from this analysis.

The scorecard presents 4 modalities: Visual (Colour plus Appearance), Odour, Texture and Aroma/Taste with a compliance score. For each of these modalities the individual variables have been correlated with the relevant compliance score (Table 1a-e).

Note that for Colour (but not Appearance), Odour, Texture and Aroma/Taste there is an intensity measurement followed by some descriptors related to defects.

The next step in the analysis is to "predict" each of the four compliance scores from all the other sensory data. For each of the four Compliance Scores there are twenty one possible explanatory factors and yet only twenty four sample values. In such circumstances multiple regression analysis is known to be problematic. One solution to this problem is to use principal components regression (PCR). First the explanatory data is summarised by the principal components scores on the much smaller number of principal dimensions (typically 2, 3 or 4), which summarise the data, and these scores are then used in the regression instead of the initial data. The multiplier for each variable of the initial data can be obtained using the regression coefficients plus the loadings of the initial data on the relevant dimension. However, we have chosen to use Partial Least Squares (PLS) regression, which has many similarities with PCR. PLS (MARTENS and NAES, 1989) is an iterative technique and has been found to produce more effective prediction equations in most circumstances.

The number of (PLS) dimensions (1, 2, 3....) was determined by the computationally intensive technique of cross-validation. A value of Predictive Residual Error Sum of Squares (PRESS) was calculated by taking each unit of data in turn and forming a prediction equation from the remaining units of data using 1, 2, ... dimensions. Ostens (OSTENS, 1988) test of significance was used to judge how many PLS di-

Table 2a - Predicting each of the Compliance variables in turn from the total Sensory Data.

Variable	COMPLIANCE			
	VISUAL	ODOUR	TEXTURE	AROMA/TASTE
Mean	4.96	4.55	4.68	4.38
No of PLS dimensions	2	1	3	2
% Variance accounted for PRESS	76.7	28.4	91.4	73.6
Predictive Error Sum of Square	2.858	2.125	1.708	2.229
Standard error of prediction	0.345	0.298	0.267	0.305

mensions (Table 2a) were required. Once the number of dimensions were determined the predictive equations were found (Table 2b). The Genstat (VSN International) statistical package was used.

RESULTS

In previous work (ZANNONI and HUNTER, 2013) the scorecard was evaluated by fitting a mixed model to each attribute and by using Generalised Procrustes Analysis for each modality. The results showed good discrimination between samples and good agreement between assessors.

Correlations

The correlations of descriptors scores for each sample with the corresponding compliance score e.g. odour intensity, rancid, rind, sour correlated with odour compliance. Table 1 shows that 13 out of the 21 descriptors

Table 2b - Predictive equations for the Compliance variables from PLS, the predicted Compliance Scores are calculated using the equation: Predicted Score = Const + $Coeff^{1}$ * Colour Intensity (Colour) + ……+ $Coeff^{n}$* Descriptor n …+ $Coeff^{21}$* Rind (Aroma Taste) (n = 1….21).

n		VISUAL		ODOUR		TEXTURE		AR./TASTE	
		Const 4.58	%Y 76.7	Const 4.42	%Y 28.4	Const 3.58	%Y 91.4	Const 3.61	%Y 73.6
	Colour	Coeff	%X	Coeff	%X	Coeff	%X	Coeff	%X
1	**Colour Intensity**	0.021	0.9	0.012	1.3	-0.02	50.3	0.029	1.9
2	**Brown**	-0.024	15.4	-0.005	16.3	-0.022	22.3	-0.017	14.2
3	**Lemon yellow**	0.019	1.2	-0.002	0.0	-0.011	7.6	-0.014	8.1
4	**Other colours**	-0.013	11.5	-0.001	6.4	-0.002	19.4	-0.004	5.9
	Appearance								
5	**Particles size**	-0.066	10.3	0.013	14.7	-0.052	82.2	0.032	15.0
6	**Large grains**	-0.401	67.3	-0.025	20.8	-0.229	70.4	-0.117	21.6
7	**Long threads**	-0.213	44.0	-0.026	29.0	-0.134	37.1	-0.137	37.1
	Odour								
8	**Odour Intensity**	-0.010	5.3	0.002	1.4	-0.037	21.0	-0.014	1.7
9	**Rancid**	-0.044	14.6	-0.028	21.4	-0.055	18.0	-0.121	20.6
10	**Rind**	-0.142	51.9	-0.024	64.6	-0.171	60.3	-0.106	62.5
11	**Sour**	-0.039	0.4	-0.01	0.6	-0.030	10.7	-0.005	0.8
	Texture								
12	**Solubility**	0.184	53.2	0.046	61.2	0.391	84.3	0.226	76.6
13	**Dryness**	-0.101	43.0	-0.021	56.3	-0.248	83.2	-0.116	68.9
14	**Rind particles**	0.016	93.6	-0.035	56.7	0.004	99.6	0.010	99.1
15	**Sandy**	0.059	28.0	-0.017	45.8	-0.117	55.7	-0.096	50.9
	Aroma_Taste								
16	**Aroma/Taste Intensity**	0.059	9.7	0.013	6.5	0.114	26.6	0.058	15.6
17	**Salty**	0.021	5.6	0.002	0.6	0.047	24.5	0.019	2.0
18	**Pungent**	-0.002	3.2	-0.008	0.5	0.057	9.0	-0.006	2.9
19	**Sour**	-0.001	1.3	-0.008	0.3	0.037	3.5	-0.012	0.3
20	**Rancid**	-0.038	23.6	-0.032	35.4	-0.092	25.5	-0.146	35.0
21	**Rind**	0.016	93.6	-0.035	56.7	0.004	99.6	0.010	99.1

were significantly correlated with the relevant compliance variable. The only significant positive correlation is that of *Solubility.* Negative correlations were found, in descriptors (with a 4-points scale) considered defects: *Large grains, Long threads, Rancid Odour, Rind odour, Dry, Rind particles, Sandy, Rancid Aroma, Rind Aroma*. It is interesting to note that some of these descriptors *Large Grains, Long threads, Rind particles, Rind Aroma* are influenced by processing conditions. *Large grains* and *Long threads* are determined by the grating conditions i.e. type of grating surface, pressure applied. *Rind particles* and *Rind Aroma* are determined by the percent of rind in the cheese used for grating.

Partial Least Squares (PLS)

PLS has been used to predict each of the Compliance variables in turn from all the sensory data.

The percent variance explained for the PLS correlations showed very good results for Texture 91.4% (with 3 dimensions) and for Visual and Aroma/Taste (76.7 and 73.6 % respectively, both with 2 dimensions). The results for Texture probably occurred because this modality is very strongly positively correlated with *Solubility* and strongly negatively correlated with *Dryness*, *Rind particles* and *Sandy*. The rather disappointing results for Odour (28.4% of variance explained) showed the difficulties the panel had with this modality.

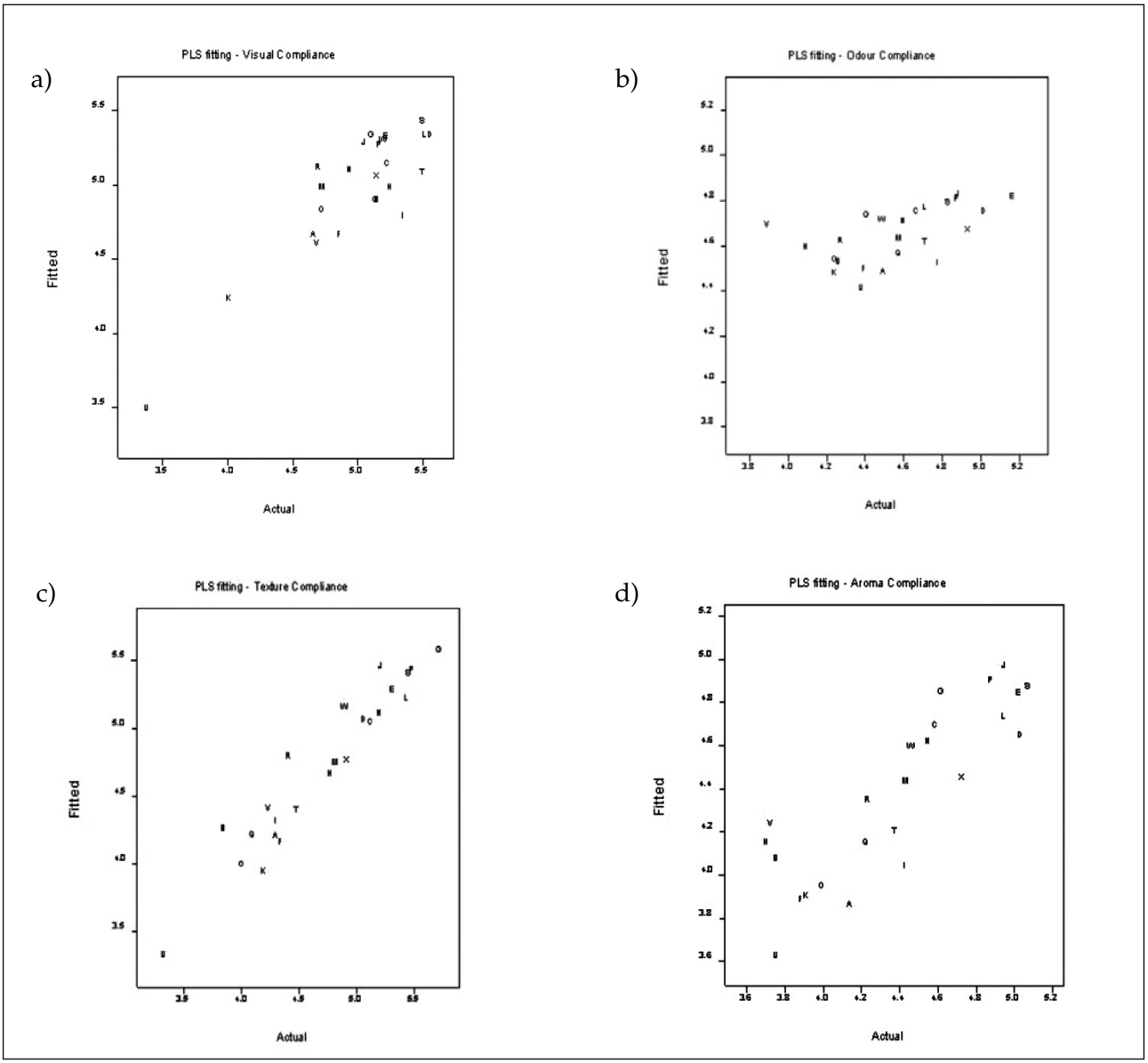


Fig. 2 - a,b,c,d : PLS: relationship between actual and predicted compliance data for each modality (visual, odour, texture, aroma). Letters represents the samples.

The equations for predicting each of the Compliance scores from all the individual descriptors allows the importance of every descriptor to be better judged. Correlations among the descriptors not directly related to a Compliance Score is logical because when we run a PCA with all the data the 4 compliances show their relationships being grouped together in an area opposed to that of descriptors considered defects which are grouped together even though they belong to different modalities. This makes sense because in cheese the presence of one defect always involves other defects. For instance if we find *Rind odour* this is an odour defect but also a texture one (i.e. *low Solubility*) and of aroma/taste (*Rind aroma* and often *Rancid*).

In predicting the ***visual compliance*** the most important predictors are *Rind aroma* and the presence of *Rind particles* in texture. Both are related to *Large grains* which is the visual descriptor correlated with the presence of rind in the grated cheese. Colour is of minimal importance in this prediction.

The most important predictor of ***odour compliance*** by far was *Rind odour* followed by other rind descriptors in other modalities.

In case of ***texture compliance*** we have the contribution of an important very large positive correlation (*Solubility*) and three substantial negative correlations (*Dryness, Rind particles, Sandy*) to the prediction.

The ***aroma/taste compliance*** is predicted mostly by the descriptor *Rind* in aroma/taste, texture and odour. This value seems to depend slightly more than the others on the quantity of cheese rind present in the samples.

The Fig. 2 shows relationships between actual and predicted compliance. It is evident that three of the four modalities are closely predicted.

CONCLUSIONS

In evaluating compliance of a PDO/PGI, it is clear that compliance cannot be separated from the descriptors. It is also true that compliance could be the results of the interaction of many sensory perceptions, not all of which are present in the scorecard. Nevertheless the scorecard has been refined by long experience in order to find the most important perceptions connected to the definition of the desired quality of the product.

PLS has allowed a better understanding of compliance evaluation of grated Parmigiano-Reggiano cheese and has confirmed the usefulness of the scorecard for official control.

Three important points come out from this work:

The most important descriptors in relationship with compliance were found to be concerned with the presence of rind in samples. The regulations allow a maximum of 18 % of rind by weight in grated cheese. The sensory control has been shown to be effective in finding rind in the product. An increase in the amount of rind causes a decrease in the sensory quality with clear disadvantages for consumers.

It is possible to satisfactorily predict compliance values using descriptor values with the exception of odour.

The compliances results expressed in a numeric manner are a practical way to show the sensory quality of this PDO cheese thus allowing its employment by the official certification body.

Another important finding of this work is the importance of descriptor *Solubility* ("positive" descriptor) in the quality assessment of a product that is normally spread over warm pasta.

Negative descriptors were, mainly descriptors connected to an excess of rind: *Large grains, Rind odour, Presence of Rind particles* and *Rind aroma.*

Extra cheese ring is readily available in plants for vacuum packed sliced Parmigiano-Reggiano because the ring flat parts of the wheel are removed before slicing the wheels into 200 or 300 g pieces. This work showed that one of the most important quality problem influencing the compliance score was the addition of extra quantities of cheese ring not belonging to the original wheels.

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PAPER

IMPACT OF BUCKWHEAT FLOUR GRANULATION AND SUPPLEMENTATION LEVEL ON THE QUALITY OF COMPOSITE WHEAT/BUCKWHEAT GINGER-NUT-TYPE BISCUITS

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ABSTRACT

Effects of gradual wheat flour substitution with buckwheat flour in ginger-nut biscuit formulation were investigated regarding dough characteristics, physical and textural characteristics of final product assessed after baking and 30 days of storage. Buckwheat flour was added at 30, 40, 50% levels and two granulations (fine and coarse). Addition of buckwheat flour significantly increased dough hardness and decreased adhesiveness. Spread significantly increased in biscuits with 40% and 50% of coarse buckwheat flour. Biscuits containing coarse flour were harder and more fracturable than the control, whereas those with fine flour tended to be softer and less fracturable. Textural properties were significantly correlated to protein stability to heat and retrogradation tendency of starch in biscuit dough as well as moisture content.

- Keywords: buckwheat; biscuit; dough; storage; texture -

INTRODUCTION

Ginger-nut biscuit (GNB) is a sweet biscuit containing honey and aromatic spices (cinnamon, ginger, cloves), which is very popular globally. By this term, variety of sweet biscuit types are described. They may range from thin (less than 3 mm thick), crisp varieties with smooth surfaces to thicker (over 3 mm thick), smaller in diameter and softer varieties with prominent cracks on top surface. The large variation in ingredients and their proportions contributed to a wide range of biscuit types. For example, the finest quality Nürnberg *Lebkuchen* does not even contain flour but nuts and candid fruits deposited on thin wafer base whereas the Polish *Pierniki Toruńskie* is made from the highest quality flour. The similar feature in all of them is that they all contain honey and spices. In Serbia, ginger-nut biscuits are traditionally produced by artisans, although their production has been industrialized (GAVRILOVIĆ, 2003). In appearance, they mostly resemble the gingersnaps from the United States: usually circularly shaped, over 6 mm thick with cracks on the top surface and soft crumb.

