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PAPER

EFFECT OF SPIRULINA (*SPIRULINA PLATENSIS*) ADDITION ON TEXTURAL AND QUALITY PROPERTIES OF COOKIES

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ABSTRACT

Nowadays scientists are looking for new food ingredients that are not seasonal and are rich in bioactive compounds, such as microalgae.
The aim of this study was to enrich wholegrain cookies with microalgae (*Spirulina platensis)* powder. 1%, 2% and 3% of spirulina was used to fortify the cookies. Their physical, textural and sensory properties were analyzed.
The addition of even small amounts of the microalgae (1%) changed color of the cookies significantly to intensive green. Decrease of moisture content and hardness of the cookies was correlated with addition of microalgae powder. It was observed that hardness measured by sensory analysis increased with the spirulina content. Microalgae had a negative impact on the overall sensory quality.

Keywords: microalgae, spirulina, biscuits, sensory analysis, texture analysis

1. INTRODUCTION

Snacks such as cookies have low water content, which protects them from microbial spoilage and provides longer shelf life. Cookies are very popular, easy-to-eat products consumed all over the world. As such, they could be great carriers of nutritionally valuable compounds. Unfortunately, such products in the market very often contain fat rich in saturated fatty acids and *trans* fatty acids. However, products with wholegrain flour and dried fruits are becoming more popular due to the consumers' increasing awareness of healthy life style. Scientific papers report possibilities of using bioactive compounds such as: dietary fibers (ONACIK-GÜR *et al*., 2015; DEMIRKESEN, 2016; ZBIKOWSKA *et al*., 2017), plant extracts (MILDNER-SZKUDLARZ et al., 2009; KOZŁOWSKA et al., 2014), fruit pomaces in bakery products (BAJERSKA *et al.* 2016) and legume flours (CHENG and BHAT, 2016). Addition of such ingredients may very often have adverse impact on textural properties and sensory characteristics. Fibers and ingredients with high protein content may increase hardness of bakery products. Fruit pomaces and plant extracts significantly change color of products. There are still a few papers presenting *Spirulina platensis* as an ingredient increasing nutritional value of such products and its influence on textural properties and quality characteristics.
Microalgae such as *Chlorella* spp., *Dunaliella* spp., *Scenedesmus* spp. and *Spirulin*a spp. are becoming more and more popular as new, highly nutritious food ingredient. They are rich in easily digestible protein, fat with a high content of unsaturated essential fatty acids, vitamins, minerals, carotenes and chlorophyll (PELIZER *et al*., 2015; KAY and BARTON, 1991). Moreover, they contain fat rich in unsaturated fatty acids, half of which is γ-linolenic acid (GLA). GLA is particularly important because it plays many important functions in the human body and it prevents and helps treatment of many diseases (BIAŁEK and RUTKOWSKA, 2015). Spirulina is also rich in A and B group vitamins (TANG and SUTER, 2011). It has an especially high content of B12 vitamin, which is particularly important for vegans since it is one of very few sources of this vitamin for people who do not consume meat and dairy products. Moreover, it may display anticancer activity because, according to an in-vitro assay, polysaccharides obtained from this microalga have strong scavenging effects in vitro on DPPH and hydroxyl radicals (KURD and SAMAVATI, 2015).
Microalgae such as Spirulina and Chlorella are sold in Europe as dietary supplements, without any kind of processing except drying. Most of them are produced in Asia. Such supplements contain (per 100g): 55-70g of protein, 2-6g of fat, 0.6-1g of chlorophyll and 0.1-0.4g of carotenoids, minerals (calcium 0.5-1g, magnesium 0.2-0.6g, iron 30-100 mg, zinc 2-4 mg, selenium 10-30 μg) and vitamins (A 100-200 mg, 1.5-4 mg B1, 3-5 mg B2, 10-30 mg B3, 0.6-0.8 mg B6, 0.05-1.5 mg B12, 5-10 mg E) (LIANG *et al*., 2004)
Spirulina (cyanobacteria) occurs naturally in subtropical lakes. This microalga has a spiral shape and green-blue color. Cyanobacteria were known and used by Aztecs hundreds of years ago to produce cakes (HABIB *et al*., 2008). Microalgae were used in other food systems such as: noodles (5g of Chlorella and Spirulina per 90g of wheat flour) (KUMORO *et al*, 2016), gluten free bread (3 and 5 % of Spirulina supplementation) (FIGUEIRA *et al*., 2011), cookies (1 and 3% of *Isochrysis galbana*) (GOUVEIA *et al*., 2008). Microalgae are used in food not only because of their bioactive compounds but also as salt and gluten-replacers. Addition of even small amounts of Spirulina and Chlorella has a strong impact on sensory characteristics. It changes color, smell, taste and texture of a product (KUMORO *et al*., 2016). That is why it can be assumed that addition of spirulina to cookie recipes may significantly influence both physical parameters and overall acceptability.
The aim of this work was to analyse physical and sensory properties of cookies supplemented with different amounts of spirulina.

2. MATERIALS AND METHODS

2.1. Materials

The research materials were cookies and cookie dough. The following ingredients were used in cookie production: flour mixture (1:1:1:1 of wheat - ash 0.5%, wholegrain wheat - ash 2%, wholegrain oat - ash 2%, wholegrain barley - ash 1.6%) 47.7% (Młyny Kruszwica, Polska), palm fat 6.2% (Bunge Poland), high-oleic sunflower oil 24,9% (Bunge Poland), sugar 20.7%, baking powder 0.1%, rapeseed lecithin 1.8% (Bunge Poland), powder spirulina – *spirulina platensis* (MyVita natural supplements), 5% water. The cookies were made in four variants: 0%, 1%, 2% and 3% addition of spirulina to the whole dough weight as a flour replacer. Spirulina powder is rich in bioactive compounds and its daily intake should not exceed 2.5 g. Palm fat, oil, lecithin and water were mixed for approximately 5 minutes with a kitchen processor Braun Multiquick (type 4644) until the emulsion was fully homogenized. Sugar was then added and the whole dough was mixed for another 5 minutes. The remaining dry ingredients were mixed and kneaded until the consistency of dough became uniform. The dough was subsequently flattened with a rolling pin down to a 6 mm sheet and cut into 55mm diameter circular shapes. The cookies were baked in a convection oven (UNOX, Italy) at 170°C for 10 minutes. The cookies were packed in polyethylene bags and stored for 9 weeks at room temperature without access to light.

2.2. Density of dough

Density of the dough was measured by pressing the dough into a glass-weighting bottle with a capacity of 30 cm^3. Then the bottle with the dough was weighed and the density was calculated (ONACIK-GÜR *et al.*, 2015). The above assay was repeated 3 times for each sample.

2.3. Texture analysis of cookie dough

The texture of cookie dough was calculated by penetration test using a texture analyzer TA.XT plus (Stable MicroSystems, UK, 5 kg load cell). 110 g of the dough was formed into a ball and put on a metal dish from a dough preparation set A/DP. The firmness was measured with an edged cylinder with a diameter of 6 mm (P/6), which plunged the sample 20 mm deep with a test speed of 3mm/s. The firmness was defined as resistance to the penetration and measured by the maximum force (in newtons). The adhesive force was the maximum negative measured force needed to take the plunger out of the dough with a speed of 3 mm/s. The test was conducted in triplicate.

2.4. Physical characteristics of cookies

After baking and cooling down to ambient temperature, the thickness (T) and diameter (D) of cookies were measured. The spread ratio (D/T) was calculated by dividing the diameter by thickness (DEMIRKESEN, 2016).
The density of cookies was calculated from the weight and volume of eight cookies. The volume was determined by rapeseed displacement method (REHMATI and TEHRABI, 2014). The above assay was performed 3 times for each sample.