Formulations of GNB typically include 35-50% honey, 28-32% sugar, 0-5% fat on a flour weight basis (GAVRILOVIĆ, 2003), whereas the levels of major ingredients in common sweet biscuits are 30-75% sugar, 15-50% fat and 7-20% water (MANLEY, 2000). According to PYLER (1998), basic ratios of flour, fat and sugar in cookies vary from 100:30:30; 100:50:50 to 100:50:variable depending on processing type but may considerably deviate. GNB flour can be specified as a wheat flour with good bread-making performance (quality group B, peak amylogram value 300 B.U.), preferably of higher extractions (ash contents 0.8-1.15% d.b.) due to higher protein, hydrocolloid and enzyme content as opposed to the quality requirements in common biscuit production. The flour in common biscuits is usually soft wheat flour with lower protein content and moisture absorption and ash contents 0.34-0.38% d.b. (PYLER, 1998) and weak gluten (PAREYT and DELCOUR, 2008). In contrast to common biscuits whose final moisture content is low and ranges between 1-5% (CHEVALLIER *et al.*, 2000, 2002), the lowest recommended moisture content in GNB is 7% (GAVRILOVIĆ, 2003). Below this value, the quality deteriorates due to increased hardness, fracturability and crumbling. The crumb of GNB is porous with denser or looser pore structure, soft and elastic. Its upper surface is cracked which is a common pattern with formulations high in sugar and low in fat and is attributed to sugar recrystallization on the surface area (PAREYT *et al.*, 2009).

GNB is traditionally perceived as healthier in relation to other types of sweet biscuits probably because they contain honey and low amount of fat. As such, they can be seen as a convenient medium for providing further improvement in nutritional value and functionality by replacing a part of wheat flour with other nutritionally more valuable cereal or non-cereal flour. One such ingredient having an excellent reputation for its nutritious quality and abundance in bioactive compounds is common buckwheat. Buckwheat is most commonly used for producing flour and groats. While groats are mainly used for porridge and in various ethnic dishes, buckwheat flour is used as an ingredient in a variety of baked, cooked and extruded products: breads made from wheat-buckwheat flour blends at different ingredient proportions, flat bread, pasta (pizzoccheri in Italy), noodles (soba in Japan, extruded noodles in China and Korea), pancakes, breakfast cereals (based on extrudates made from buckwheat, rice, and/or corn blends), and biscuits (buckwheat added at 20-30% wheat flour basis).

Results from previous studies (FILIPČEV *et al.*, 2012) revealed that buckwheat flour has a potential as an ingredient in GNB formulation but a main disadvantage was related to the coarse granulation of commercially available buckwheat flour which reflected gritty texture of the final product. Therefore, this study was conducted to investigate the effect of substitution level and granulation of wholegrain buckwheat flour on dough characteristics and GNB physical and textural characteristics. The effects of three substitution levels (30%, 40%, 50%) and two flour granulation sizes (fine (FBF) and coarse (CBF)) were studied.

MATERIALS AND METHODS

Materials

Commercially available wheat flour (WF) type 850 (ash content 0.81% dry basis, moisture content 12.53%), and wholegrain buckwheat flour (ash 2.20% d. b., moisture content 12.31%) were used in the experiment. Other ingredients honey, vegetable fat (from sunflower), sugar, $NaHCO_3$, lecithin and spice (cinnamon) were obtained from a local food store (Novi Sad, Serbia). The purchased buckwheat flour was coarsely granulated. To obtain finer granulation, it was re-milled on a Falling Number 3100 mill (Perten Instruments). The granulation of used flours is given in Table 1.

Water absorption capacity of flours

Flour sample (5 g) was mixed with an excess of distilled water (25 ml), kept at ambient temperature for 30 min and then centrifuged at

2000 x g for 15 min. Water absorption capacity was expressed as g of water bound by 100 g of dry matter.

Syneresis degree of wheat and buckwheat flours

The method described by SINGH *et al.* (2003) was used. Flour suspensions (6 % w/v) were heated in a water bath to 90°C and held 20 min at this temperature. Cooled flour paste (30 mg) was poured into a centrifuge tube. The tubes were stored for 1 and 10 days at 4°C. Syneresis was measured as % of water expelled after centrifugation of sample.

Biscuit making

GNB was prepared by substituting wheat flour with buckwheat (0, 30, 40, 50 %). Formulae are found in Table 2. Firstly, a basic dough was formed by warming a mixture of honey, sugar and water to 80°C and adding part of the flour (75% of total) to a hot mixture. The mixture was mixed to obtain a thick, homogenous mass. After cooling to 40°C, basic dough was sprinkled over with flour, covered with a plastic foil, and left to rest at ambient temperature for two days. Other ingredients (remaining flour amount, spices, raising agents dissolved in water, fat and lecithin) were added and mixed using a kitchen mixer with a spiral hook for 10 min. The amount of added water was adjusted to obtain a maximally soft dough with acceptable handling characteristics. The consistency of dough was evaluated subjectively by an experienced baker. Dough moisture content ranged between 17.8-21.7%. After mixing, the dough was sheeted on a pastry break to 10-mm thickness (Sfogliatrice Mignon, Maestrino, Pd, Italy). The dough was cut to a diameter of 60 mm and baked for 15 min in a deck oven at 170°C. After 1 hour of cooling at room temperature, the biscuits were packed in polyethylene bags and stored at room temperature.

Texture profile analysis of biscuit dough

Dough characteristics were evaluated using a texture analyzer (TA.XTPlus Stable Micro Systems Ltd., Surrey, UK) equipped with a 30-kg load cell. Texture Profile Analysis (TPA) was employed to measure dough properties as described by (GALLAGHER *et al.*, 2005). Doughs were prepared as for the baking test and cut into round pieces (60 mm). A 36-mm cylindrical aluminium probe was used in two compression cycles at test speed 1.0 mm/s. Pre and post-test speeds were 2.0 mm/s. The force was measured at 45% compression. The recovery period between the strokes was 5 s. The following 4 parameters were recorded: hardness, adhesiveness, cohesiveness and springiness. Seven measurements per each biscuit type were made. Measurements were performed on dough after the resting period.

Table 1 - Particle size distribution of used flours.

Particle size (weight %)	Wheat flour (WF)	Coarse buckwheat flour (CBF)	Fine buckwheat flour (FBF)
>350 mm	0.00	55.30	5.74
>250 mm	0.02	9.40	11.32
>180 mm	0.40	4.76	11.11
>150 mm	1.32	3.80	8.90
>105 mm	22.54	10.14	18.86
>85 mm	26.85	9.80	30.76
bottom	48.87	6.80	13.31

Table 2 - Ginger nut biscuits formulations.

Ingredients, g	Control	Biscuit supplemented with CBF[a]			Biscuit supplemented with FBF[a]		
Wheat flour	100	70	60	50	70	60	50
Buckwheat flour	0	30	40	50	30	40	50
Honey	50		50			50	
Sugar	10		10			10	
Vegetable fat	5		5			5	
$NaHCO_3$	2.1		2.1			2.1	
Spice blend	2		2			2	
Lecithin	1		1			1	
Water	20	18	17	16	20	21	22

[a] CBF-coarse buckwheat flour; FBF-fine buckwheat flour

Analysis of thermo-mechanical properties of biscuit dough by Mixolab

Mixing and pasting properties of the ginger nut biscuit doughs were studied on Mixolab (Chopin, Tripette and Renaud, Paris, France). This device measures in real time the torque produced by dough during mixing at conditions of controlled temperature regime which include dough heating to 90°C, maintenance at constant temperature and dough cooling to 50°C. In this way, the behaviour of both proteins and starch under dual mechanical shear stress and temperature constraint can be measured (ROSELL *et al.*, 2006). Usually, individual flours or flour blend slurries are analysed in this way. In our study, previously prepared GNB dough was subjected to analysis using a modified Mixolab protocol. For the assays, 80 g of dough was inserted to Mixolab bowl, followed by the next regime: mixing speed 80 rpm, tank temperature: 30°C, 1st plateau temperature 30°C, duration of first plateau 5 min, heating rate 4.0°C/min, 2nd plateau temperature 90°C, duration of 2nd plateau 7 min, cooling rate 4.0°C/min, 3 rd plateau temperature 50 °C, duration of 3rd plateau 5 min.

Main derived parameters from the Mixolab curves are: development (C1) or maximum torque reached during mixing at 30°C, protein weakening (C2) or the minimum torque produced as a consequence of heating and mechanical stress, maximal torque (C3) produced during the heating stage as a consequence of starch gelatinization, minimal torque at the stage of cooling (C4) and the torque after cooling at 50°C (C5).

Measurements were replicated twice for each biscuit dough type after the resting period.

Textural analysis of ginger nut biscuits

Hardness and fracture of GNB were measured 24 h post-bake by penetration test on TA.XTPlus Texture Analyzer (Stable Micro Systems, England, UK). The test mode was force in compression. A 5 kg load cell was used. The sample was placed on the platform with a holed plate and centrally punctured with a 2 mm cylinder probe through the sample at test-speed 0.5 mm/s. Hardness of the sample was calculated from the area under the curve whereas fracturability was calculated from the linear distance. Four biscuits from each treatment were punctured five times in an 'X' pattern avoiding the outer 1 cm to prevent from edge effects.

Biscuit dimensions and density

Diameter (width) and height (thickness) of biscuits were measured using a vernier calliper. Diameter was calculated as an average of long and short diameter. Spread was calculated from the ratio of width and height. For the measurements, twelve randomly chosen biscuits were taken. Density was calculated as a ratio of biscuit mass and volume. Since biscuits had a regular shape, their volume was approximated to the volume of cylinder according to formula $V=\pi*(R/2)^2*h$ where h is biscuit height and R is average biscuit diameter.

Moisture content

Moisture was calculated according to AOAC Method 926.5 (2000). Two composite samples of each biscuit type were analyzed in duplicates. The composite samples were prepared by homogenization of six individual biscuit samples.

Statistical analysis

An analysis of variance (ANOVA) of data was performed by using a Statistica 7.1 statistical software package (StatSoft Inc., Tulsa, Oklahoma). Tukey's post-hoc test was used to compare the means at 95% confidence interval. Correlation analysis was conducted using Spearman's rank correlation coefficient applied to mean values for each biscuit.

RESULTS AND DISCUSSION

Water absorption capacity (WAC) and syneresis degree of used flours

Buckwheat flour showed higher WAC in comparison to WF (Table 3). This might be due to various reasons: higher hydration capacity of buckwheat starch in comparison to wheat starch (QIAN *et al.*, 1998); presence of other hydrophilic constituents in the wholegrain buckwheat flour (fibers). In contrast, BALJEET *et al.* (2010) reported that buckwheat flour had lower WAC, higher oil absorption capacity, higher foaming capacity and higher least gelation concentration than wheat. WAC of FBF increased but not significantly in comparison to CBF which might be explained by the fact that milling does not tend to increase the amount of damaged starch in buckwheat. It has been shown by QIAN and co-workers (1998) that

Table 3 - Water absorption capacity (WAC) and syneresis (%) of wheat and buckwheat flour.

Flour	WAC (g/100 g dry matter)	Syneresis (%)	
		1 day	10 days
Wheat flour	63.96[a]	9.27[a]	14.57[a]
Buckwheat flour, coarse	101.10[b]	39.27[b]	46.86[c]
Buckwheat flour, fine	104.14[b]	40.20[b]	42.67[b]

[a,b,c] Figures followed by the same letters in a column are not significantly different (p>0.05).

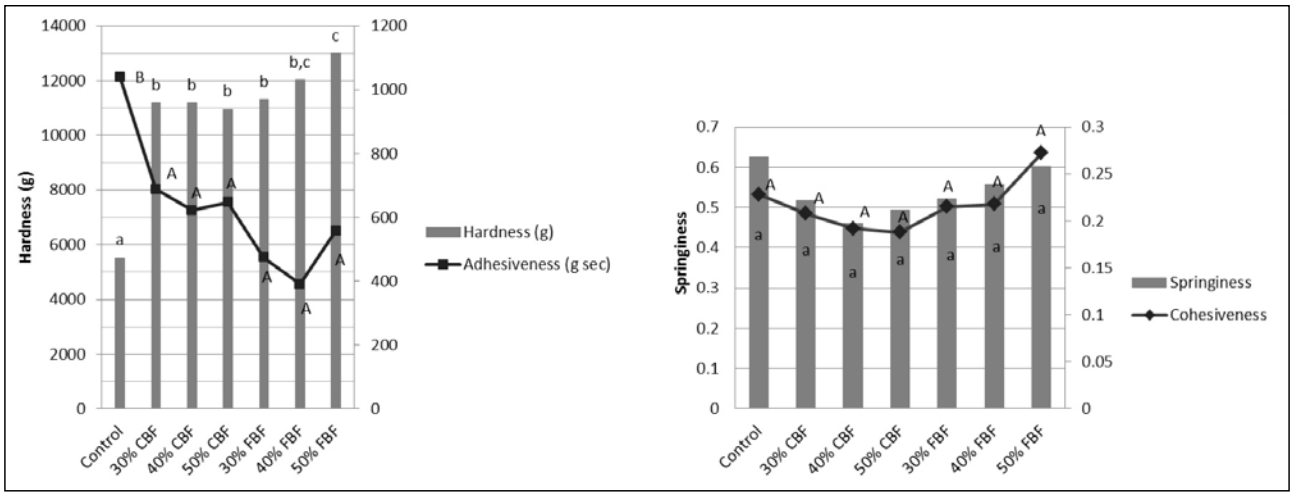


Fig. 1 - Dough hardness, adhesiveness, cohesiveness and springiness for different formulations of GNB.
[a,b,c .... A, B,....] Values followed by same letters of the same case are not significantly different (p>0.05).

buckwheat flour has lower amounts of damaged starch than wheat.

The syneresis value (%) of cooked pastes from wheat and buckwheat flour significantly differed. WF showed much less syneresis than buckwheat. During storage, syneresis of the pastes increased. The highest syneresis was observed in the paste made from CBF (46.9%), followed by FBF (42.7%). The retrogradation properties of the flours are generally attributed to composition, ratio and interactions of flour constituents: proteins, starch, lipids, fibers.

GNB dough characteristics

The addition of buckwheat significantly increased dough hardness in comparison to the control ($p<0.05$) (Fig.1). The dough hardness increased remarkably in the case of finely ground buckwheat flour. TPA adhesiveness showed a declining trend which was significant in comparison to the control. Dough springiness and cohesiveness also tended to decline with increased proportion of buckwheat, but there were no significant differences between the samples. FBF increased dough cohesiveness in comparison to CBF.

These effects can be related to the particularities of buckwheat flour and flour particle size. Buckwheat is characterized by resistant starch, which may contribute to low viscoelastic properties of dough and increased hardening (QIAN *et al.*, 1998; DE FRANCISCHI *et al.*, 1994; LI *et al.*, 1997; QIAN and KUHN, 1999; YOSHIMOTO *et al.*, 2004; HATCHER *et al.*, 2008). The observed variations in dough hardness and adhesiveness were mainly due to the effect of buckwheat flour granularity. Even though WAC between FBF and CBF did not significantly differ, it seems that finer flour particles were able to absorb more water during mixing, thereby forming more cohesive and harder dough. Lowering of dough adhesiveness and cohesiveness as a consequence of increasing amounts of coarse wheat flour was reported earlier (SINGH GURJAL *et al*. 2003). This coincides with our findings. Furthermore, dough made with wholegrain buckwheat flour was reported to have lower adhesiveness in comparison to the majority of other buckwheat flour fractions (IKEDA and KISHIDA, 1992).

Parameters related to thermo-mechanical behaviour of GNB dough made with coarse and fine buckwheat flour is given in Table 4. Buck-

Table 4 - Dough mixolab parameters for different formulations of gnb (CBF-coarse buckwheat flour; FBF-fine buckwheat flour).

Sample	C1 (Nm)	C2 (Nm)	C3 (Nm)	C4 (Nm)	C5 (Nm)	C1-C2 (Nm)	C5-C4 (Nm)
Control	3.45^{a}	2.73^{e}	3.04^{f}	1.65^{e}	1.66^{b}	0.72^{a}	0.005^{a}
CBF							
30%	3.99^{b,c}	1.20^{d}	1.40^{c}	1.39^{c}	2.71^{e}	2.79^{c}	1.32^{d}
40%	4.31^{c}	0.86^{c}	1.53^{c}	1.54^{d}	2.24^{d}	3.45^{e}	0.70^{b}
50%	3.68^{a,b}	0.68^{a}	1.81^{d}	1.62^{e}	2.69^{e}	3.00^{d}	1.06^{c}
FBF							
30%	4.22^{c}	1.30^{d}	1.30^{b}	0.97^{b}	1.85^{c}	2.92^{d}	0.88^{c}
40%	4.19^{c}	0.75^{b}	2.00^{e}	0.91^{b}	1.52^{b}	3.44^{e}	0.61^{b}
50%	5.32^{d}	0.85^{c}	1.10^{a}	0.63^{a}	0.95^{a}	4.47^{e}	0.32^{b}

[a,b,c ....] Figures followed by the same letters in a column are not significantly different (p>0.05).

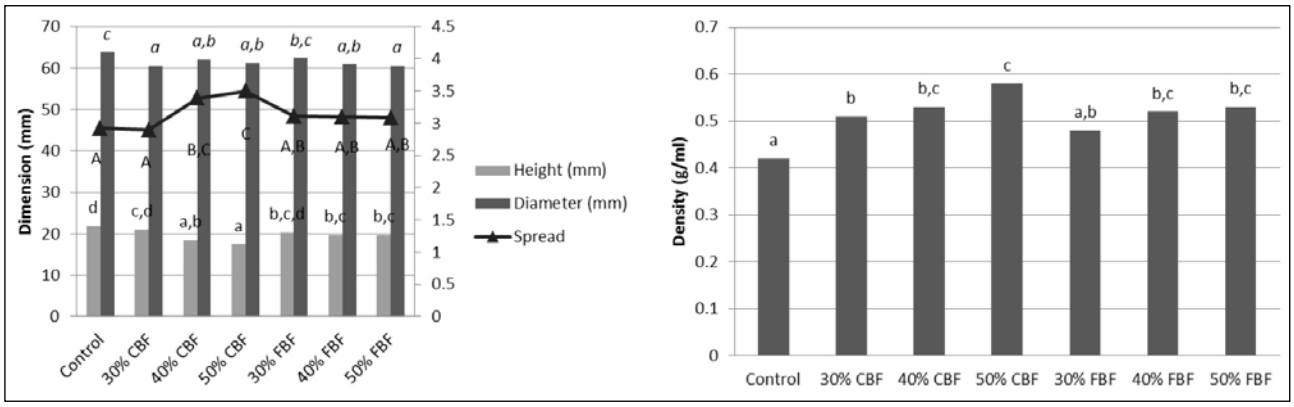


Fig. 2 - Effect of GNB formulation on biscuit dimensions (height, diameter, spread) and density.
[a,b,c ,... A, B,...] Values followed by same letters of the same case are not significantly different (p>0.05).

wheat supplemented dough was characterized with higher maximal torque during mixing (C1) and lower resistance to thermal and mechanical stresses (low C2 and high C1-C2) than the control dough. These effects seem to be more pronounced in the case of dough supplemented with coarse buckwheat flour. Both buckwheat flours affected these parameters in a similar way. This behaviour is probably due to dilution of gluten by addition of non-glutenous buckwheat flour which contributed to the formation of weaker protein network. The control dough showed higher maximal peak during heating (C3) than the buckwheat supplemented doughs. The decrease in peak viscosity of the buckwheat supplemented doughs may be attributed to lower swelling of starch granules and poorer gelatinization which is probably due to native characteristics of buckwheat starch and limited water amount in GNB doughs. Similar results were reported by HADNAĐEV and co-workers (2008), even in systems with higher moisture content, resembling bread dough. The buckwheat doughs exhibited lower breakdown torque (C4) which indicates lower stability of warm gel. Final torque (C5) and total setback values (C5-C4) are parameters used to characterize starch retrogradation that occurs during cooling to 50°C. Final torque (C5) was lower in the control dough and dough made with fine buckwheat flour. The total setback was the lowest in the control dough followed by FBF dough. Hence, regarding the ability to resist retrogradation, the tested GNB doughs follow the order: wheat dough > fine buckwheat flour dough > coarse buckwheat flour dough.

Biscuit dimension, density and spread

Data related to the biscuit dimension and density is displayed in Fig. 2. Majority of the variables showed significant differences as compared to the control especially at higher replacement levels (40% and 50%). The height and diameter decreased with increasing levels of both FBF and CBF. Despite general shrinkage, the interrelation between both dimensions was such that spread increased significantly at 40% and 50% of CBF addition whereas in other cases, spread did not significantly vary from the control. There was a strong inverse correlation between spread and height ($r=-0.84$, $p<0.01$) showing that reduction in height dominantly affected spread whereas spread and diameter were not significantly correlated ($r=-0.17$, $p>0.01$). The spread of the biscuit during baking is caused by expansion of dough by leavening and gravitational flow (HOSENEY and ROGERS, 1994). It depends on factors that control dough viscosity: the amount of water free to act as solvent and the strength of dough (RAM and SINGH, 2004). It also depends on partitioning of available water between the ingredients. The lesser the amount of water held by ingredients, the more the amount of water is available to dissolve sugar, decrease dough viscosity and increase spread (PAREYT and DELCOUR, 2008). Coarse flour has been reported to contribute to greater spread (SINGH GURJAL *et al.*, 2003; MANLEY, 1991). Substitution of wheat flour with a finely ground light and dark buckwheat flour (granulation size 100-150 μm) in sugar-snap cookie formulations lowered the cookie spread (MAEDA *et al.*, 2004). It was also found that buckwheat flour of similar granulation dosed at 10-40% level (flour basis) decreased the spread in sugar snap cookies (BALJEET *et al.*, 2010). Results obtained in this study confirmed the relation between spread and flour granularity: in relation to the control, the addition of CBF increased the spread. Higher biscuit spread is considered advantageous in biscuit making (PAREYT and DELCOUR, 2008). But, in contrast to majority of biscuits, the main quality requirement for GNB is well developed and soft crumb (GAVRILOVIĆ, 2003). Therefore, high spread may not be necessarily considered as preferable in GNB making since the increased thickness (height) is needed to develop a porous and soft crumb.

Regarding biscuit density, a general trend was

that density increased in the combined formulations. There was a strong inverse correlation between the biscuit dimensions and density r=-0.97 ($p<0.01$) and -0.76 ($p>0.05$) for height and diameter, respectively, confirming that higher density reduces biscuit development.

Biscuit moisture content

Minimal moisture content required for the retention of freshness in GNB is 7%. Moisture contents below this value render biscuits unacceptable owing to their increased tendency to dry out during storage. The moisture content of biscuits was significantly affected by the level of flour substitution with buckwheat flour as well as its granulation: increased buckwheat doses and coarse flour tended to decrease it whereas FBF increased the moisture content (Fig. 3). As a result, the composite biscuits with 40% and 50% of CBF were significantly lower in moisture (8.00-8.50%, $p<0.05$, respectively). Other formulations had higher moisture contents with the highest registered in GNB made with FBF and the control (≥ 10.0% moisture content). The water content in GNB formula was significantly correlated to biscuit hardness (r=-0.84, $p<0.01$), dough cohesiveness (r=0.84, $p<0.01$) and C5 (r=-0.92, $p<0.01$). These results indicate that higher moisture content is advantageous in this category of biscuits as it contributes to softer texture, more cohesive and stable dough. Over time, a decrease in moisture contents was observed in all samples. The most marked moisture content decrease (by 8%-10.7%, $p<0.05$) were in the biscuits substituted with FBF and much less in other samples (by 2.6%-3.8%), in spite of the lower syneresis degree of FBF. Although subjected to the high moisture loss, these biscuits remained higher in moisture content than the control due to higher initial moisture. During storage, moisture migration and evaporation occurs because gluten and starch undergoes such transformations which result in water release (WILLHOFT, 1971; SENTI and DIMLER, 1960).

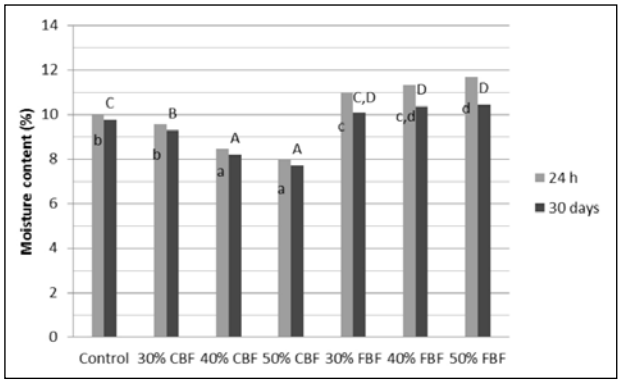


Fig. 3 - Effect of GNB formulation and storage time on biscuit moisture content.
a,b,c .... A, B,.... Values followed by same letters of the same case are not significantly different ($p>0.05$).

Biscuit texture characteristics

Granulation of buckwheat flour affected hardness and fracturability of GNB. In general, two trends were observed: as the level of CBF increased, hardness also increased, whereas the addition of FBF decreased hardness (Fig. 4). However, the produced results were similar to the control except in the case of GNB made with 50% CBF, which produced significantly higher hardness. Fracturability showed a similar trend (Fig. 4). BALJEET and others (2010) said that the addition of finely ground buckwheat flour decreased the hardness of composite biscuits.

Increased hardness has been usually related to increased association of wheat proteins as correlated by GAINES (1990) in sugar snap cookies and HATCHER *et al.* (2008) in the buckwheat supplemented soba noodles. In contrast to common biscuits, good quality wheat flour is required in the production of ginger nut biscuits. Consequently, its texture is related to the quality of flour proteins with the ability to provide unique viscoelastic and network-forming properties. The results of this study showed a significant correlation (r=0.74, $p<0.05$) between the biscuit hardness and a Mixolab parameter

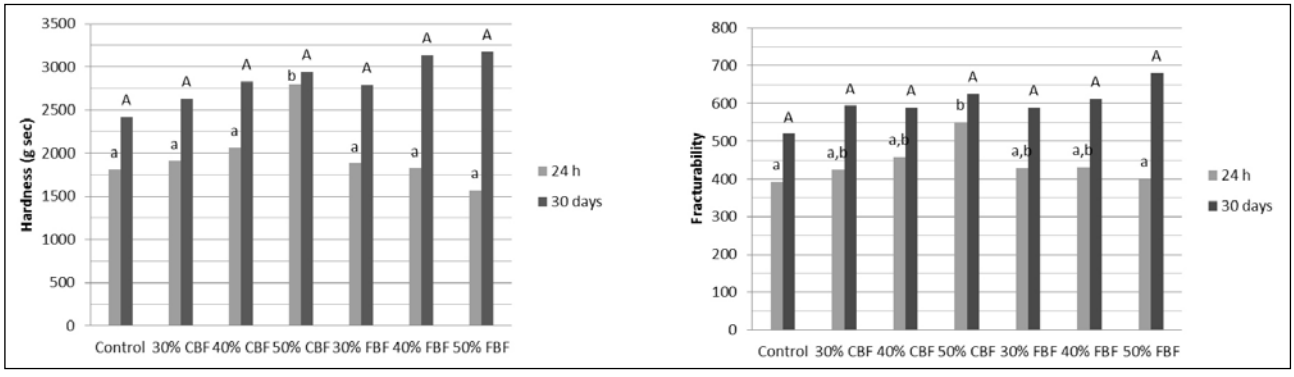


Fig. 4 - Effect of GNB formulation on biscuit hardness and fracturability measured after 24 h and 30 days.
a,b,c .... A, B,.... Values followed by same letters of the same case are not significantly different ($p>0.05$).

related to the quality of proteins, C1-C2. As already noted, C1-C2 indicates protein weakening during heating: higher difference means increased protein weakening i.e. lower stability to heat.

GAVRILOVIĆ (2003) suggested that GNB hardness is also affected by starch pasting properties. In this study, significant correlation (r=0.84 and 0.81, p<0.01) was found only between hardness and Mixolab parameters which indicates starch gel retrogradation (C5 and C5-C4 (total setback), respectively) i.e. higher biscuit hardness can be related to higher setback and C5 values which are indicators of higher susceptibility to staling. The ability of the buckwheat starch to increase hardness of GNB can be counteracted by using ingredients able to retain more water (such as an observed decreasing trend for hardness in the biscuits made with FBF (Fig. 4)). Actually, it was found that the moisture content significantly affected biscuit hardness; there was a significant inverse correlation between hardness and formula water content i.e. biscuit moisture content (r=-0.84 (p<0.01) and -0.74 (p<0.05), respectively).

According to GAVRILOVIĆ (2003), fracturability of GNB can be related to the presence of partially dehydrated gluten in the dough matrix. CHARLES and co-workers (2004) proposed that the presence of discontinuous phase in gluten network was related to an increase in fracturability in flaky snack. Here in the study, significant correlation was found between the biscuit fracturability and two Mixolab parameters related to the behaviour of protein during heating (C2 and C1-C2): C2 denotes the minimum torque produced during heating as a consequence of the beginning of protein weakening (r=-0.84, p<0.01) and C1-C2 can be associated with protein stability (r=0.95, p<0.01). Lower value of C2 and higher value for C1-C2 indicate lower protein stability. In other words, increasing fracturability of GNB can be related to all such factors that may lower the protein stability or discontinue the gluten network such as the addition non-gluten ingredient like buckwheat, especially in the form of coarse flour. There was also a significant correlation between biscuit fracturability and setback value (C5-C4) (r=0.78, p<0.05).

Over the 30-day trial period, a significant increase in hardness and fracturability occurred for GNB made with FBF whereas all others showed insignificant increases as related to the initial values (Fig. 4). But, there were no significant differences in hardness and fracturability within all biscuits measured after 30 days of storage.

On the basis of the above mentioned high correlation between the texture and Mixolab parameters that indicate starch susceptibility toward retrogradation, it could be thought that the lower the susceptibility of starch in biscuit dough to retrograde, the lower the biscuit hardness, fracturability and presumably its tendency towards staling. But, the results obtained after storage showed that GNB made with FBF, which initially gave the softest biscuit and which paste had lower syneresis degree, significantly increased in hardness (in relation to the initial values). This could be due to higher moisture evaporation and more compact structure in comparison to the granular structure of GNB made with CBF. It is also worth noting that GNB dough represents a low-moisture system containing up to 25% moisture and, in addition, interfering ingredients like sugar and fat which might have caused different behaviour and tendencies regarding starch pasting. Interestingly, dough hardness was strongly positively correlated with biscuit hardness and fracturability after storage (r=0.84 and r=0.81, p<0.01).