2.4. Cookie moisture

5 grams of crushed cookies were dried in a laboratory convection dryer (SUP 100, Poland) at 130 °C for 1 hour. Samples were weighed before and after drying. The moisture of cookies was calculated from the difference and expressed in %. Above assay was run in triplicate (ONACIK-GÜR *et al.*, 2015).

2.6. Texture of cookies

Texture analysis of cookies was conducted by a three-point bending test (HDP/3PB edge), carried out at ambient temperature with a TA.XT plus Texture Analyzer (Stable MicroSystems, UK). The span length was 40 mm and the compression test speed was 3 mm/s. The end result was an average of 9 repetitions, hardness determined in N and fracturability in mm.

2.7. Color

The cookie and cookie dough color was measured with a chromameater Konica Minolta CR-200 in CIE L*a*b* system. The color parameters were determined by: L* lightness (0-black, 100-white), a* (-a* green, +a* red) and b* (-b* blue, +b* yellow). The cookies were scanned at three different points to determine the average as an end result.
The following parameters were calculated based on the results (Chroma – color saturation, BI – browning index, ΔE – total color differences) (BAL *et al.*, 2011):

$$\Delta E = \sqrt{(L_c - L_s)^2 + (a_c - a_s)^2 + (b_c - b_s)^2}$$

where: L_c – parameter L* of control cookies, L_s – parameter L* of analyzed cookies; a_c – parameter a* of control cookies, a_s – parameter a* of analyzed cookies, b_c – parameter b* of control cookies, b_s – parameter b* of analyzed cookies.

$$Chroma = \sqrt{a^{*2} + b^{*2}}$$

$$BI = \frac{100\,(X - 0.31)}{0.17}$$

where:

$$X = \frac{a^* + 1.75\,L^*}{5.645L^* + a^* - 3.01b^*}$$

2.8. Sensory analysis

The profile method was used to determine sensory properties of the cookies. 20 individuals evaluated the cookies. All the panelists had completed sensory analysis classes and passed the sweet and salty threshold test and were trained for profiling method. The sensory analysis was conducted in laboratory conditions. Each person was given one cookie of each variant and an evaluation card with instructions concerning the evaluation procedure. 10 cm unscaled line was used to rate each of the discriminants: typical odour

(typical for wholegrain cookies), algae odour, browning uniformity, dark color, hardness, crispiness, taste (typical for wholegrain cookies), algae taste and overall sensory quality (all sensory properties which influence acceptability and quality). Intensity of the discriminants was increasing from left to right side of the line. Samples were presented in the same containers in randomized order and labeled with three digit random numbers.
First, the evaluators examined odour and appearance of the cookies. After the first bite the hardness of the product was evaluated. Hardness is the first experienced sensation and then crispness, which is noticeable 10 seconds after the first bite. Crispness is a sensation of brittleness in the mouth, when the teeth crack the product during mastication, with multiple fractures at low force loads. After chewing, soaking the bite in saliva and swallowing, when all the taste substances reached taste buds, the panelists evaluated the taste of cookies. In the end, the evaluators rated the overall quality of products, taking into account all the discriminants (LAGUNA *et al.*, 2013; ONACIK-GÜR *et al.*, 2015).

2.9. Statistical analysis

Statistical analysis was performed by means of a computer program Statistica 12.0 (StatSoft, USA). The data was subjected to ANOVA including post hoc comparison Tukey's test, at the probability level α = 0.05 to determine significant differences. Moreover, Person's correlation was carried out for the results, p-value ≤ 0.05.

3. RESULTS AND DISCUSSIONS

3.1. Physical properties and texture of cookie dough and cookies

Addition of spirulina did not have significant effect (Table 1) on cookie dough and cookie density. No significant differences were observed in geometry of cookies, either. However, it was found that addition of microalgae powder decreased the spread ratio of cookies. Other researchers found that addition of fiber may have such impact on spread ratio of cookies (GUPTA *et* al., 2011).

Table 1. Physical properties of cookie dough and cookies.

Spirulina addition [%]	Cookie dough density [g/cm^3]	Diameter [mm]	Thickness [mm]	Cookie density [g/cm^3]	Spread ratio [D/T]	Water content [%]
0	1.08±0.04^a	58.56±1.43^a	8.02±0.28^a	0.85±0.01^a	7.31±0.12^b	4.16±0.05 ^{b}
1	1.12±0.09^a	58.86±1.06^a	8.33±0.27^a	0.91±0.01^a	7.05±0.16^a	4.05±0.05 ^{b}
2	1.11±0.09^a	58.70±1.00^a	8.31±0.21^a	0.94±0.05^a	7.06±0.15^a	3.83±0.09 ^{a}
3	1.11±0.09^a	58.64±1.18^a	8.27±0.36^a	0.93±0.11^a	7.08±0.14^a	3.79±0.06 ^{a}

*a, b, c - describes homogenous groups.
p-value ≤ 0.05.

However, the addition of microalgae decreased the moisture content of the baked product (Table 1). Decreasing water content in the product can be related to a partial reduction of flour, which was replaced by spirulina. Wholegrain flours used to produce these cookies, since they were rich in fiber, which increased the water absorption (SOBCZYK, 2012).

Addition of spirulina did not significantly affect the firmness and adhesive force of cookie dough. Even when the enrichment level of microalgae powder increased, the dough had the same textural parameters (Fig. 1).

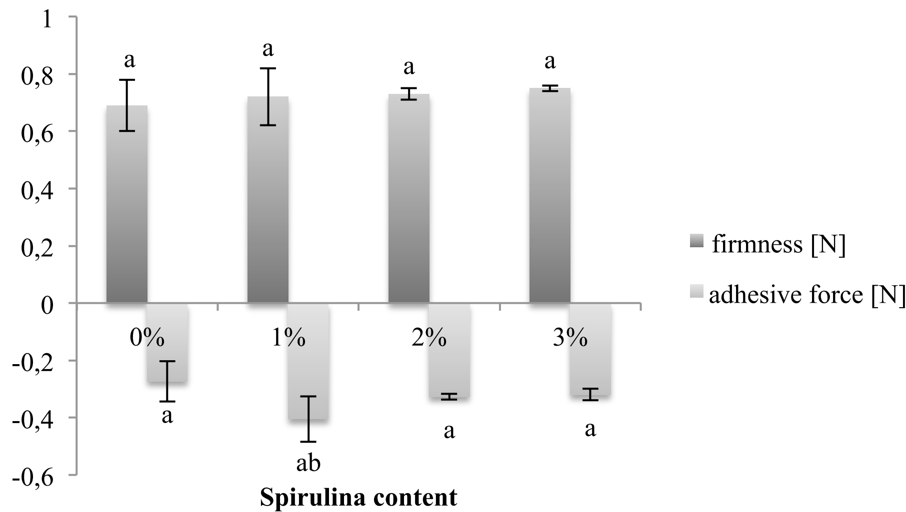

Fig. 1. Textural properties of cookie dough.
* a, b - describes homogenous groups, p-value ≤ 0.05.