In the literature, there is little data on the functional properties of buckwheat flour; more data exist on buckwheat starch. Furthermore, data on the ability of buckwheat starch to retrograde are rather contrasting. In the study of QIAN and colleagues (1998), besides increased hardness and stability, buckwheat starch gels exhibited lowered retrogradation as compared to cereal starches, even after storage. Lower susceptibility to retrogradation was also suggested in reports on thermomechanical properties of buckwheat flour slurries or wheat/buckwheat blends slurries (Chopin's Mixolab User's Manual 2005, BANU *et al.*, 2010). In another study, however, a stronger retrogradation peak was observed in buckwheat thermogram than that in wheat (LIU *et al.*, 2006). ZHENG and associates (1998) reported that buckwheat starch had a higher peak viscosity and setback value than maize and wheat starches which may suggest higher retrogradation tendencies in buckwheat starch since setback viscosity indicates the degree of starch retrogradation, mainly its amylase fraction. Starch pasting properties depend on the hydration level (ZHOU *et al.*, 2009). It was concluded that buckwheat starch gelatinization temperatures and enthalpies increased along with the decrease of water content, which can be associated with an increased retrogradation tendency.

Moreover, there is little data to relate starch pasting behaviour with the final properties in a biscuit-like products. It was reported that the addition of corn flour produced harder cookies than did the addition of potato flour, although corn flour had been shown to give lower syneresis (retrogradation) than potato flour (SINGH *et al.*, 2003). Some authors did not find any correlation between pasting properties of batters and characteristics of layer cakes (GÓMEZ *et al.*, 2010). Others suggested that dehulled buckwheat flour, although showing a tendency to retrograde, can be used in buckwheat enriched products (MARIOTTI *et al.*, 2008). IN-

GLETT and his team (2009) proposed that only specialty buckwheat flour with low paste viscosity is suitable for mixing with wheat flour to produce bread and cookies. Much earlier, LORENZ and DILSAVER (1982) mentioned that inclusion of native buckwheat starches in cake formulations did not produce cakes of acceptable quality.

CONCLUSIONS

The results indicated that the addition of buckwheat significantly increased dough hardness and decreased dough adhesiveness. Springiness and cohesiveness of buckwheat doughs were reduced but no significant difference within the samples was observed.

The dimensions of GNB decreased, but since the biscuit height was more affected by the rising doses of buckwheat, the spread increased in the composite biscuits (significant difference was noted with CBF at 40% and 50% replacement level). Hardness and fracturability of GNB increased with increasing doses of CBF whereas FBF decreased hardness and fracturability. However, a significant change was noted only with the addition of 50% CBF.

Hardness and fracturability of GNB significantly correlated to the quality of proteins; lower stability of proteins to heat was associated to increased hardness and fracturability in GNB. Tendency of starch gel in biscuit dough to retrograde was found to be in significant positive correlation with biscuit hardness and fracturability, however, after 30 days of storage, hardness and fracturability increased most markedly in the biscuits made with FBF which were initially softer than the others. Biscuit hardness was in significant inverse relation with the formula water content which might additionally support the importance of protein quality and starch pasting behaviour to the quality of GNB.

Comparing GNB and common biscuits made from short and semi-sweet dough from literature, it seems that GNB exhibits different behaviour. Better quality characteristics are obtained when more water is added to the dough and increased spread cannot be regarded as positive since well-developed crumb is more advantageous for better textural properties.

The addition of FBF to GNB formulation is appropriate at the investigated doses. However, since they have been inclined to excessive drying out, the use of humectants or suitable edible coatings would be appropriate.

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PAPER

EFFECT OF SULFITES ON THE *IN VITRO* ANTIOXIDANT ACTIVITY OF WINES

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ABSTRACT

The objective of this study was to assess the contribution of SO_2 to the overall antioxidant activity of wines. In this study, white, red, and model wines, with increasing sulfite content, were used. The radical scavenging activity of the wines was evaluated by ABTS and DPPH assays, while the reducing capacity of the wines was assessed by the FRAP assay. SO_2 positively affected the antioxidant properties of the wines and, in some cases, its contribution to the overall antioxidant activity of wines was higher than that of naturally occurring antioxidants. Depending on the assay, SO_2 showed both synergistic and antagonistic effects with the antioxidants naturally present in wines.

- Keywords: antioxidant activity, ABTS, DPPH, FRAP, wine, sulfites -

INTRODUCTION

Wine is one of the most important dietary sources of antioxidants with both *in vitro* and *in vivo* antioxidant activity (MANZOCCO *et al.*, 1998; FRANKEL *et al.*, 1995; VINSON and HONTZ, 1995; SERAFINI *et al.*, 1998; TSANG *et al.*, 2005). The *in vitro* antioxidant activity of wine, which has been studied in depth for decades, is highly correlated to its phenolic content (SIMONETTI *et al.*, 1997; BURNS *et al.*, 2000; ALONSO *et al.*, 2002; FERNÁNDEZ-PACHÓN *et al.*, 2004; YILDIRIM *et al.*, 2005). *In vivo* studies have shown that the consumption of wine modulates the serum non-enzymatic antioxidant capacity in humans. However, the direct antioxidant effect of polyphenols *in vivo* is still under debate (SERAFINI *et al.*, 2011; HOLLMAN *et al.*, 2011).

Even though the antioxidant activity of wines has been mostly attributed to the presence of phenolic compounds (MANZOCCO *et al.*, 1998; YILDIRIM *et al.*, 2005; BURNS *et al.*, 2001; VILLAÑO *et al.*, 2006), exogenous antioxidants, such as sulfites, are added during the wine-making process. Sulfur dioxide is one of the most commonly used additives in the food and beverage industries (WHO, 1998) due to its antioxidant, antiseptic, and preservative properties (BRANEN *et al.*, 2002). In wines, sulfur dioxide has positive effects by inhibiting oxidation and microbial growth, increasing pigment extraction, and reducing color loss and phenolic polymerization (RIBÉREAU-GAYON *et al.*, 2000). However, the use of sulfites in certain food products has either been banned (FDA, 1986) or strictly limited (EEC, 1995) and is currently under regulation due to its allergenic effects in hypersensitive individuals (EFSA, 2004).

Even though sulfites have reducing and antioxidant properties, there are contradictory findings on the contribution of sufur dioxide to the overall antioxidant capacity of wines. Some studies have reported that sulfur dioxide reacts with DPPH radicals and improves the radical scavenging activity of wines (ABRAMOVIC *et al.* 2015). Other studies have found that sulfur dioxide plays a minor role in the antioxidant capacity of wines (MANZOCCO *et al.*, 1998; CIMINO *et al.*, 2007). Additionally, as reported by KILMARTIN *et al.* (2001), the contribution of sulfur-containing antioxidants is lost when their reducing properties are determined by cyclic voltammetry methods equipped with glassy carbon electrodes.

On the other hand, authors have reported that sulfur dioxide might play a significant role in the antioxidant activity of beverages and sauces (LONG *et al.*, 2000; LACHMAN *et al.*, 2009; MITSUHASHI *et al.*, 2001), especially of white wines, which contain high sulfite levels and low natural antioxidant levels. Additionally, the *in vivo* effect of sulfites on the antioxidant activity of foods is still unknown (CAMPANELLA *et al.*, 2004; LAGGNER *et al.*, 2005).

The objective of this study was to evaluate the contribution of sulfur dioxide to the overall *in vitro* antioxidant capacity of wines using the ABTS, DPPH, and Ferric Reducing Antioxidant Power (FRAP) assays. These assays differ in several properties including mechanism of action (radical or redox reaction) and environmental conditions (solvent polarity and pH). In this study, three wine types (white wine, red wine, and a model wine) were used. This experimental approach was used to assess possible matrix effects which, to the best of the authors' knowledge, have not been evaluated.

MATERIALS AND METHODS

Materials

Three types of wines were analyzed: white wine (Trebbiano d'Abruzzo Pietrosa, 2003 vintage, winery Dora Sarchese), red wine (Montepulciano d'Abruzzo, 2004 vintage, Miglianico social winery), and a model wine made from distilled water, ethanol (12% v/v), and tartaric acid (0.033 M, pH 3.6). The content of alcohol, total polyphenols, and sufur dioxide, and the pH value of the three wines are shown in Table 1. Different levels of sodium metabisulphite ($K_2S_2O_5$) were added to the wines. All reagents used in this study were of analytical grade.

Chemical and chemico-physical analyses

Alcohol content, total and free sulfur dioxide content, and pH values were determined by official EU methods (EEC, 1990). Total polyphe-

Table 1 - Total and free sulphur dioxide content, pH, alcohol amount, total polyphenol index and total dry extracts of the white and red wines under investigations.

Samples (mg l^{-1})	$SO_{2\,TOT}$	$SO_{2\,FREE}$ (mg l^{-1})	pH	Alcohol (%v/v)	TPI (mg GAE l^{-1})	Total dry extract (g l^{-1})
white wine	77	65	3.24	12.90	1052	22.80
red wine	70	54	3.27	13.05	1837	23.55
Data coefficient of variation <2%.						

nol content was determined by the method reported by SINGLETON and ROSSI (1965).

ABTS assay

The radical-scavenging activity of the samples was determined by the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical decolorization assay (RE *et al.*, 1999). The bleaching rate of the ABTS radical in the presence of sample was monitored at 734 nm. ABTS radical solution (2.97 mL; Abs = 0.70±0.02) was mixed with 30 μL of diluted wine samples (1:2, 1:5, 1:10, and 1:20) using the model wine as diluent. ABTS radical bleaching was monitored at 25°C for 60 min; the decoloration degree after 5 min was used as an indicator of antioxidant activity. In the dilution range considered, the ABTS radical bleaching was proportional to the concentration of sample added to the medium; a dose-response curve was fitted to a linear model. Antioxidant activity, which was calculated as the ratio between the regression coefficient of the dose-response curve of the sample and the regression coefficient of the dose-response curve of Trolox (hydrophilic homologue of tocopherol), was expressed as μmoles of Trolox equivalents per mL of sample ($TEAC_{ABTS}$: Trolox Equivalent Antioxidant Capacity).

FRAP assay

The reducing activity of the samples was determined according to the method described by BENZIE and STRAIN (1996), with slight modifications. Sample (0.1 mL) was mixed with FRAP reagent (2.9 mL) obtained by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solubilized in 40 mM HCl, and 20 mM $FeCl_3$ in a 10:1:1 ratio. Absorbance was measured at 593 nm for 6 min. A calibration plot was generated based on $FeSO_4$ $7H_2O$; the results were expressed as mM Fe^{2+}.

DPPH$^{\bullet}$ assay

The antiradical activity of the samples was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) decolorization assay as reported by BRAND-WILLIAMS *et al.* (1995), with slight modifications in data computation. A dose-response curve was generated by adding 0.1 mL of sample at different dilutions (1:2, 1:5, 1:10, and 1:20) to 2.9 mL of a $6.1 \cdot 10^{-5}$ M DPPH-methanol solution. Radical bleaching was monitored at 25°C for 60 min. Dilutions were performed with the model wine as diluent. The $TEAC_{DPPH}$ value was calculated as the ratio between the regression coefficient of the dose-response curve of the sample and the regression coefficient of the dose-response curve of Trolox and expressed as μmoles of Trolox equivalents per ml of sample.

Statistical analyses

Three aliquots were sampled from each wine; each aliquot had different levels of sodium metabisulfite. All analytical determinations were carried out in triplicate. Data were reported as mean ± standard deviations. Linear regression was applied to assess the relationship between sulfite content and antioxidant activity; the goodness of fit was evaluated by the coefficient of determination (R^2). The antioxidant activity of wines in the absence of sulfites was obtained by extrapolation of the intercept value; the accuracy of the predicted values was assessed from the standard deviation. All statistical analyses were performed with Statistica® for Windows (Statsoft, Tulsa, OK).

RESULTS AND DISCUSSION

The proximate composition and sulfite content of the wines are shown in Table 1. The polyphenol content of the white wine was quite high because the wine was processed by cryo-maceration, while that of red wine was relatively low because it was a 'cerasuolo-type' red wine. These two types of wine were selected for this study because they had similar alcohol and total dry extract contents.

The sulfite content of the three wines increased with increasing sodium metabisulfite addition. The total sulfur dioxide content, which was assessed by titration, was 50, 100, 150, and 200 mg L^{-1} in the model wine; 77, 113, 125, 153, 185, and 209 mg L^{-1} in the white wine; and 70, 100, 125, 150, 175, and 200 mg L^{-1} in the red wine. The amount of sodium metabisulfite added to the wines was calculated using data in Table 1. The free sulfur dioxide content was measured immediately and 2 h after sodium metabisulfite addition; no significant changes in bound sulfilte levels were obtained between these two time points. This time lapse is usually required for antioxidant activity determinations.

Antioxidant activity was determined by ABTS (TEAC method), DPPH, and FRAP assays. The ABTS and DPPH assays have similar mechanism of action towards AR-OH, because they can be neutralized either by direct reduction via electron transfer or by radical quenching via H atom transfer (PRIOR *et al*, 2005), even though in the case of DPPH radical, the hydrogen atom removal from AR-OH could be considered as a marginal reaction because it occurs very slowly in strong hydrogen bond-accepting solvents such as methanol (HUANG *et al.*, 2005). The environmental conditions of the two radical scavenging assays are quite different because the ABTS assay is performed in aqueous media versus pure methanol in the DPPH assay.

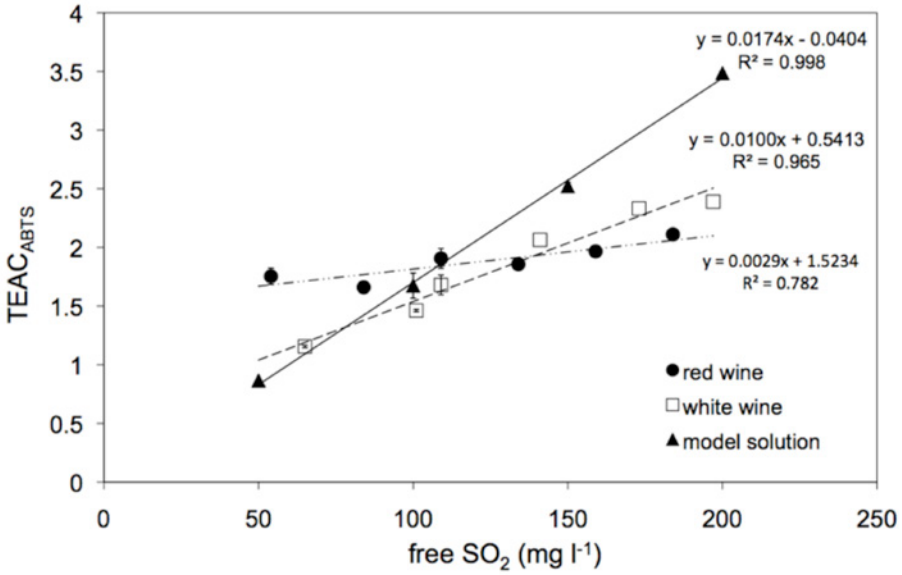


Fig. 1 - Antiradical activity as evaluated by the ABTS radical decolorization assay of the model wine solutions and of the white and red wines as a function of free SO_2 concentration.

Radical scavenging activity as determined by the ABTS radical decolorization assay

The antiradical activity of the wines, as determined by the ABTS decolorization assay, is shown in Fig. 1. Antiradical activity improved with increasing sulfur dioxide concentration. The model wine containing 50 ppm SO_2, an amount that is likely to occur in real wines, was characterized by a $TEAC_{ABTS}$ value of 0.85 μmoles Trolox equivalents per ml of sample, while the 200 ppm model wine had a $TEAC_{ABTS}$ value of 3.48 μmoles Trolox equivalents per ml of sample.

Taking into account the fact that the antioxidant activity of the white wine measured by the ABTS assay may vary between 0.8 and 4.24 μmoles Trolox equivalents per mL (ALONSO *et al.*, 2002; DE BEER *et al.*, 2003; VILLAÑO *et al.*, 2004), these results suggest that sulfites may play a more significant role in the antiradical properties of wines than polyphenols. However, all wine samples had SO_2 added in its free form; commercial wines are likely to have SO_2 bound to different compounds.

To evaluate the effect of sulfur dioxide on the antioxidant capacity in a real wine, the antiradical activity determinations were carried out in white wine with different contents of total sulfur dioxide. The results revealed that the white wine, with a total SO_2 content of 77 mg L^{-1}, was characterized by a $TEAC_{ABTS}$ value of 1.16 μmoles of Trolox equivalents per ml. Taking into account that 77 mg L^{-1} of sulfur dioxide in the model wine exerted a TEAC value of 1.30, it can be hypothesized that most of the antioxidant capacity of white wine is attributed to its sulfur dioxide content. By extrapolating the antioxidant activity of white wine without sulfites from the regression curve (Fig. 1), the wine had a $TEAC_{ABTS}$ value of 0.40 μmoles of Trolox equivalents per ml of sample. Therefore, sulfur dioxide contributed to the antioxidant activity of white wine to such an extent that an amount of 50 mg L^{-1} sulfur dioxide can double the $TEAC_{ABTS}$ value. These results were in agreement with those obtained by LONG *et al.* (2000), who reported that small quantities of sulfites can affect the total antioxidant activity of the product.

In white wine, an increase in sulfur dioxide concentration from 77 to 200 mg L^{-1} doubled its antioxidant activity. This result is quite significant because most wine research studies have not evaluated sulfite interference or sulfite contribution to the overall wine antioxidant activity (VILLAÑO *et al.*, 2006; LACHMAN *et al.*, 2009; 24, VILLAÑO *et al.*, 2004; ARNAO, 2000), even when the fractionation of polyphenolic compounds could not explain the overall antioxidant activity of the samples (FERNÁNDEZ-PACHÓN *et al.*, 2004). The regression coefficient of the dose-response curve of the white wine was lower than that of the model wine (Fig. 1), which could be attributed to matrix effects.

To further investigate the matrix effect on the antioxidant capacity of sulfur dioxide, the antiradical activity was also determined in red wine. Fig. 1 shows that the red wine, with a sulfur dioxide content of 70 mg L^{-1}, had a $TEAC_{ABTS}$ value of 1.70 μmoles of Trolox equivalents per ml. Considering that the model wine with similar sulfur dioxide content had a $TEAC_{ABTS}$ value of 1.18, it could be hypothesized that a considerable percentage of the antiradical activity of red wine is attributed to its sulfur dioxide content. However, when the antioxidant activity of the red wine with no sulfites was extrapolated in the regression curve (Fig. 1), the $TEAC_{ABTS}$ value was 1.52. Taking into account the regression equa-

tion, the sulfur dioxide contribution to the overall antiradical capacity was 10–38% in the tested concentration range, which was lower than that of white wine.

In decreasing order of regression coefficient magnitude, the wines were model wine > white wine > red wine. This result confirmed the presence of a matrix effect on the determination of antioxidant activity; this matrix effect was higher in the red wine than in the white wine. It has been extensively reported that sulfites in wine can bind to several compounds such as acetaldehyde and polyphenols. Polyphenol content is usually much higher in red wines than in white wines (ALONSO *et al.*, 2002; DE BEER *et al.*, 2003). Additionally, red wines contain a high amount of anthocyanins, which bind to sulfur dioxide (ANTONELLI and ARFELLI, 1993; TIMBERLAKE and BRIDLE, 1967). However, in this study, the free sulfite content was taken into consideration (Fig. 1); therefore, in our experimental conditions it could be assumed that natural antioxidants and sulfites interfered with the antioxidant activity assays. In fact, both synergistic and antagonistic effects among antioxidants were observed in different *in vitro* antioxidant activity assays.

Reducing activity as determined by the FRAP method

The antioxidant properties of the wines were evaluated with the FRAP assay (BENZIE and STRAIN, 1996). In contrast with the previously described methods, this method is based on the reducing capacity of a compound rather than its antiradical activity.

The FRAP values of the model, white, and red wines are shown in Fig. 2. The model wine had low reducing activity; however, the addition of sulfites (70–200 ppm) resulted in a threefold increase relative to the initial value. The addition of sodium metabisulfite to the white and red wines increased their reducing power. The higher the sulfite content, the higher the reducing properties, likely due to the protective role of sulfur dioxide against polyphenol oxidation. An increase in sulfur dioxide from 71 to 200 ppm contributed to a 73% and 158% increase in the reducing capacity of the red and white wines, respectively.

In the red and model wines, it was possible to extrapolate the FRAP value without sulfite addition. Based on the results, sulfur dioxide is responsible for most of the reducing power of the wine samples. However, with respect to the white wine without sulfite addition, the experimental data did not allow an accurate estimation of the FRAP value because of a non-linear response (Fig. 2). This result could be attributed to synergistic effects between natural antioxidants and sulfur dioxide. The synergistic effects between natural antioxidants and sulfur dioxide could account for a non-linear response between the FRAP assay and the SO_2 dose, which was evident in the red and white wines (Fig. 2). If the individual effect of an antioxidant on FRAP is linear within a certain concentration range, the synergistic effect of two antioxidants could show an increase or decrease in the response due to variations in their molar ratios (HIDALGO *et al.*, 2010).

In order of decreasing regression coefficient magnitude, the wines were red wine >white wine > model wine. There were no negative matrix effects in the red and white wines. Contrary to the results obtained from the ABTS assay, sulfites

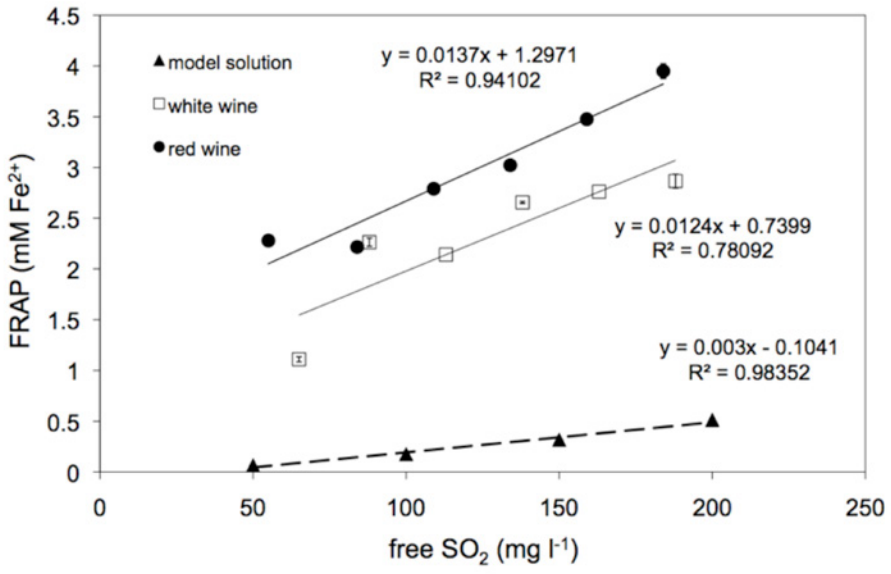


Fig. 2 - Reducing capacity as evaluated by the FRAP method of the model wine solutions and of the white and red wines as a function of free SO_2 concentration.

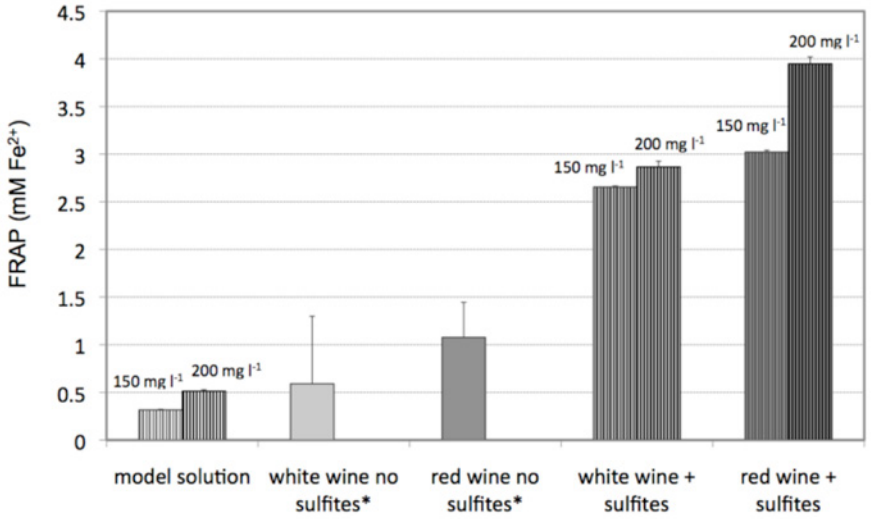


Fig. 3 - Reducing capacity of the samples with no sulfites (*obtained by extrapolation of data linear regression) and in the presence of 150 and 200 mg l^{-1} total sulphur dioxide content.

and naturally occurring antioxidants (i.e., polyphenols) had a synergistic effect on the reducing power of wines (Fig. 3). This result may be attributed to several factors: (i) in the experimental conditions of the FRAP assay (pH= 3), there is a lower amount of bound SO_2 (RIBÉREAU-GAYON *et al.*, 2000) than in the ABTS assay (pH= 7); (ii) free SO_2 could scavenge hydrogen peroxides produced via the Fenton reaction from catechols; and (iii) polyphenols could prevent the prooxidant action of peroxomonosulfate radicals resulting from Fe(III)-initiated bisulfite oxidation (DANILEWICZ, 2007; DANILEWICZ *et al.*, 2008). The synergistic effect between sulfites and polyphenols support the facts that SO_2 and catechols are not individual antioxidants, and that the antioxidant activity of wine is a result of multiple antioxidants (DANILEWICZ *et al.*, 2008).

Radical scavenging activity as determined by the DPPH• radical cation decolorization assay

The antiradical activity of sulfur dioxide was evaluated by the DPPH decolorization assay (BRAND-WILLIAMS *et al.*, 1995), which relies on a methanol-soluble stable radical in an amphiphilic environment. Dose response curves were generated with different dilutions of hydroalcoholic solutions containing increasing amounts of total sulfur dioxide. Fig. 4 shows the antioxidant activities of the model wine, expressed as $TEAC_{DPPH}$, plotted against the total sulfur dioxide content (25–200 mg L^{-1}).

The model wine containing sulfites had limited antiradical activity in the amphiphilic environment (MANZOCCO *et al.*, 1998). Water-metha-

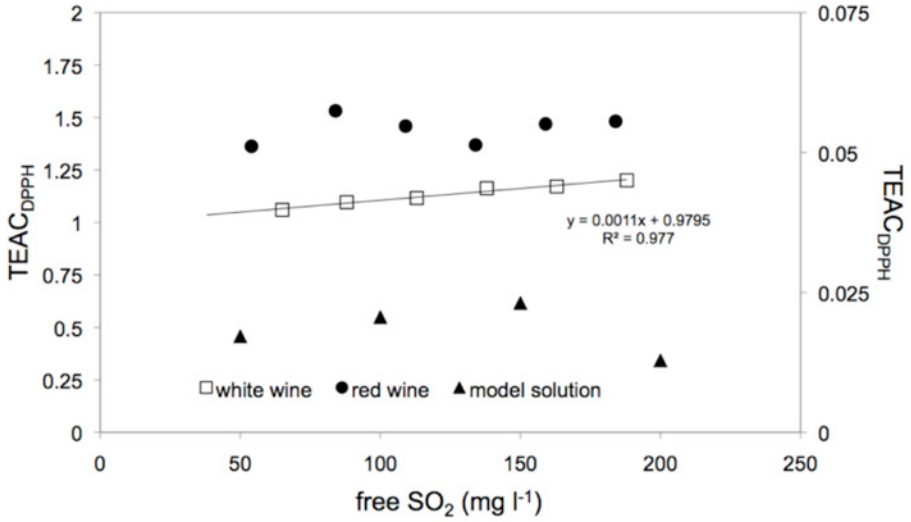


Fig. 4 - Antiradical activity as evaluated by the DPPH radical decolorization assay of the model wine solutions (secondary y axe) and of the white and red wines (primary y axe) as a function of free SO_2 concentration.

nol mixtures are not ideal solutions; in such mixtures, small volumes of water in methanol result in the stabilization of methanol clusters as a result of hydrophobic and hydrogen bond interactions (TAKAMUKU *et al.*, 2000; WAKISAKA *et al.*, 1998; OKASAKI *et al.*, 1984), resulting in a phase separation that could limit the contact between the water soluble antioxidant (sulfites) and the methanol-soluble radicals with negative effects on the estimation of the antioxidant capacity.

Fig. 4 shows the dose response curves of the white and red wines. In the white wine, increasing sulfur dioxide content from 77 to 200 mg L^{-1} caused a slight increase in the antiradical activity (+13%). In the red wine, the increase in sulfur dioxide contributed to increased but fluctuating antiradical activity values. A reduction in antioxidant activity due to sulfite addition (>150 mg L^{-1}) was observed in the model wine (Fig. 4).

Based on the results obtained from the DPPH• assay, there were no negative matrix effects on the antiradical activity of sulfur dioxide. White and red wines, as opposed to the model wine of this study, contain phenolic compounds, which exhibit a surface activity that affects their radical scavenging efficiency in multiphasic systems (DI MATTIA *et al.*, 2009; 2010). A possible explanation for this result is that amphiphilic compounds like polyphenols could have acted as surfactants allowing sulfur dioxide to exert its antiradical activity with positive effects on the $TEAC_{DPPH}$ value. In this case, the interfacial effect of polyphenols may justify the positive combined effects between the two antioxidants. Another possible explanation is the synergistic effects between polyphenols and SO_2 on reducing activity.

The synergistic effects between natural antioxidants and sulfur dioxide could account for a non-linear response between the DPPH assay results and the SO_2 dose, which was evident in the red wine (Fig. 4). In the case of the FRAP assay, a non-linear response was observed in the white wine, which contains less polyphenols (Fig. 2), while in the case of the DPPH• assay, a non-linear response was evident in the red wine, which contains more polyphenols (Fig. 4). This result could be due to the different solvents used in the two assays: hydrophilic solvents in the FRAP assay and amphiphilic solvents in the DPPH• assay. Additionally, the different activities of phenolic antioxidants in the two assays is pH dependent (JOVANOVICH *et al.*, 1994).

CONCLUSIONS

Sulfur dioxide had antioxidant activity in the white, red, and model wines. In some cases, the contribution of sulfur dioxide to the overall antioxidant capacity of the wines was higher than that of naturally occurring antioxidants. Moreover, in wines, sulfur dioxide had both antagonistic and synergistic effects with naturally occurring antioxidants on the total antioxidant activity of wines.