However, the addition of algae decreased the hardness of cookies. Statistically significant differences were not observed among additions at the levels of 1, 2 and 3% of spirulina. However, a tendency was found showing that the hardness of cookies decreased in line with microalgae powder content (Fig. 2). GOUVEIA et al. (2007) arrived at opposite conclusions in their study, where the hardness of cookies was increasing with the microalgae level enrichment. In our study, microalgae powder was added to the recipe as a flour replacer, which is rich in fiber and absorbs high quantities of water, while spirulina is rich in protein (60%) (PELIZER *et al.*, 2015). A partial replacement of flour caused a decrease in fiber content, which significantly changed the water absorption. As a consequence, the structure was less compact and the hardness decreased (GOUVEIA *et al.*, 2008; SUDHA *et al.*, 2007). SHYU and SUNG (2010) observed a decrease in hardness with increasing addition of γ-polyglutamic acid obtained from *Bacillus* spp.
Addition of spirulina did not change significantly the fracturability of cookies on the first day after baking. However, the difference was visible during storage. In the 6th and 9th weeks, it was found that the fracturability of cookies decreased with spirulina enrichment level (Fig. 3).
Texture of cookies during the storage did not change significantly. Products with 1% addition of spirulina had a slightly lower hardness in the 9th week. Nonetheless, it was found that fracturability of cookies decreased at the time of storage.

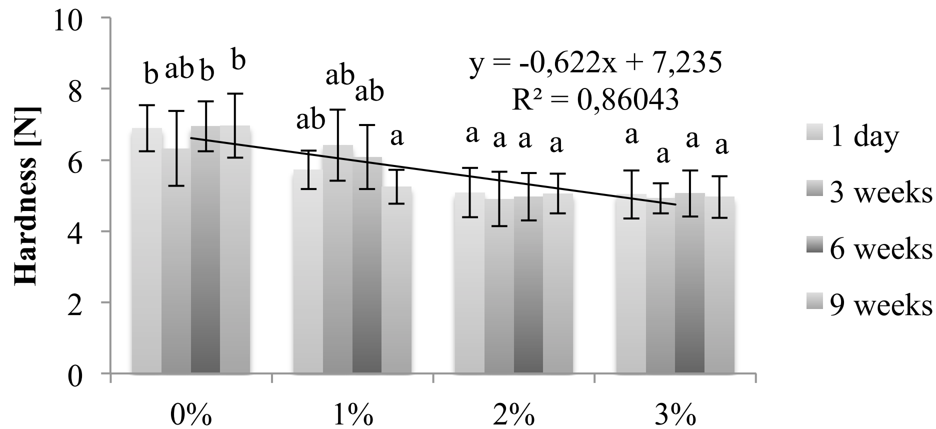

Fig. 2. Hardness of cookies.
* a, b - describes homogenous groups, p-value ≤ 0.05.

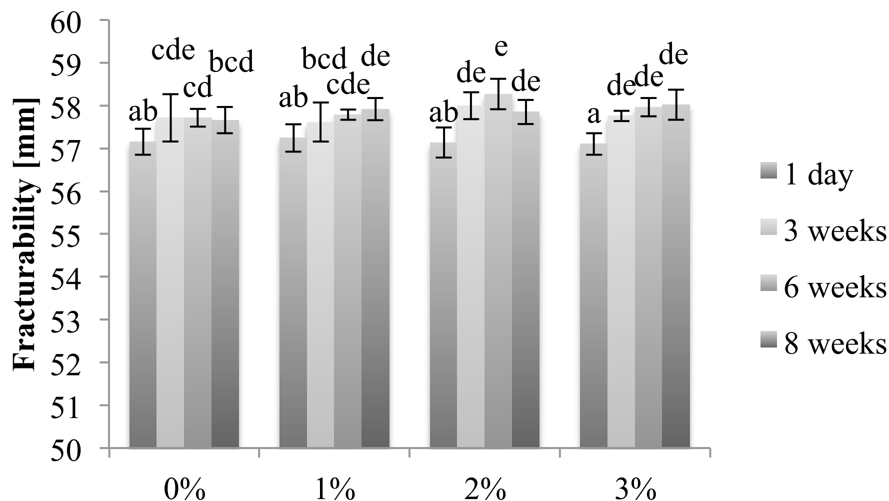

Fig. 3. Fracturability of cookies.
* a, b, c, d, e - describes homogenous groups, p-value ≤ 0.05.

3.2. Color of cookies and cookie dough

Color parameters of the cookie dough and cookies changed statistically significantly depending on the amount of spirulina addition. Parameters L* (white/black), a* (red/green) and b* (yellow/blue) were decreasing for cookie dough and cookies with increasing content of microalgae powder. It shows that baked products and dough were becoming more green-blue with increasing content of spirulina in the recipe. Similar results were obtained by GOUVEIA *et al*. (2008) who added microalgae *Isochrysis galbana* to cookies.

ΔE value increased in products before and after baking by adding higher amounts of spirulina. Chroma indicates the saturation – intensity of color (BAL *et al.,* 2011) and the highest values were obtained for the control sample (without a spirulina addition) despite

a strong green color of samples with microalgae addition. The sample of cookie dough with the lowest addition of spirulina (1%) has the lowest color saturation value, which increased with larger quantities of this additive. Color saturation results for the baked cookies were the reverse, as chroma decreased with increasing addition of spirulina.
Browning index (BI) indicates the purity of brown color, which is particularly important when products are dried (BAL *et al.*, 2011) or, in the case of cookies, baked. This index decreased with the addition of spirulina and rose after the same samples were baked (Table 2).

Table 2. Color parameters of cookie dough and cookies.

Spirulina addition		Color parameters L*	a*	b*	ΔE	Chroma	BI
Cookie dough	0%	49.18±2.40 c	4.97±0.12 c	9.45±0.72 c	0	10.68	28.35
	1%	39.80±2.20 b	-0.39±0.66 b	0.48±0.28 a	14.04	0.62	0.48
	2%	37.34±0.94 ab	-0.96±0.49 a	-1.70±0.49 b	17.31	1.95	-6.15
	3%	36.58±0.73 a	-1.84±0.26 a	-2.51±0.64 b	18.66	3.11	-10.01
Cookie	0%	61.89±2.77 c	5.46±0.31 c	20.45±1.00d	0	21.17	45.83
	1%	50.61±1.02 b	0.83±0.71 b	14.01±1.58 c	13.79	14.03	32.85
	2%	45.89±1.68 a	-0.35±0.44 a	9.62±1.07 b	20.18	9.63	22.37
	3%	42.96±1.59 a	-0.44±0.70 a	6.75±0.59 a	24.10	6.76	15.91

*a, b, c - describes homogenous groups.
p-value ≤ 0.05.

3.3. Sensory analysis

Microalgae powder had a positive impact on uniformity of browning. Color of the cookies became darker with the increasing addition of algae, which was confirmed in instrumental analysis. Cookies with the highest 3% addition of spirulina were the hardest, while the control sample 0% (without addition) and the one with lowest addition of spirulina (1%) were the softest. The results were not confirmed with the instrumental texture analysis, where the hardness of cookies decreased with the content of microalgae powder. Hardness in sensory analysis could be affected by the density and thickness of cookies. In the 3-point blend test, a cookie breaks when first touched by a probe, while biting teeth are going through the whole cookie. That is why in some studies it was found that hardness measured instrumentally is more correlated to crunchiness or crispiness than hardness of the first bite (KIM *et al.*, 2011). Another explanation for the difference of sensory hardness perception in relation to instrumental hardness can be due to the fact that an instrumental test always measures the force in the middle of the cookie, while the first bite is usually taken from a side. In the case of crispness, no influence of spirulina addition was observed (Table 3). Fracturability, which is measured instrumentally, should by definition be comparable with crispness and no significant differences were found for both of them. The intensity of sensing the taste and odour of algae between cookies with 2% and 3% of spirulina was similar.

Table 3. Sensory analysis of cookies with spirulina.

Spirulina powder content	Typical odour	Algae odour	Browning uniformity	Dark color	Hardness	Crispness	Typical taste	Algae taste	Overall sensory quality
0%	7.08±2.05 ^{b}	0 ^{a}	6.38±2.82 ^{a}	3.46±0.82^{a}	4.09±2.54^{a}	7.35±1.57^{a}	6.64±2.32^{a}	0 ^{a}	7.24±1.66^{a}
1%	5.62±2.2 ^{ab}	2.64±1.80 ^{ab}	6.22±2.60 ^{a}	4.79±1.08^{a}	4.15±1.67^{a}	6.73±1.90^{a}	4.86±1.44^{a}	4.11±1.66^{b}	5.64±2.21^{ab}
2%	3.88±1.95 ^{a}	4.55±1.14 ^{b}	7.25±1.68 ^{a}	7.10±1.68^{b}	4.75±1.82^{a}	7.72±1.15^{a}	3.42±1.32^{ab}	6.35±1.02^{b}	5.48±2.38^{ab}
3%	3.50±2.25 ^{a}	4.88±1.70 ^{b}	7.62±1.54 ^{a}	7.62±1.54^{c}	5.8±2.40^{a}	6.57±1.60^{a}	3.09±1.97^{b}	6.56±1.25^{b}	5.10±1.99^{b}

*a, b, c - describes homogenous groups, p-value ≤ 0.05.

Table 4. Correlation of variables – properties of cookie dough and cookies, p-value ≤ 0.05.

	spirulina	Dd	Dc	h	d	m	Fd	Fc	ΔEc	chroma c	BI c	Q	hard
Dd	0,59	1,00	0,84	0,98	0,85	-0,56	0,73	-0,96	0,78	-0,73	-0,68	-0,89	0,31
Dc	0,87	0,84	1,00	0,91	0,46	-0,90	0,90	-0,95	0,96	-0,95	-0,93	-0,96	0,63
h	0,66	0,98	0,91	1,00	0,79	-0,66	0,77	-0,99	0,84	-0,79	-0,75	-0,92	0,35
d	0,08	0,85	0,46	0,79	1,00	-0,05	0,26	-0,69	0,33	-0,25	-0,19	-0,51	-0,23
m	-0,97	-0,56	-0,90	-0,66	-0,05	1,00	-0,93	0,76	-0,95	0,97	0,98	0,87	-0,87
Fd	0,98	0,73	0,90	0,77	0,26	-0,93	1,00	-0,86	0,98	-0,99	-0,99	-0,96	0,87
Fc	-0,87	-0,96	-0,95	-0,99	-0,69	0,76	-0,86	1,00	-0,91	0,87	0,84	0,96	-0,49
ΔEc	0,96	0,78	0,96	0,84	0,33	-0,95	0,98	-0,91	1,00	-0,99	-0,99	-0,98	0,79
chroma c	-0,98	-0,73	-0,95	-0,79	-0,25	0,97	-0,99	0,87	-0,99	1,00	0,99	0,96	-0,84
BI c	-0,99	-0,68	-0,93	-0,75	-0,19	0,98	-0,99	0,84	-0,99	0,99	1,00	0,94	-0,87
Q	-0,90	-0,89	-0,96	-0,92	-0,51	0,87	-0,96	0,96	-0,98	0,96	0,94	1,00	-0,70
hard	0,93	0,31	0,62	0,35	-0,22	-0,87	0,87	-0,74	0,79	-0,84	-0,87	-0,70	1,00
crisp	-0,32	-0,40	-0,10	-0,26	-0,30	0,09	-0,42	0,29	-0,29	0,28	0,26	0,39	-0,39

Legend: Dd – density of cookie dough, Dc – density of cookies, h – height of cookies, d – diameter of cookies, m – moisture content, Fd – hardness of cookie dough, fr – fracturability of cookies, Fc – hardness of cookies (measured instrumentally), ΔEc – total color difference of cookies, chroma c – color saturation of cookies, BI c – browning index of cookies, Q – overall sensory quality, hard – hardness (sensory analysis), crisp – crispness.

Spirulina addition has adversely influenced sensory quality of cookies. It caused a decrease in typical aroma, typical taste and overall sensory quality – acceptability. In the study by SHARMA and DUNKWAL (2012), the acceptability of biscuits with addition of 10% of spirulina powder did not result in low acceptability. Scores obtained for the control biscuits and spirulina based biscuits were similar. In comparison, the microalgae content was much higher than in the study presented in this paper. High overall acceptability in the study conducted by SHARMA and DUNKWAL (2012) can be due to the fact that eating habits and flavor preferences are different in India and in Poland.

3.4. Correlation between qualitative parameters of cookie dough and cookies

It was observed that addition of spirulina had an effect on the analyzed parameters of cookies and cookie dough. Only crispness, cookie geometry and density of cookie dough were weakly correlated with the amount of microalgae powder addition. Statistical analysis indicated a statistically significant correlation between hardness measured by texture analyzer, color parameters and overall sensory quality ($p<0,05$). Color was strongly and significantly dependent on the spirulina content in the recipe. Based on the statistical analysis, it can be assumed that crispness and fracturability of cookies with spirulina did not depend significantly on any of the analyzed variables (Table 4). The variables describing properties of cookie dough had strongly correlated with those describing the baked product. This may indicate that by knowing properties of the cookie dough before baking, we can anticipate features of the final product. Hardness of cookies measured with texture analyzer was correlated with spirulina content, which was also confirmed by linear regression (Fig. 2).

4. CONCLUSIONS

Spirulina has influence on most physical and sensory properties of cookies. Its even minor addition had strong impact on change of color to green of the cookie dough and baked products. Addition of microalgae caused a decrease in moisture content and hardness measured by texture analyzer. It was observed that the color and hardness of products had an impact on the overall sensory quality. More brown color and less green improved acceptance of a product.

Because of increasing consumers' interest in novel food ingredients such as microalgae, production of cookies with spirulina addition may be a forward-looking development. Due to high content of bioactive ingredients and low sensory acceptability 1% addition of spirulina powder seems to be optimum. Unfortunately, larger quantities of spirulina caused significant changes in some parameters affecting quality of cookies.

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PAPER

EFFECT OF HYDROGENATED FAT REPLACEMENT WITH WHITE SESAME SEED OIL ON PHYSICAL, CHEMICAL AND NUTRITIONAL PROPERTIES OF COOKIES

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ABSTRACT

Sesame seed oil has known antioxidant properties that may improve both nutritional importance and shelf-life of the product. Three aims of the study were to: a) examine nutritional value and physicochemical properties of white sesame seed oil (WSSO) and hydrogenated vegetable fat (HVF) cookies, b) compare the antioxidant potential of the cookies and c) determine the effects of storage and treatment conditions on palatability of the cookies. Results showed that energy and fat% were significantly higher ($P < 0.05$) in WSSO than HVF cookies. At 60th day, mean moisture, peroxide value, and acidity were higher ($P < 0.05$) in HVF cookies. Over time, protein and fiber% decreased significantly ($p < 0.05$) in both cookies but remained higher ($P < 0.05$) in WSSO at 60 days. WSSO cookies had longer shelf life, greater palatability, improved physical properties and greater antioxidant potential.

Keywords: antioxidant potential, food product development, palatability, physicochemical properties, sesame oil

1. INTRODUCTION

Food industries are interested in developing plant products that provide both functional as well as nutritional value (JISHA *et al.*, 2009; OOMAH and MAZZA, 1998; TRIPATHY *et al.*, 2003). In addition, the increasing trend toward consuming "pre-packaged" "ready-to-eat" products have increased the need for improving the nutritional quality, palatability and shelf life of these food products (NANDITHA and PRABHASANKAR, 2009).