The role of sulfur dioxide on the total antioxidant capacity of wines is of utmost importance to assess the technological and potentially health-promoting properties of different products. Future studies should evaluate the *in vivo* antioxidant effects of sulfur dioxide in wines.

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PAPER

ADEQUACY OF MINERAL CONTENTS OF RAW AND PLAIN STICKY SAUCE OF COMMON AND BUSH OKRA

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ABSTRACT

In Nigeria, common okra (*Abelmoschus esculentus L.)* and bush okra **(***Corchorus olitorius L.)* are popular mucilage vegetables used as sticky sauce for easy consumption of starchy staples. Both raw vegetables and sticky sauce of common as well as bush okra were estimated for their potential in the provision of daily dietary allowance of important minerals. Modified methods of the Association of Official Analytical Chemists (AOAC) were used to estimate the assessed minerals. The results showed that the raw and sticky sauce of assessed common and bush okra contained appreciable levels and essential minerals, but are not adequate to meet recommended dietary allowance, except for Fe and Cu. Comparatively, the two species of okra varied significantly in their mineral content of the raw and plain sauce. There was also a negative effect of cooking on the mineral contents, which reduced significantly to an average of 30% on a dry weight basis. Therefore, the two vegetables, either as a fresh or sticky sauce, require additional sources of P, K, Na, Mg, Ca, Mn, and Zn to meet recommended dietary allowance. Furthermore, dried mucilage sauce, though, could be an appreciable post harvest management and storage but not without a loss of about one-third mineral content in the process. However, the sauce of common okra and bush okra are good sources for any of the assessed mineral restricted diets.

- Keywords: bush okra, common okra, mineral content, recommended dietary allowance, sticky sauce -

INTRODUCTION

Vegetables are nutritious foods that provide sufficient amount of nutrients needed for normal body functions, maintenance and reproduction. Also, their intake in different combination is essential for the maintenance of healthy life and normal body function (RUMEZA *et al.*, 2006). Vegetables have a lot of health benefits, which include reduced cancers, diabetes and cardiovascular diseases (COX *et al.*, 2000). Leafy vegetables are major sources of nutrients in rural areas where they contribute substantially to protein, mineral, vitamins, fiber and other nutrients which are usually in short supply in daily diets. Besides their use as food, they also add flavor, variety, taste, color and aesthetic appeal to what would otherwise be a monotonous diet (MEPBA *et al.*, 2002).

Thousands of leafy vegetables abound in Nigeria and are used primarily as food and medicine. These vegetables are diverse in species from different families and orders, and many of which have specific regional or local area of domestication (FAFUNSO and BASSIR, 1987; MEPBA *et al.*, 2007). However, they have crossed regional or local barriers through migration and exchange of goods. Presently, they are being cultivated throughout the country but are concentrated in their domesticated regions or localities. As a consequence, they bear different local names from one region or locality to the other in order of increasing distance. For example, bush okra in the southern part of Nigeria, is known as "ewedu" in Lagos and Ogun state, whereas, it is called, "ooyo" in Osun and Oyo state. Leafy vegetables are seasonal and in abundance shortly after the rainy seasons but become scarce during the dry season. They are sold in many Nigerian markets to meet daily demand as an important complement of staple dishes (FAFUNSO and BASSIR, 1987; MEPBA *et al.*, 2007).

Two of the abundant vegetables in Nigeria are common okra (*Abelmoschus esculentus*) and bush okra (*Corchorus olitorius*); common okra is popularly grown at every nooks and crannies of Nigeria while bush okra is concentrated in southwest Nigeria. Common okra and bush okra are largely cultivated by both men and women for domestic and commercial purposes. Common okra, *Abelmoschus esculentus* (L.) Moench (synonyms, *Hibiscus esculentus* L.), belongs to the family of Malvaceae, and is known as lady's finger in English. It was believed to have originated in south-East Asia and have spread widely in tropical, subtropical and warm temperate regions, but is particularly popular in West Africa (HAMON and SLOTEN, 1995). On the other hand, bush Okra (*Corchorus olitorius* L.) belongs to the family of Tiliaceae, its English name is Jew's mallow. Genetic diversity points to Africa as its first centre of origin (SINGH, 1976**).** At present, *Corchorus olitorius* has widely spread all over the tropics and probably occurs in all countries of tropical Africa (EDMONDS, 1990). It is a leading leaf vegetable in many African countries such as Côte d'Ivoire, Benin, Cameroon, and Nigeria (SCHIPPERS, 2000).

Common and bush okra are mucilaginous vegetable, both the fruit and leaves of these popular vegetables are used as food in Nigeria. Their young immature fruits are important vegetable, consumed cooked or fried. In Nigeria, they are usually boiled in water to make slimy sticky soups and sauces. The young leaves of common okra are commonly used as spinach and sometimes as cattle feed (BURKILL, 1997) while that of bush okra are more valued as cooked slimy sticky sauce, compared to common okra fruit. In Nigeria, sticky sauces from these two vegetables are found suitable for easy consumption of starchy balls made from cassava, yam or millet (AKORODA, 1988). The very small fruits of common okra can fetch a higher price, being of prime quality while Jew's mallow is a high quality leafy vegetable in market value, consumers' preference and nutritional value. The two vegetables are grown under rain fed conditions, the immature fruits of common okra and fresh leaves of bush okra can be conserved by drying, whole or chopped, or by pickling and sell as dried ground for the preparation of this slimy sauce during the dry season.

Common and bush okra mucilage is suitable for medical and industrial applications. Common okra mucilage has been used as a plasma replacement or a blood volume expander and its leaves are sometimes used as basis for poultices, as an emollient and to treat dysuria. Okra mucilage is added as size to glaze paper and is also used in confectionery. Roasted common okra seeds are used in some areas as a substitute for coffee. Tests conducted in China suggest that an alcohol extract of *Abelmoschus* leaves can eliminate oxygen free radicals, alleviate renal tubular-interstitial diseases, improve renal function and reduce proteinuria (TOMODA *et al.*, 1980). On the other hand, Jute mallow has been the most widely used packaging fibre for more than 100 years because of its strength and durability, low production costs, ease of manufacturing and availability in large and uniform quantities. In Kenya, the root scrapings of Jew's mallow are used to treat toothache; in Congo, the root decoction is a tonic and leafy twigs is used against heart troubles; in Tanzania, an infusion of the leaves is taken against constipation; while in Nigeria, the seeds are used as a purgative and febrifuge (EDMONDS, 1990; BURKILL, 2000).

Fresh common okra can be transported quite easily in bulk and kept for a few days without much loss of quality. However, Jew's mallow

leaves cannot be kept long. Mostly, the product is sold on the harvest day, and it is constantly kept wet. If cooled to 20°C it can be kept for about 1 week, in cold storage for several weeks. If the leaves are dried and pounded to powder, the product can be stored for at least half a year (AKORODA, 1988). Since, both vegetables are prepared as a sticky slim sauce by boiling in water and common okra is considered more stable in quality, then it is assumed that a lesser proportion of its nutrients would be lost through the boiling process when compared to Jew's jute. Therefore, the present study was designed to estimate the mineral content of raw and sticky sauce of common and bush okra for recommended daily dietary intake and to compare mineral nutrient loss during preparation of common okra and bush okra sauce. The study will enhance knowledge on boiled and dried mucilage as a means of post harvest handling and storage for maintaining mineral constituents.

MATERIALS AND METHODS

Samples collection and preparation

Indigenous fresh green moderate size of Common okra fruits and leafy Bush okra samples were purchased from three retailers, as three replicates, in three major markets at Ijebu-Ode (New market, Oke-Aje and Ita-Ale). The samples were transported in properly labeled polythene bags to the Chemistry Laboratory, Tai Solarin University of Education, Ijagun, Ijebu-Ode, Ogun State Nigeria. Twenty grams of wholesome samples were handpicked from each of the common okra and bush okra samples, destalked, rinsed thrice in deionized water, drained, chopped into smaller pieces, and divided into two portions.

Preparation of raw vegetables

The first portions were air dried in an air-circulating oven at 65°C for 5hrs for bush okra samples while common okra was dried for 10 hrs. The dried samples were ground in a sample attrition mill (model no. ED-5), sieved into 4mm particle sizes, kept at 4°C and labeled as R (raw) samples.

Preparation of plain sticky sauce samples

The second portions were further chopped into smaller pieces and boiled for 5 min to slim sticky sauce as usually prepared as complement to stew for consumption of starchy staples in the southwest of Nigeria but without salt and then air dried at 55°C until constant weight was assumed, ground and sieved into 4mm particle sizes and labeled S (sauce) samples.

Assay methods

All chemical Analyses were carried out in triplicates using modified methods of the Association of Official Analytical Chemists (AOAC, 2005). A gram of 4mm particle size of common and bush okra samples were combust at 500°C for 5 hrs in a cool muffle furnace and left overnight to cool to room temperature, the residue was weighed as ash content and kept at 4°C for mineral analysis.

Elemental analysis

A half-gram ash of each sample was, subsequently, digested in 2.5 mL selenium/H_2SO_4 mixture (3.5 g Se/1L H_2SO_4) at 200°C. The residue was re-suspended in selenium/H_2SO_4 mixture; Na, Mg, Ca, and K were determined using Jenway Digital Flame Photometer (PFP7 model), phosphorous by the Vanodo-molybdate method, while Fe, Mn, Cu, and Zn were determined using Buck Scientific Atomic Absorption Spectrophotometer (BUCK 210VGP model).

Statistical analysis

Estimation of precision was measured using the statistical analysis system software package [19]. Detection of variation between vegetables, raw and cooked, and purchased markets for minerals were based on Analysis of variance (ANOVA) using General Linear model (GLM) and useful relationships between minerals were estimated using Pearson correlation analysis, in the same SAS software package.

RESULTS

Mineral levels in raw and sticky sauce of common okra and bush okra

Samples of Nigerian common okra and bush okra by different retailers were obtained from three different major local markets in Ijebu-Ode and were assessed for nine macro and micro minerals in both raw and sticky sauce. The precision measures the nine assessed minerals, across all samples of raw and sticky sauce of common and bush okra as estimated in SAS provided in Table 1. In overall samples, the decreasing order of abundance of minerals were as follows: K>Na>Ca>P>Fe>Mg>Zn>Mn>Cu, with the following mean values on a dry weight basis, 376.03±25.37 mg/100g, 250.2±8.58 mg/100g, 176.73±19.88 mg/100g, 82.11±7.47 mg/100g, 4.23±0.44 mg/100g, 2.91±0.33 mg/100g, 2.39±0.26 mg/100g, 0.88±0.12 mg/100g and 0.75±0.08 mg/100g, respectively. The results indicate the abundance of macro minerals over micro minerals but with a fall in Mg. On a clos-

Table 1 - Descriptive statistics between the raw and boiled common okra and bush okra in Ijebu-Ode.

Variable	Mean mg/100g	SD	SE±	CV	Range	Minimum	Maximum	N
Raw-common Okra								
Sodium	258.77	2.5	1.02	0.97	7	255.1	262.1	9
Potassium	351.32	2.4	0.98	0.68	6.5	347.9	354.4	9
Calcium	95.42	2.28	0.93	2.39	5.7	92.4	98.1	9
Phosphorus	60.32	2.3	0.94	3.82	5.9	57.2	63.1	9
Iron	4.24	0.99	0.4	23.25	2.21	3.1	5.31	9
Zinc	2.67	0.99	0.4	36.93	2.23	1.52	3.75	9
Magnesium	2.48	0.72	0.3	29.12	1.62	1.74	3.36	9
Manganese	0.87	0.5	0.2	57.4	1.08	0.42	1.5	9
Copper	1.05	0.21	0.08	19.76	0.48	0.78	1.26	9
Sauce-common Okra								
Sodium	223.17	2.32	0.95	1.04	6	220	226	9
Potassium	235.17	2.79	1.14	1.19	8	231	239	9
Calcium	87.67	2.25	0.92	2.57	5.2	84.9	90.1	9
Phosphorus	47.94	2.32	0.95	4.85	6.05	44.75	50.8	9
Iron	2.1	0.99	0.4	46.96	2.23	0.95	3.18	9
Zinc	1.56	0.99	0.4	63.08	2.28	0.39	2.67	9
Magnesium	1.32	0.72	0.3	54.64	1.62	0.58	2.2	9
Manganese	0.63	0.5	0.2	79.17	1.1	0.17	1.27	9
Copper	0.67	0.21	0.08	31.11	0.47	0.4	0.87	9
Raw-bush okra								
Sodium	278.17	2.79	1.14	1	8	274	282	9
Potassium	487.52	2.4	0.98	0.49	6.5	484.1	490.6	9
Calcium	289.62	2.33	0.95	0.8	6.1	286.4	292.5	9
Phosphorus	127.27	2.25	0.92	1.77	5.2	124.5	129.7	9
Iron	7	1	0.41	14.21	2.46	5.74	8.2	9
Zinc	3.39	0.99	0.4	29.13	2.31	2.2	4.51	9
Magnesium	4.99	0.72	0.3	14.48	1.62	4.25	5.87	9
Manganese	1.43	0.5	0.2	35.12	1.11	0.96	2.07	9
Copper	0.7	0.19	0.09	27.27	0.48	0.38	0.86	9
Sauce-bush okra								
Sodium	264.87	2.46	1	0.93	6.8	261.3	268.1	9
Potassium	457.57	2.61	1.06	0.57	7.4	453.7	461.1	9
Calcium	234.92	2.58	1.05	1.1	7.3	231.1	238.4	9
Phosphorus	96.94	2.32	0.95	2.4	6.05	93.75	99.8	9
Iron	3.55	0.99	0.4	27.77	2.23	2.4	4.63	9
Zinc	1.98	0.99	0.4	49.75	2.3	0.8	3.1	9
Magnesium	2.8	0.72	0.3	25.84	1.63	2.05	3.68	9
Manganese	0.6	0.5	0.2	83.83	1.11	0.13	1.24	9
Copper	0.46	0.21	0.08	44.88	0.46	0.2	0.66	9

SD, standard deviation; SE, standard error; CV, coefficient of variation.

er observation made from the visualized bar graphs, levels of minerals were higher in both raw and sticky sauce of bush okra than common okra except Cu for raw, Mn and Fe for cooked. Levels of all the minerals in the two vegetables were reduced upon boiling (Fig. 1). Based on simple calculations of the estimates obtained in SAS, Common okra lost more of Na, K, Mg, and Cu on cooking than bush okra while the latter lost more of P, Ca and Mn than the former but both lost almost the same amount of Fe and Zn**.** The minimum estimated loss in common okra on cooking and drying was 8.12% for Ca and the maximum was 50.49% for Fe with a mean loss of 30.91% while for bush okra, the loss ranged from 4.78% for Na to 58.10% for Mn with a mean loss of 30.63%. The difference in the overall loss in minerals from the two vegetables could be considered small and insignificant. Therefore, one third of the minerals are lost in the cooking and drying process when packaged as a post harvest handling or

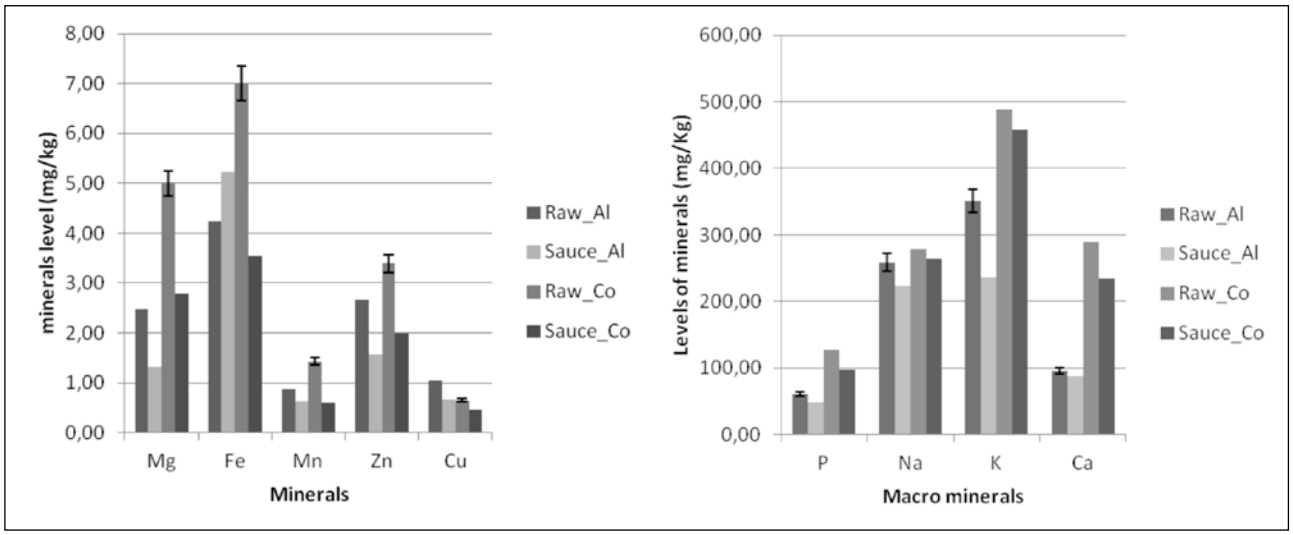


Fig. 1 - Trends of minerals levels in raw and sauce of common and bush okra on dry weight basis.
Raw_Al, raw common okra; Sauce_Al, common okra sauce; Raw_Co, raw bush okra; Sauce_Co, bush okra sauce.

management for later use as slimy sauce. The trend in mineral loss per vegetable in cooking is provided in Fig. 2.