Along with proteins and carbohydrates, fats are a critical component of a healthful diet. Fats are important for improving both the taste and texture of food as well as stimulating neurological sensory signals of "fullness" after consumption (ROLLS, 1995). Some oil containing foods are rich in antioxidants, which may be able to decrease harmful inflammatory conditions resulting from oxidative stress. These same antioxidative properties may also be able to increase the shelf life of food products by reducing the undesirable progression of oxidation that causes rancidity (SIMS and FIORITI, 1977). Furthermore, increasing shelf life also influences the economic cost of the product by reducing the waste of discarding unused "out of date" products (REDDY *et al.*, 2005). Finally, there is an increasing need to replace partially hydrogenated fats that are now known to be unhealthy, with healthy replacements without affecting the physical and sensory properties of the end products (WANG *et al.*, 2016).

White sesame seed oil (WSSO) (*Sesamum indicum L.*) specifically has been recognized for its potential role in a healthful diet. The oil in sesame seeds contains appreciable amounts of bioactive components with powerful antioxidant properties identifying it as a promising nutraceutical in the treatment of chronic inflammatory conditions such as cardiovascular disease and diabetes (BHUVANESWARI and KRISHNAKUMARI, 2011; HEMALATHA and GHAFOORUNISSA, 2004; LATIF and ANWAR, 2011).

WSSO is readily cultivated in tropical and sub-tropical regions of the world and has been used in food preparation and baking and for centuries (RESHMA *et al.*, 2010). Sesame oil (SO) is considered as an extremely stable oil against oxidation because of the high proportion of natural antioxidants or lignans, such as sesamin and sesamolin (ANILKUMAR *et al.*, 2010). The presence of these antioxidants may also improve the oxidative stability of SO (EL-ADAWY, 1997; LATIF and ANWAR, 2011; RESHMA *et al.*, 2010). It is plausible that exchanging the fat source in cookies from standard hydrogenated vegetable fat with SO, would improve the antioxidant potential, shelf life and palatability of cookies. SOWMYA *et al.*, (2009) described the effect of fat replacement with SO, hydrocolloids and emulsifiers on changes in the fatty acid profile and microstructural qualities of cakes. They determined that a combination of 50% SO combined with hydroxypropylmethylcellulose and emulsifiers greatly improved the palatability of the product and provided better results than the control (vegetable fat) cake in all aspects. In fact, replacement of fat with SO also decreased the saturated fatty acid content of the cake (SOWMYA *et al.*, 2009). LIM and LEE (2015) described that that by incorporating black sesame powder into cookies, the functional properties of the cookies was improved without affecting the consumer acceptability. In addition, cookies in which butter was replaced with an oil emulsion, showed better fracture properties and higher consumer acceptability and potentially overall healthier properties (GIARNETTI *et al.*, 2015). Finally (RANGREJ *et al.*, 2015) noted that the replacement of hydrogenated fat with seed oil improved the physical, textural and sensory properties of cookies. To our knowledge, no previous studies have examined the effect of replacing the fat source in cookies with SO. It is currently unknown whether replacing the fat source in cookies would improve the nutritional, antioxidative or perhaps more importantly, the taste and palatability of the cookies. The purpose of this investigation was to study the differences in nutritional

properties as well as the palatability of cookies made with SO compared to cookies made with hydrogenated vegetable fat (HVF).
The specific aims of this study were three-fold: 1) to examine differences in nutritional value and physicochemical properties of cookies made with SO compared to HVF, 2) to compare the antioxidant potential of the cookies and 3) to determine the effects of time and oil type on palatability of the cookies.

2. MATERIAL AND METHODS

2.1. Materials

Two types of cookies were prepared, one with 100% hydrogenated vegetable fat (HVF) and other with 100% white sesame seed oil (WSSO). Vegetable fat (VF) was the hydrogenated fat and it was the blend of soybean, palm and canola vegetable oil. Cookies were prepared according to the method given by the American Association of Cereal Chemists (AACC, 2000) with modifications to fat replacement and baking time & temperature. Baking ingredients were procured from the local market. All ingredients were weighed as per their percentage in the recipe that included: fine (cake) flour (45%), whole wheat flour (10%), white sesame seed oil (33%) or vegetable fat (hydrogenated fat composed of soybean, palm and canola vegetable oil), salt (0.30%), egg (5%), stevia (0.15%), baking powder (5%), sugar (1.5%), and vanilla extract (0.05%). Cookies were prepared in a commercial bakery unit. The white sesame seed oil was extracted from seeds (PB-Till 90) that were procured from the Ayub Agriculture Research Institute Faisalabad, Pakistan. Sesame seed oil was extracted from seeds through solvent extraction method (LATIF and ANWAR, 2011) then this oil was used for the preparation of cookies.
Dry ingredients (flour, whole wheat flour, salt, sugar, stevia and baking powder) were placed in a mixer at low speed for 2-3 minutes to ensure thorough mixing. Next, eggs were added to the dry mixed ingredients during mixing. The fat source (either SO or HVF) was then added in small amount to facilitate thorough blending of fat with the ingredients and the mixer speed was increased up to 50-60 rpm (medium) and the ingredients were mixed for additional 4-5 minutes. Then vanilla extract was added. The whole mixing process was completed in approximately 10-15 minutes. Two batches of 15 kg each, one from SO and the other from HVF were made. Cost of recipe for both type of cookies was almost same and these was not much difference.
The mixture was dispersed onto a cookie sheet in 12-15 gm aliquots to produce a total of 950-970 cookies. Cookies were baked in a preheated commercial oven at 175°C for approximately 15-20 minutes until a golden color was achieved. Baked cookies were then allowed to cool on a rack and the weight of each cookie was noted. Cookies were then divided into 12 air-tight glass containers (6 with SO and 6 with HVF) holding 160-170 cookies each and placed in a cabinet in the laboratory at an ambient room temperature (25 ± 5°C) away from sunlight for a period of 60 days. One container of each type of cookie (SO and HVF); were opened and the cookies were analyzed at each data collection period (baseline, 30th and 60th day).

2.2. Methods

2.2.0. Proximate analysis of cookies

Proximate composition analyses followed the specific methodology as described by AACC, (2000). These analyses included, moisture (Method No. 44-15A), protein (Method

No. 46-30), fat (Method No. 30-25), fiber (Method 32-10), ash (Method No. 08-01). Energy (kcal) was estimated by calculating kcal per gram of the individual macronutrients.

2.2.1 Moisture

Moisture was determined by following the method (method no. 44-15A) AACC (2000). Air dried oven (Blodgett; CTB/CTBR, USA) was used to determine the moisture. The percentage of moisture was calculated according to the expression given below.

$$\text{Moisture (\%)} = \frac{W1 \times 100}{W}$$

Where,
W1 = Loss in gm of the material on drying
W = Weight in gm of the material taken for test

2.2.2 Protein

Protein content in each sample was estimated according to the Kjeldahl's method (method no. 46-30) as described in AACC, (2000).

2.2.3 Fat

Fat (%) content in each sample was determined by taking 5 gm dried sample and running through Soxhlet apparatus for 04 hours using n-hexane as a solvent by following the procedure described in AACC, (2000) method no. 30-25. The percentage of fat was calculated according to the expression given below.

$$\text{Fat (\%)} = \frac{\text{Wt. of fat}}{\text{Wt. of sample}} \times 100$$

2.2.4 Fiber

The crude fiber was estimated according to the procedure as outlined in AACC, (2000) method 32-10. Muffle furnace (Thermo scientific thermolyne F48010-33, USA) was used to determine the fiber. The percentage of fiber was calculated after igniting the samples according to the expression given below.

$$\text{Fiber (\%)} = \frac{\text{Weight loss}}{\text{Weight of sample}} \times 100$$

2.2.5 Ash

Ash was estimated according to the procedure as outlined in AACC, (2000) method no. 08-01. Ash content was determined by high temperature incineration in an electric muffle furnace (Thermo scientific thermolyne F48010-33, USA).

$$\text{Ash (\%)} = \frac{A-B}{C} \times 100$$

Where:
A = weight of crucible with sample (gm) B = weight of crucible with ash (gm) C = weight of sample (gm)

2.3. Antioxidant potential

Antioxidant potential was determined by measuring peroxide value, total acidity, energy, nitrogen free extract, and thiobarbituric acid value according to their respective methods described in AACC, (2000). Furthermore, the lignan content (sesamin, and sesamol) of the cookies were assessed following the method of SCHWERTNER and RIOS, (2010).

2.3.1 Peroxide value

Ash was estimated according to the procedure as outlined in AACC, (2000) method. Sample was melted and filtered through the filter paper to remove any impurities. A blank reading was taken under the similar conditions at the same time.
The peroxide value was calculated by using the relationship

$$\text{Peroxide value} = \frac{(B-A) \times N \times 1000}{W}$$

B = Vol. of $Na_2S_2O_3$ used for blank
A = Vol. of $Na_2S_2O_3$ used for sample
N = Normality of $Na_2S_2O_3$
W = Weight of the oil taken.

2.3.2 Total acidity

Total was estimated according to the procedure as outlined in AACC, (2000) method. Total acidity was calculated according to the expression given below.

Calculation:

Acid value = 56.1VN,
Where:
V = Volume in ml of standard KOH or NaOH used
N = Normality of the KOH solution or NaOH solution; and
W = Weight in gm of the sample

2.3.3 Nitrogen Free Extract (NFE)

The NFE was calculated by the following expression.
NFE% = 100 – (moisture% + ash%+ fat%+ fibre% + protein%)

2.3.4 Thiobarbituric Acid Value

TBA reagent (0.2883g/100mL of 90% glacial acetic acid), heated in water bath for 35 min with a blank sample. The tubes were cooled in water for 10 min and absorbance (D) against blank sample was taken by adjusting spectrophotometer (Cecil CE-7200, UK) on 538nm wavelength (AACC, 2000).
TBA no. was calculated by using the following expression:

TBA no. (mg malenaldehyde per Kg sample) = 7.8 x D

2.3.5 Lignans

Lignans (sesamin and sesamol) were analyzed by adopting the method of (SCHWERTNER and RIOS, (2010); SCHWINGSHACKL and HOFFMANN, (2012))with little modifications in it. A 2ml of sesame seed oil sample was taken in glass tubes and poured in it 20ml of methanol and vortex it for 30 minutes. The sample was then centrifuged at 2500 rpm for 30 minutes. After centrifugation, the upper layer was separated and again extraction was done by adding methanol again in it. The two extractions were combined and then evaporated under nitrogen. Then 2 ml of methanol was added to reconstitute it and vortex it. 20 µl of sample was injected into HPLC (Model: Perkin Elmer series 200 USA) equipped with C18 (4.6mm X 150 mm). The mobile phase was a mixture of methanol and water (70:30, v/v) and the flow rate was 1 ml/min. The UV detector was set at 288 nm. Sesamin, sesamolin and sesamol were quantified by comparing with standards.

2.4. Physical properties and palatability

To evaluate the palatability of cookies, taste tests were conducted using a sensory evaluation assessment tool described by MEILGAARD *et al.,* (2007). Briefly, 100 men and women were recruited from University student, faculty, and staff members, and the local community to participate in a series of consumer taste tests. The same individuals were asked to evaluate the cookies at each time period. Among these volunteers, 75 people completed the testing at all three-time points. Cookies were placed on a table in small cups coded by type. Both researchers and panelists were blinded to the coding scheme. Panelists were provided water to neutralize the taste after chewing and between tasting each cookie. Each panelist was asked to taste 3 cookies of each type and asked to evaluate the cookies for colour, flavor, taste, texture, crispness and overall acceptability using a 9-point (1= extremely poor to 9 = excellent), hedonic scale (MEILGAARD *et al.,* 2007). The physical properties such as cookie size and diameter, thickness, and spread factor were determined by investigators on three cookies from each group at the three time points (AACC, 2000).

2.5. Statistical Analyses

Data was expressed as mean ± SE. For composition analyses and physical properties, a total of 3 cookies from each group (HVF or SO) were evaluated at each time point. For palatability studies, evaluation data from 75 subjects was compared for each variable at each time point. All data were analyzed by repeated measures ANOVA. Where significant effects occurred, Tukey post-hoc analyses were performed. A p-value of $\leq$ 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSIONS

3.1. Proximate analysis and antioxidant potential of cookies

As previously described, two types of cookies were prepared, one with 100% SO and other with 100% HVF and both were stored for up to 60 days in an air tight container. A description of the proximate composition of each of the cookies type for each time period is shown in Table 1. At baseline, both SO and HVF cookies had similar properties of moisture, fiber and ash. Compared to HVF, SO had significantly higher initial percentages of protein (6%), fat (8.5%), and energy kcal/100gm (15.4%).

By the end of the 60 days of storage time, moisture content in SO cookies increased approximately 34% ($p < 0.05$), while other components decreased significantly ($p < 0.05$) over time; (protein: -0.2%, fat: -3%, fiber: -5.5%, and ash: -7.9%). In HVF cookies, a similar trend was observed. In HVF cookies, moisture increased by about 52% ($p < 0.05$), while other components decreased ($p <0.05$); (protein: -2.5 %, fat: -3.4 %, fiber: -6.9 %, and ash: -16.4 %) from baseline to 60 days of storage. Energy (kcal/100gm) did not change over time in either cookie group.

Table 1. Cookies proximate analysis.

Component	Day 0	Day 30	Day 60
Moisture (%)			
SSO	2.49 ± 0.04 (2.63)	3.19 ± 0.05 (2.87)^{a}	3.35 ± 0.05 (2.60)^{a,b,c}
HVF	2.41±0.02 (1.66)	3.08±0.03 (1.49)^{a}	3.66±0.06 (2.96)^{a,}
Protein (%)			
SSO	9.11±0.02 (0.33)^{c}	9.05±0.04 (0.83)^{c}	9.00±0.03 (0.62)^{a,c}
HVF	8.57±0.02 (0.40)	8.43±0.03 (0.54)^{a}	8.36±0.02 (0.43)^{a}
Fat (%)			
SSO	39.62±0.59 (2.57)^{c}	38.92±0.04 (0.18)^{c}	38.41±0.31 (1.40)^{c}
HVF	36.51±0.39 (1.83)	35.82±0.07 (0.33)	35.31±0.05 (0.22)
Fiber (%)			
SSO	2.09±0.02 (1.27)	2.01±0.02 (1.32)^{a,c}	1.98±0.03 (2.31)^{a,c}
HVF	2.01±0.02 (1.99)	1.92±0.03 (2.90)^{a}	1.88±0.04 (3.72)^{a}
Ash (%)			
SSO	0.95±0.01 (2.11)	0.93±0.02 (2.84)^{c}	0.88±0.02 (4.10)^{a,c}
HVF	0.92±0.02 (3.92)	0.83±0.03 (5.52)	0.79±0.03 (6.70)
Energy (Kcal/100gm)			
SSO	535.67±2.97 (0.96)^{c}	535.58±0.24 (0.08)^{c}	534.42±2.59 (0.84)^{c}
HVF	464.33±2.96 (1.11)	463.89±2.77 (1.03)	463.12±2.82 (1.06)

Mean±SE (%Coefficient of Variance); the values are replicate of at least three.

Sig. $^{a}p<0.05$ from Day 0;

Sig. $^{b}p<0.05$ from Day 30;

Sig. $^{c}p<0.05$ sesame seed oil cookies vs vegetable fat cookies.