Interaction of vegetable type, purchased market and cooking on mineral content

Analysis of variance (ANOVA) based on General Linear Model (GLM) in the same SAS package, in overall, showed a very large variation in all the assessed minerals ($P<0.0005$) but less variation was observed for Cu ($P<0.005$) and Zn ($P< 0.05$) while Mn was not significantly varied. The variations observed in the nutrients were highly contributed by the differences in the vegetable type and raw/cooking (processing). However, only raw/cooking contributes to the variation observed in Zn. Manganese, though, not generally significant but was significantly varied between raw or cooked vegetable. Therefore, there was a significant difference between common okra and bush okra for Na, P, K, Ca, Mg, Fe, and Cu but no significance difference was observed between the two vegetables for Zn and Mn. The results also showed that cooking has a significantly negative effect on the levels of all the minerals assessed, that is, the levels of minerals reduced on cooking. Effect of market was also tested for only the three micro minerals; Zn, Mn and Cu were significantly varied while the rest were not. Duncan multiple grouping provides a visual variation of the nutrients between the vegetables, raw/boiled, and markets, based on the significant difference between their means, by assigning them into groups using letters A, B and C. A detailed ANOVA measures and Duncan grouping were also given in Table 2.

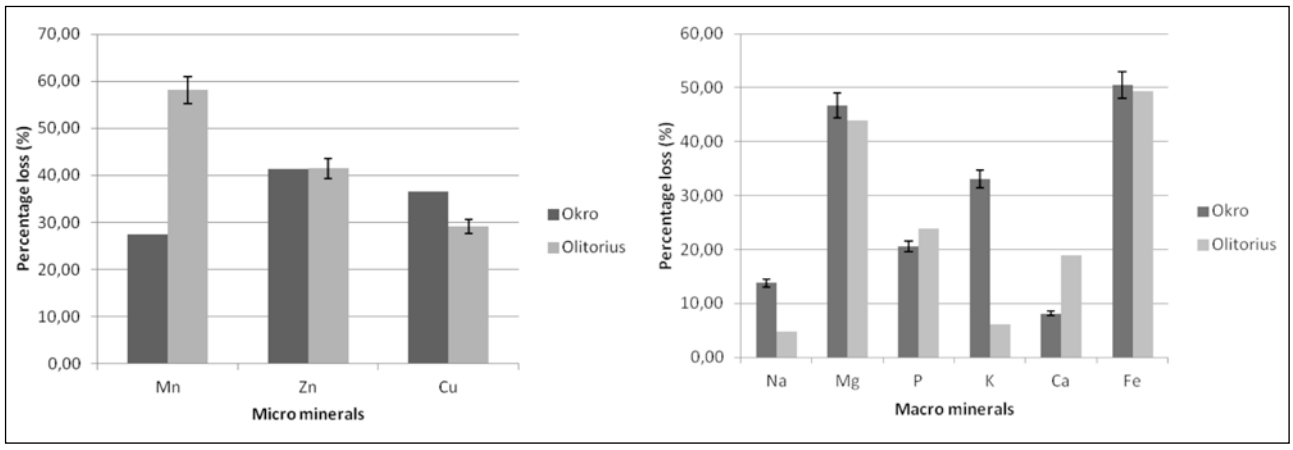


Fig. 2 - Proportion of mineral loss by common okra and bush okra upon cooking at 100°C for 5 min Okro, common okra; Olitorius, bush okra.

Table 2 - Effect of Species type, cooking and purchased market on mineral levels based on GLM-ANOVA.

	Model					Species		Raw/Sauce		Market	
	Mean	CV	SS	R^2	Pr>F	Pr>F	SS	Pr>F	SS	Pr>F	SS
Macro-mineral mg/100g											
Na	256.24	2.58	9187.47	0.91	***	***	5599.81	***	3586.81	ns	101.3
K	382.89	6.20	224919	0.95	***	***	192890	****	32017	ns	101.3
P	83.12	6.5	22901	0.97	***	***	20168	***	2734	ns	101.3
Ca	176.9	7.83	180737	0.98	***	***	174882	***	5850	ns	101.3
Mg	2.9	26.78	40.70	0.77	***	***	23.32	***	16.88	ns	10.45
Micro-minerals											
Fe	4.22	24.39	73.62	0.77	***	**	26.65	***	46.96	ns	19.41
Cu	0.70	30.27	1.04	0.53	**	**	0.55	*	0.50	**	0.85
Mn	0.88	59.64	2.12	0.27	ns	ns	0.40	*	1.71	***	5.02
Zn	2.4	41.22	11.4	0.37	*	ns	1.94	**	9.45	***	19.41
Duncan Grouping	Na	K	Ca	P	Mg	Mn	Fe	Zn	Cu		
By Species											
CommonOkra	B	B	B	B	B	A	B	A	A		
bush okra	A	A	A	A	A	A	A	A	B		
By Process											
Raw	A	A	A	A	A	A	A	A	A		
Sauce	B	B	B	B	B	B	B	B	B		

ns, not significant; *, P<0.05; **, P<0.005; ***, P<0.0005, SS, sum of square.
Means with the same letter are not significantly different.

Table 3 - Correlation Matrix among the minerals based on Pearson model.
Veg_C is degree of common consumption (okra<ewedu), Proc_C, raw to cooking Vice versa.

	Proc_C	Na	K	Ca	P	Fe	Zn	Mg	Mn	Cu
Veg_C	ns	0.74***	0.90***	0.97***	0.92***	0.50*	ns	0.65**	ns	-0.46*
Proc_C	1	-0.58**	ns	ns	ns	-0.68***	-0.53**	-0.54**	-0.46*	-0..56**
Na		1	0.95***	0.79***	0.86***	0.74***	0.45*	0.77***	0.42*	ns
K			1	0.91***	0.93***	0.68***	ns	0.76***	ns	ns
Ca				1	0.98***	0.63**	ns	0.76***	ns	ns
P					1	0.72***	ns	0.82***	0.43*	ns
Fe						1	0.89***	0.97***	ns	ns
Zn							1	0.83***	ns	ns
Mg								1	ns	ns
Mn									1	ns

ns, not significant; *, P<0.05; **, P<0.005; ***, P<0.0005.

Relationship among minerals

Pearson correlation analysis was used to identify useful associations among the nutrients. The matrix generated (Table 3) revealed that Na was strongly significantly correlated with all the other macro minerals (P<0.0005) and Fe but slightly with Zn and Mn. Therefore, as the level of Na increases, the level of all P, Ca, K, Mg, Fe, Zn and Mn also increases and vice-versa. Potassium and calcium were independently correlated with all the macro minerals and Fe but both were insignificant with the rest of micro minerals. Therefore, as levels of K and Ca increases, level of other macro minerals and Fe increases. A strong positive relationship was observed among Fe, Zn and Mg (P<0.005). Manganese on the other hand was correlated with only Na and P while Cu had no relationship with any of the minerals. The correlation matrix also showed that the levels of the minerals were higher in bush okra than common okra except Cu that was inversely correlated while Zn and Mn were insignificant. In addition, the levels of minerals were observed to be inversely correlated with cooking except that of P, K and Ca. Therefore,

cooking reduces the level of these minerals in the assessed vegetables, thereby supporting the results obtained from ANOVA.

DISCUSSION

Adequacy of the Minerals in Common and bush okra

The adequacy of mineral content for estimated average requirements (EAR) and recommended daily allowance / adequate intake (RDA/AI) in both the raw and sticky sauce of common and bush okra per hundred grams were judged using DRIs (DRIs, 2004). The mean levels of potassium (235.51 - 487.52 mg/100g) and sodium (223.17 - 278.17 mg/100g) estimated in the raw and sticky sauce of both common okra and bush okra were too low to meet the RDA/AI for K (4700 mg) and Na (1500 mg). Therefore, the content of sodium in both the raw and sauce of common bush (223.2 - 258.8 mg/100g) and bush okra (264.9 - 278.1 mg/100g) is considered safe due to health impairments such as increased urinary calcium loss and hypertension that could result from excess intake of sodium (WARDLAW and KESSEL, 2002). Since, cooking reduces Na level, then, common okra and bush okra sauces are good in restricted Na diets. Neither the raw nor the sauce of the two vegetables could also provide the daily EAR of calcium (800 mg), although bush okra could provide about 30% estimated average requirement (800 mg) while common bush Okra could only provide about 11%. Therefore, additional supplementary sources of Ca such as fortified cereal and condensed cow's milk (USDA, 2010) were required in the walnut diets. The magnesium level, either in the raw or sauce of the two vegetables (0.67 - 4.99 mg/100g) were too low for any Mg provision in the body when compared with the daily EAR of 330 mg. So, the phosphorus content of the two vegetables (48 - 127 mg/100 g) was also inadequate to provide for 580 mg EAR. Estimated average requirements for Mn is yet to be evaluated but the RDA/AI is 2.3 mg and each of the raw vegetable and sauce could provide an approximately proportion of 25% of the allowance except for 1.43 mg obtained in raw bush okra that could provide about 62%. Both the raw of common okra (1.05 mg/100 g) and bush okra (0.7 mg/100 g) had an adequate EAR content of Cu (700 ug) while their sauce had a proportion of 96 and 66%, respectively. However, raw common okra contains Cu level that is slightly higher than the 900 ug RDA but lower than tolerable upper limit level of 10000 ug, [20], which is reduced upon cooking by 36% (0.67 g) and therefore posed, no health hazard. None of the vegetables, either raw or cooked sauce could meet the daily average requirement and allowance for Zn intake of 9.4 and 11 mg, respectively. Only the raw bush okra met the estimated average requirement of Fe (6 mg), which is 77.7% of daily allowance intake (8 mg) but was reduced upon cooking by 34%, providing about 76.5% EAR while raw common okra could provide for 70.1% EAR, which was also reduced upon cooking by 30%. In overall, plain sauce of these vegetables contains appreciable levels of minerals assessed but did not meet the daily dietary allowance of these minerals except for Cu and Fe. Therefore, the sauce should be taken to complement carbohydrate based meal or soup supplement that contains additional sources of minerals or should be made into a proper soup with top sources of minerals for provision of adequate daily dietary intake of these minerals for health implications.

Interaction of species, cooking and market on mineral levels

Boiling was observed to reduce all the mineral contents in both the common and bush okra with a variation between the two okra vegetables, common okra lost more in macro minerals than bush okra (except in Ca and K) while bush okra lost more of micro minerals. By simple estimation, based on average, about one-third of the mineral content was lost on dry weight basis, when considered for use as a mucilage sticky sauce after boiling. Similar loss due to boiling was reported in both common and bush okra and many other vegetables (MEPBA *et al.*, 2002; AUTA *et al.*, 2011; AYE, 2012; YAKUBU *et al.*, 2012; ILELABOYE *et al.*, 2013). There are also reports on species differences for loss in minerals as presently observed (MEPBA *et al.*, 2002, ILELABOYE *et al.*, 2013; SHAHNAZ *et al.*, 2003). Varying degree of mineral loss due to cooking / boiling were reported in similar studies, a proportion of 6 to 30% mineral loss (ILELABOYE *et al.*, 2013), 25 to 55% (AYE, 2012) and 30 to 54% (YAKUBU *et al.*, 2003). In addition, SHAHNAZ *et al.* (2003) reported a pronounced effect of peeling combined with cooking on mineral loss of some vegetables (14 to 74%). The mineral loss in the present study ranged between 4.0%, for Na in common okra, to 58%, for Mn in bush okra, which is comparable to the previous reported losses. The differences in ranges reported could be largely due to differences in species type, region, analytical methods, varying degree of cooking time and temperature. Whatsoever, there is loss of minerals due to either boiling or cooking, which is as a result of mineral leaches from the vegetables into the cooking water during the process. Most of the proteins that contain the inorganic elements such as prosthetic groups are denatured during the process with loss of these minerals into the boiling water. Market effect was limited to three micro minerals, Mn, Cu and Zn which could be attributed to diverse agricultural practices from different

sources, soil fertility, environments and use of fertilizers. Similar useful associations obtained among the minerals have earlier been observed in food crops. The strong association obtained between Fe and Zn in the present study corroborates that of similar studies in plants (BEEBE *et al.*, 2000; SILVA *et al.*, 2012).

CONCLUSIONS

The study detected appreciable levels of P, Na, Mg, K, Ca, Fe, Mn, Cu, and Zn in both common and bush okra per 100g which were reduced upon cooking at 90-100°C for 5 min. The two species differed widely in their levels of mineral content in both raw vegetable and sticky sauce. They also lost specific mineral on cooking at different proportion but had an overall equal proportion of a mean percent loss. There was also an influence of market differences on Cu Mn and Zn. Dried mucilage as a postharvest management for later use such as sticky sauce reduced the mineral content by a mean proportion of about 30%. Plain soup of these vegetables either fresh or after storage requires additional sources of these minerals, except Fe and Cu, to meet recommended daily intake but are good sources for Na and any of these minerals restricted diets.

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PAPER

CHILDREN PREFERENCES OF COLOURED FRESH CHEESE PREPARED DURING AN EDUCATIONAL LABORATORY

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ABSTRACT

Choices among young consumers are mainly driven by food preferences; in particular, a connection between appearance and acceptance of food has been highlighted, together with a general lack of knowledge of food processing. For these reasons, educational activities are important to increase scientific knowledge and awareness. The cheese-making educational laboratory described herein involved children, adolescents, and their parents/teachers in the preparation of fresh and naturally-coloured cheeses. At the end of the activity, both the colour preference and possible relation between preference and colour of cheese prepared were investigated administering a short questionnaire.