At 60 days there were significant ($p < 0.05$) differences between groups. Moisture was significantly higher in HVF verses SO, whereas all other components were significantly ($p < 0.05$) lower in HVF group compared to SO group; (protein: -7.6%, fat: -9%, fiber: -5% and ash: -11 %).

Table 2 shows antioxidant potential of both SO and HVF cookies. At baseline, both SO and HVF had similar properties of nitrogen free extract and thiobarbituric acid value. Compared to SO, HVF had significantly higher initial percentages of peroxide value (37.5%) and acidity (20%). Lignans with antioxidant potential were detected in SO but were absent in HVF. Over time, from baseline to 60 days, peroxide value increased approximately 252% in SO cookies. Additionally, in SO, acidity, nitrogen free extract, and thiobarbituric acid values increased (35%, 3%, 54% respectively), while bioactive components, sesamin and sesamol, decreased significantly ($p < 0.05$) over time (i.e., -0.22% and -1.2% respectively). A similar trend was observed in HVF cookies. In HVF cookies significant ($p < 0.05$) increases were observed in peroxide (+182.5%), acidity (+24%), nitrogen free extract (+5%) and thiobarbituric acid (+ 53%) from baseline. There were no bioactive components detected in HVF cookies.

Table 2. Antioxidant potential of cookies.

Component	Day 0	Day 30	Day 60
Peroxide value (meq/kg)			
SSO	0.133±0.01 (17.3)^{c}	0.341±0.00 (1.83)^{a,c}	0.469±0.00 (0.93)^{a,b,c}
HVF	0.183±0.01 (8.67)	0.428±0.00 (0.62)^{a}	0.517±0.00 (1.02)^{a,b}
Acidity (%)			
SSO	0.142±0.01 (11.3)^{c}	0.188±0.01 (4.88)^{a}	0.192±0.00 (4.26)^{a,c}
HVF	0.171±0.00 (0.58)	0.198±0.00 (1.34)^{a,b}	0.212±0.00 (1.70)^{a}
Nitrogen free extract (%)			
SSO	41.27±1.01 (4.23)	42.21±1.24 (5.10)	42.57±1.21 (4.93)
HVF	41.87±0.51 (2.11)	42.45±0.70 (2.84)	43.94±0.58 (2.29)
Thiobarbituric acid value (mg malonaldehyde/kg-oil**)**			
SSO	0.046±0.00 (8.70)	0.068±0.01 (14.0)^{a}	0.071±0.01 (18.3)^{a}
HVF	0.055±0.00 (6.56)	0.062±0.00 (8.98)	0.084±0.00 (3.15)^{a,b}
Sesamin (mg/kg)			
SSO	8.093±0.00 (0.01)	8.077±0.00 (0.09)^{a}	8.075±0.00 (0.09)^{a}
HVF	NA	NA	NA
Sesamol (mg/kg)			
SSO	18.64±0.05 (0.46)	18.48±0.02 (0.20)^{a}	18.42±0.02 (0.20)^{a}
HVF	NA	NA	NA

Mean±SE (%CV) the values are replicate of at least three
Sig. $^{a}p<0.05$ vs Day 0; $^{b}p<0.05$ vs Day 30; $^{c}p<0.05$ vs HVF

The moisture content of both the cookies increased over the 60 days of storage period in both cookies. However, it increased significantly more in the HVF cookies. During storage, the rise in moisture content in cookies and cakes has been well documented by studies conducted by (LEELAVATHI and RAO, 1993; NAGI *et al.*, 2012; ROBERTSON, 1993). The hygroscopic nature of the dry ingredients of cookies is known to influence moisture content during storage. Additionally, this rise in moisture can influence the shelf life of the products by increasing peroxide value, acidity, nitrogen free extract, and thiobarbituric

acid value; while decreasing protein, fat, fiber and ash content. Ash helps with the overall absorption of moisture. Thus a reduction of ash results in a corresponding increase in moisture. This storage phenomenon whereby a decrease in ash results in increasing moisture, has been well documented (PASHA *et al.*, 2002; REDDY *et al.*, 2005; SHARIF *et al.*, 2003; WAHEED *et al.*, 2010). When compared with other vegetable oil; sesame seed oil has high degree of monounsaturated and polyunsaturated fatty acids *i.e.*, is approximately 39% and 46% respectively that collectively makes almost 85% of unsaturated oil and low saturated fatty acids *i.e.*, is approximately 14% (SCHWINGSHACKL and HOFFMANN, 2012). Replacement of normal shortening in cookies with vegetable oils showed significant effect on moisture, fat content and NFE during storage, while change in fiber and ash content was not significant (SHARIF *et al.*, 2005). Bioactive components (i.e., sesamin, sesamolin, and sesamol) in the SO, were also affected during the storage period. High temperature (160-250°C) does not affect the bioactive components of sesame seed oil especially lignans and their concentration almost remains the same that is the reason behind the strong antioxidant potential of sesame seed oil even at high temperature it sustain its properties (YOSHIDA and TAKAGI, 1997). Nutritional improvement is not in a sense that it increases anything, but here it's in a context that it increases the stability of WSSO cookies against oxidation during study period due to antioxidant potential of white sesame seed oil. Antioxidant potential is evident from the results. Storage in polythene bags showed a significant change in moisture, peroxide value and overall acceptability of cookies (RANGREJ *et al.*, 2015). As the moisture increased with storage time, the antioxidant properties decreased. However, although moisture increased in both groups of cookies, because SO cookies had greater antioxidant potential to begin with, the SO cookies showed more stability than HVF cookies. NANDITHA and PRABHASANKAR, (2009) presented in their studies that natural antioxidants are very effective in enhancing the shelf life of bakery products. SHARIF *et al.*, (2003) made cookies from oil extracted from natural source that has functional properties and natural antioxidants, which increased the shelf life of cookies by enhancing their antioxidant potential. Similar to our findings various studies reported that SO cookies are not only more stable in their proximate composition analysis but also in the antioxidants available by day 60 (QUILEZ *et al.*, 2006; REDDY *et al.*, 2005).

3.3. Sensory evaluation

A total of 75 people completed the study at all three time periods. Before sensory evaluation, training and instructions were given to the all participants so that they can evaluate the products for sensory evaluation in the right way. The mean rating scores of the 9 points (low to high) sensory evaluation scale of colour, flavour, taste, crispness and overall acceptability are shown in Table 3. Table 3 indicates that at baseline, SO cookies had significantly ($p < 0.05$) higher evaluation for colour (16%), flavour (10%), taste (5%), texture (9%), crispness (9%) and overall acceptability (12%) compared to HVF cookies.

Over the period from baseline to 60 days, the mean rating on each attribute decreased significantly ($p < 0.05$) for each cookie type. For SO cookies, colour decreased by about -5.5%, flavour -8%, taste -16%, texture -11.6%, crispness -8% and overall acceptability by -14%. A similar trend was observed in HVF cookies. In HVF cookies, the mean rating for colour decreased -9%, flavour decreased by -11%, taste decreased by -11%, texture decreased by -12%, crispness decreased by -7% and overall acceptability decreased by -5.5%.

By day 60, there were significant ($p < 0.05$) differences in the sensory rating between groups. Compared to the HVF group, colour, flavor, texture and crispness were rated

higher in the SO group (range 8-20%). Taste and overall acceptability were lower than baseline but were not significantly different between groups by day 60.

Table 3. Palatability of cookies.