- Keywords: cheese preference, children food preference, colour preference, gender, neophobia, nutrition education -

INTRODUCTION

Food preference has a fundamental role in driving consumer's choices and habits, especially in children (COOKE, 2007; NICKLAUS *et al.*, 2004, LAUREATI *et al.*, 2015a). Therefore, the number of food products specifically developed for children is growing and this market is acquiring increasing importance (ISSANCHOU, 2015).

Consequently, the youngest consumers are frequently involved in research and development programs, since their food habits will influence choices as they grow older and because their preferences, even if partly driven by advertisements (USTJANAUSKAS *et al.*, 2014), seem to be strictly related to the sensory aspects of food (PAGLIARINI *et al.*, 2005; MUSTONEN and TUORILA, 2010).

In 1994, Moskowitz (MOSKOWITS, 1994) highlighted how a visually pleasing product tends to be more appreciated by children, as visual attributes seem to be, among different sensory characteristics, those that mainly determine its success (KILDEGAARD *et al.*, 2011; TOPCU, 2015). Indeed, children tend to create an "ideal picture" of each food product that can be related to their own idea of "good"; this picture represent a sort of reference point that can be used to dislike products whose appearance is not close to their expectation (MUSTONEN and TUORILA, 2009).

This phenomenon, called food neophobia, is defined as the fear of eating new or unfamiliar food (PLINER and HOBDEN 1992) and is related to both the quality and variety of diet (LAUREATI *et al.*, 2015b).

Nutritional education and food-related diseases prevention activities can be used for children and teenagers in order to: *i)* reduce the impact of poor food habits on health, and *ii)* avoid food neophobia, mainly responsible for refusing consumption of fruits and vegetables (WARDLE *et al.*, 2003). Moreover, a lack of awareness of procedural knowledge, both in terms of food processing and nutritional values, must be taken into account (WORSLEY, 2002), highlighting the link between traditional food preparation and sensory properties (LAUREATI *et al.*, 2006).

Nowadays, food education is mainly based on the social-cognitive theory (SCT) (BANDURA and ADAMS 1977), which incorporates the interaction of personal, environmental and behavioural factors. According to this theory, principles that are highly influential in establishing changing food behaviour in children are both models and repeated exposure.

Among the models, those that have been found to be effective in children include cartoon characters, peers, mothers, unfamiliar adults and teachers; moreover, children seem to be influenced more by the behaviour of multiple rather than single models (LOWE *et al.*, 2004). Furthermore, according to Zajonc's "mere exposure" theory (ZAJONC, 1968), repeated exposure to a specific food increases the liking and consumption of that food (COOKE *et al.*, 2011; WARDLE *et al.*, 2003B) through a mechanism that is believed to be a "learned safety" behaviour (KALAT and ROZIN, 1973). According to this theory, repeated ingestion of an unfamiliar food without negative consequences leads to increased acceptance of that food.

Several educational interventions for food have investigated all these factors, reporting promising results, especially for consumption of fruits and vegetables (EVANS *et al.*, 2012; LAUREATI *et al.*, 2014; REVERDY *et al.*, 2008). Both principles of imitation and repeated exposure characterise food laboratories aimed at involving children in food preparation, thus familiarising them with food ingredients and technologies. Furthermore, it has been reported that the involvement of children in food preparation can contribute to increasing their acceptance for that food, according to the principle of repeated exposure (CHU *et al.*, 2012).

For all these reasons, food educational interventions can play a relevant role in driving choice, interest and preference of children through better awareness of both sensory characteristics and technological process of traditional food products (MUSTONEN and TUORILA, 2010).

The educational cheese-making activity reported herein, called *Cheese making in one hour*, was conceived by a committee of referees as part of the Festival della Scienza 2014 (which took place in Genoa, Italy, from October 24 to November 2, 2014) and is considered as a "teaching tool" in order to increase scientific information about the cheese-making process.

This practical laboratory took the form of a family entertainment during which children and parents or teachers could share principles of traditional cheese making, spending some family or educational time away from daily work, but related to increased knowledge of food science.

The activity involved children, teenagers and adults (age from 6 to 87 years) in the traditional preparation of a fresh "*primo sale like*" cheese. Cheeses were naturally coloured (using turmeric, rocket and beetroot juice), and in order to keep the attention of participants, each was actively involved in the process by preparing his/her personal small cheese.

At the end of the activity, both the colour preference and possible relation between the colour of cheese prepared and preference were investigated by administration of a short and simple questionnaire.

MATERIALS AND METHODS

The cheese-making activity involved 738 participants (395 females and 343 males) with an age between 5 and 87 years and divided in groups of no more than 30 individuals. Both families and school classes could participate in the activity by asking the Festival staff to make

Table 1 - Natural dyes and their amounts are reported together with the final colour of each cheese and its visual aspect.

	Cheese colour and appearance	Dye	Amount (g/L of milk)
	Yellow	Turmeric	5
	Pink	Beetroot juice	20
	White and green	Rocket	20

a reservation; given the popularity of the Festival, mainly represented by children and partly by parents/teachers, a large number of participants were enrolled. Each participant had an active role in the laboratory, giving his/her contribution to one of the 4 phases of cheese making: 1) adding starter, rennet and natural flavour to milk; 2) curd cutting and synaeresis; 3) curd breaking and purge; 4) cheese shaping. During the Festival, the laboratory was repeated at least 3 times a day by 4 previously-trained scientific facilitators. Due to the fact that the cheese-making laboratory should last only one hour (related to the timing of the Festival), one pot (Bartscher GmbH, Germany) was dedicated to each of the 4 main phases; in this way, participants could move from one pot to the next instead of proceeding step-by-step using a single pot such that each of the phases could be observed in a short length of time.

Cheese-making phases

1) Starter, rennet and milk natural flavouring

Fresh whole cow milk was heated to 36° C and 17 g/L of natural whole yoghurt was added as a starter. In order to produce coloured cheeses, different natural dyes were added (rocket, turmeric and beetroot juice) as reported in Table 1. While turmeric and beetroot juice were added to the milk 30 minutes after the addition of starter, together with 0.5 ml/l milk of liquid rennet (80% chymosin, 20% pepsin, provided by Graco), rocket was added during the last step of cheese shaping.

2) Curd cutting and synaeresis

After enzymatic coagulation, due to the addition of liquid curd, the gel obtained was cut into 4 × 4 cm squares, and 30 minutes later participants could observe the phenomena of curd synaeresis.

3) Curd breaking and purge

The squares of curd were broken into pieces of approximately 1.5 cm; during this phase, rocket was added (20 g/l milk) for preparation of green cheese.

4) Cheese shaping

Finally, the small pieces of curd were collected in dedicated shaper (60 g cheese shaper in polypropylene and polyethylene, Tecnolatte srl, Italy) by each participant as shown in Fig. 1.



Fig. 1 - Children at the cheese-making educational laboratory, during collection of a yellow-curd into a dedicated shaper.

5) Questionnaire

At the end of the cheese-making laboratory, each participant was administered a dedicated and simplified questionnaire. The questionnaire contained a first part regarding personal information: age and gender. Next, each participant was asked to provide information on: *i)* which colour of cheese he/she preferred and *ii)* which one he/she prepared. The filled questionnaires were collected in three dedicated paper boxes, one for each cheese colour.

STATISTICAL ANALYSIS

Preference data were analysed according to chi square test ($p>0.05$). Statistical analysis of data was carried out using the SAS/STAT statistical software package version 9.3.1. (SAS Institute Inc., Cary, USA).

RESULTS

The cheese-making laboratory was visited by 1900 participants. Many questionnaires were incomplete, while some participants preferred to not give their opinion. Data from 738 participants was collected considering the gender dimension and 5 age subgroups (1: 5-7 years old, 77 participants; 2: 8-10 years old, 142 participants; 3: 11-13 years old, 182 participants; 4: 14-18 years old, 203 participants; 5: >18 years old, 134 participants); subsequently, the visual preference for one of the three coloured cheeses was considered, as reported in Table 2. Data were divided in the 5 subgroups reported above, selected mainly according to different stages of cognitive development (GUINARD, 2001). In addition, different food preferences and consumption behaviour observed in these age groups confirmed that this type of age subdivision is relevant (LAUREATI *et al.*, 2015).

Considering colour preference, regardless of age, the majority of participants preferred the white and green cheese (333 vs. 243 yellow and 162 pink; chi square=59.49; $p<0.0001$), which could be considered as the most "traditional" because it is present on the Italian market, rather than pink or yellow cheeses, and probably seen for the first time during the laboratory activity by the most of the respondents.

As reported in Table 2, sorting by age, it could be seen that the youngest participants (between 5 and 7 years old) chose the pink cheese as their favourite, while next oldest age subgroup (8 to 10 years) preferred yellow. Subjects from 11 years old and older chose the white and green cheese as their favourite, as did the overall population.

This result may be associated with both the attractive effect of colours on the children and to the tendency of the youngest, as observed by Moskowitz (MOSKOWITZ, 1994), to prefer food products with a pleasing appearance. Starting from the age of 11, children started to prefer, in accordance with adults, the white cheese mixed with rocket.

Moreover, the youngest children's preference can be explained by food neophobia, which is particularly high for fruits and vegetables, reaching its highest level between 2 and 6 years (PELCHAT and PLINER, 1995; PLINER, 1994; PLINER and LOEWEN, 1997; LAUREATI *et al.*, 2015c). It then tends to decrease when children move towards adolescence (ADDESSI *et al.*, 2005), finally becoming relatively stable in adulthood, probably because of an increased number of food experiences (COOKE and WARDLE, 2005).

On the other hand, the preference of older respondents for a white cheese enriched with a vegetable (rocket), may be linked to a low acceptance of adults for an innovative food, while its dislike among the younger interviewees could be related to the visual presence of rocket inside cheese (RUSSEL and WORSLEY, 2008; LAUREATI *et al.*, 2015).

It has already been demonstrated that traditional food products have been always recognised by consumers as linked to a specific geographical origin and highest sensory quality (GUERRERO *et al.*, 2009); as a consequence, this cheese was probably considered much more similar to product already on the Italian market compared to the pink and yellow ones.

Table 2 - Non-parametric statistical analysis of data to identify significant differences in preference of cheese colour by age. Data were analysed using a Chi square test.
Interviewees were subgrouped by age: 5-7, 8-10, 11-13, 14-18, >18 years.

Age subgroup (years)	Preferred cheese			Total	Chi square	df	P value
	Yellow	Pink	White and green				
5-7	27	35	15	77	7.9	2	0.02
8-10	69	28	45	142	17.9	2	0.00
11–13	59	34	89	182	25.0	2	0.00
14-18	57	44	102	65	27.4	2	0.00
>18	31	21	82	134	47.9	2	0.00
All	243	162	333	738	59.5	2	0.00

Table 3 - Non-parametric statistical analysis of data to identify significant differences in preference of cheese colour by gender and age. Data were analysed using a Chi square test.
F= female; M=male; df=degrees of freedom. Interviewees were subgrouped by age: 5-7, 8-10, 11-13, 14-18, >18 years.

Prefered cheese	Gender	Age subgroup (years)					
		5-7	8-10	11-13	14-18	>18	Total
Yellow	F	10	24	31	18	25	108
	M	17	45	28	39	6	135
	Total	27	69	59	57	31	243
	Chi square	1.8	6.4	0.2	7.7	11.6	3.0
	df	1	1	1	1	1	1
	pvalue	0.2	0.0	0.7	0.0	0.0	0.1
Pink	F	29	23	22	23	18	115
	M	6	5	12	21	3	47
	Total	35	28	34	44	21	162
	Chi square	15.1	11.6	2.9	0.1	10.7	28.5
	df	1	1	1	1	1	1
	p value	0.0	0.0	0.1	0.8	0.0	0.0
White and green	F	4	20	47	47	54	172
	M	11	25	42	55	28	161
	Total	15	45	89	102	82	333
	Chi square	3.3	0.6	0.3	0.6	8.2	0.4
	df	1	1	1	1	1	1
	p value	0.1	0.5	0.6	0.4	0.0	0.5

Sorting by gender (Table 3), no differences in preferences were seen regarding the white and green cheese, except for the oldest group (>18 years). A clear gender-related difference was, however, found for the pink and the yellow cheeses: the former seemed to be much more appreciated by females (all age classes except 14–18 years, but only if $p<0.10$ is considered as marginally significant), while the latter was favoured among males (especially from 8-10, 14-18).

In order to determine if this could influence the visual preference of participants, they were also questioned about the colour of the cheese they prepared; however, a significant association between cheese preferred and colour preference for one of the three coloured cheeses was not seen, even if some significant differences were seen among the youngest consumers (Table 4).

In particular, when sorting by age, the subgroup from 8 to 10 years who prepared the yellow cheese more frequently preferred this cheese over the others (26 of 52, chi square=7.5 p=0.02). Likewise, the subgroup from 5 to 7 years who prepared the pink cheese preferred it more frequently than others (12 of 21 choices, chi square=7.1 p=0.03). However, the older participants did not show a consistent trend of preferred cheese over than prepared.

Data from literature suggest that 7–8 exposures are needed to produce a learning effect

Table 4 - Non-parametric statistical analysis of data to assess if the colour of sample prepared could affect the overall colour preference. Data were analysed using Chi square test.
F= female; M=male; df=degrees of freedom. Interviewees were subgrouped by age: 5-7, 8-10, 11-13, 14-18, >18 years.

Prepared chesee	Age subgroup	Preferred cheese						
	(years)	Yellow	Pink	White and green	Total	Chi square	df	P value
Yellow	5-7	15	14	8	37	2.3	2	0.31
	8-10	26	10	16	52	7.5	2	0.02
	11-13	21	9	37	67	17.7	2	0.00
	14-18	18	17	30	65	4.8	2	0.09
	>18	14	9	39	62	25	2	0.00
Pink	5-7	7	12	2	21	7.1	2	0.03
	8-10	19	10	9	38	4.8	2	0.09
	11-13	9	12	18	39	3.2	2	0.20
	14-18	22	16	37	75	9.4	2	0.01
	>18	6	6	19	31	10.9	2	0.00
White and green	5-7	5	9	5	19	1.7	2	0.43
	8-10	24	8	20	52	8	2	0.02
	11-13	29	13	34	76	9.5	2	0.01
	14-18	17	11	35	63	14.9	2	0.00
	>18	11	6	24	41	12.6	2	0.00

that can influence consumer appreciation regarding a product (MAIER *et al.*, 2007). Thus, it might well be that the single exposure during the cheese-making educational activity was not enough to influence children choice.

CONCLUSIONS

The results of this study highlight how visual preference for a food, in terms of colour, changes during different stages of life. Indeed, the data demonstrates how children can be influenced by food appearance and how the aspect of a product can be related to its acceptance, especially among younger individuals; in fact, the youngest participants tended to prefer intensively coloured cheese vs. the white and green version, probably because of the presence of rocket. Moreover, the cheese-making laboratory was considered to be a useful tool in order to catch and keep participants' attention, involving them in a practical activity while sharing educational and scientific information related both to food processing (fresh cheese production) and sensory (visual) aspects.

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Acknowledgments. Acknowledgments of assistance are appropriate provided they are not related to analyses or other services performed for a fee. Financial support, thanks for assistance, artic1e number or thesis fulfilment may be included.

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