Characteristic	Day 0	Day 30	Day 60
Color			
SSO	8.51±0.07 (6.80)[c]	8.21±0.05 (5.02)[a,c]	8.05±0.03 (2.81)[a,c]
Vegetable oil	7.35±0.10 (12.2)	6.91±0.09 (10.7)[a]	6.71±0.09 (11.4)[a]
Flavor			
SSO	7.51±0.06 (6.71)[c]	7.11±0.09 (11.0)[a,c]	6.93±0.08 (9.58)[a,c]
Vegetable oil	6.80±0.09 (11.1)	6.43±0.08 (10.6)[a]	6.12±0.05 (7.09)[a,b]
Taste			
SSO	8.00±0.09 (9.86)[c]	7.15±0.09 (11.2)[a]	6.87±0.07 (8.74)[a,b]
Vegetable oil	7.65±0.09 (10.2)	7.15±0.08 (10.2)[a]	6.92±0.09 (11.6)[a]
Texture			
SSO	7.49±0.09 (9.90)[c]	6.95±0.10 (12.5)[a,c]	6.72±0.08 (9.96)[a,b,c]
Vegetable oil	6.89±0.09 (11.1)	6.25±0.05 (7.48)[a]	6.17±0.05 (6.72)[a]
Crispness			
SSO	7.40±0.10 (11.5)[c]	7.05±0.09 (11.4)[a,c]	6.84±0.07 (9.00)[a,b,c]
Vegetable oil	6.79±0.08 (9.78)	6.45±0.06 (8.17)[a]	6.33±0.06 (7.93)[a]
Overall Acceptability			
SSO	8.00±0.06 (6.82)[c]	7.40±0.09 (10.4)[a,c]	7.03±0.07 (9.05)[a,b,c]
Vegetable oil	7.12±0.08 (9.51)	6.97±0.08 (10.5)	6.75±0.06 (7.35)[a]

Scale 1= low; 9 = high
Mean±SE (%CV) the values are replicate of at least three
[a]$p<0.05$ vs Day 0; [b]$p<0.05$ vs Day 30; [c]$p<0.05$ vs. vegetable oil cookies

At each time period, both SO and HVF cookies had similar physical properties as shown in table 4. By 60 days, the diameter in HVF cookies increased significantly ($p < 0.05$) by about 3% from baseline. In both groups, the rating for colour and flavor decreased over time. During storage, a common oxidation process that is stimulated by an increase in moisture known as maillard reactions, stimulates increased oxidation of the fat and increases in free fatty acids which could affect colour and flavor of the cookie. TBARS is an important indicator for the quality of stored food (BUTT *et al.*, 2007; WADA, 1998). Slow increase in moisture of WSSO cookies caused the sustainability of total acidity and peroxide value that is directly related to antioxidant potential of WSSO cookies. As noted previously, the increase in moisture with storage resulted in increased peroxide and acidity which will negatively affect most of the sensory attributes. In fact, several authors (BENDER, 1996; SHARIF *et al.*, 2003; WAHEED *et al.*, 2010), have reported a similar trend between increasing moisture with decreasing palatability with storage. Previous research has also indicated that the addition of sesame flour improved the aroma, taste and overall acceptability of cookies and there were no significant differences from the control cookies (OLAGUNJU and IFESAN, 2013). Also, LIM and LEE reported that the addition of sesame

powder did not affect the overall acceptance of cookies (LIM and LEE, 2015). Further, when bioactive components like phytosterol, α tocopherol and β phytosterol were added, there were no changes in the sensory and chemical properties of cookies (QUILEZ *et al.*, 2006). Lastly, extra moisture is known to change the diameter, thickness, and spread factor of the cookie. During storage, the amount of moisture absorbed will increase the diameter of cookies and decrease their thickness. Physical properties are also directly related to composition of cookies. As in composition of both cookies the main difference was the WSSO and hydrogenated vegetable fat that creates the difference during storage.

Table 4. Physical properties of cookies.

Characteristic	Day 0	Day 30	Day 60
Diameter			
SSO	63.25±0.26 (0.70)^{c}	63.02±0.04 (0.11)^{c}	62.96±0.06 (0.16)^{c}
HVF	62.08±0.14 (0.38)	62.01±0.10 (0.27)	63.93±0.03 (0.08)^{a,b}
Thickness			
SSO	9.80±0.03 (0.51)	9.76±0.01 (0.20)	9.71±0.03 (0.47)
HVF	9.75±0.01 (0.21)	9.72±0.03 (0.45)	9.69±0.06 (1.00)
Spread factor			
SSO	64.54±0.42 (1.13)^{c}	64.56±0.05 (0.12)	64.84±0.08 (0.20)^{c}
HVF	63.67±0.08 (0.21)	63.79±0.23 (0.64)	63.91±0.40 (1.08)

Mean±SE (%CV) the values are replicate of at least three
$^{a}p<0.05$ vs Day 0; $^{b}p<0.05$ vs Day 60; $^{c}p<0.05$ vs. HVF cookies

5. CONCLUSIONS

The results of this study indicate that exchanging the fat source used in a cookie significantly affects its physiochemical properties, antioxidant potential, palatability, and physical properties. Although, following 60 days of storage, the overall properties of both types of cookies decreased, the cookies made with white sesame seed oil had better organoleptic properties and were found to be more palatable than the HVF cookies. Furthermore, since SO cookies have greater antioxidant potential than cookies made with HVF, they may be a healthier cookie choice. The results of this study indicate that WSSO improved the overall functional importance of SO cookies in terms of their physicochemical properties and bioactive components. The enhanced stability of WSSO cookies against oxidation and their improved antioxidant potential may be particularly important for food industries that are interested in developing cookies with functional value. By replacing the fat source with sesame oil, cookie manufacturers may be able to meet high standards for nutritional potential without sacrificing palatability or shelf life of the cookies.

ABBREVATIONS

White sesame seed oil (WSSO), Sesame seed oil (SSO), Hydrogenated vegetable fat (HVF).

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PAPER

EFFECTS OF DIFFERENT EMULSIFIERS AND REFINING TIME ON RHEOLOGICAL AND TEXTURAL CHARACTERISTICS OF COMPOUND CHOCOLATE

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ABSTRACT

The purpose of this study was to investigate the possibility of producing reduced fat dark compound chocolate in the ball mill refiner and using some selected emulsifiers. The effects of selected emulsifiers including lecithin, Polyglycerol polyricinoleate (PGPR) and citrem in two levels and two refining times on the characteristics such as moisture, particle size, hardness and rheological properties of the samples was examined. Data analysis revealed that the Casson model was appropriate to describe the rheological behavior of the samples containing lecithin and citrem; however, Power law model was appropriate for the samples containing PGPR. The results showed that citrem is the most effective emulsifier to reduce hardness and rheological parameters such as apparent viscosity; casson viscosity and casson yield value and using citrem as a part of formulation in the production of reduced fat dark compound chocolate can solve many technological problems.

Keywords: citric acid ester, Casson model, Power law, PGPR, lecithin, reduced fat compound chocolate

1. INTRODUCTION

Production of chocolate and chocolate products in ball mill refiner is currently spreading worldwide to due to lower costs and easier operational systems. The increase of diseases caused by dietary misbehaviors in industrialized countries, leads to larger knowledge for nutritional requirements by the consumer and, therefore, by food industry (DIAS *et al.* 2015). As a result, the possibility of developing a formulation for chocolate models by reducing its fat content while maintaining its richness and flavor will give the consumer a new and healthy food option to enjoy. Chocolate is a fat-based suspension with about 30%wt fat. Reducing fat content causes an increase in hardness and molten chocolate viscosity that leads to difficulties in the process and a loss of eating quality in the final product. There are, however, some technical issues that must be scrutinized to achieve successful ball mill processing. Generally, several methods are introduced to reduce the fat content of chocolate with acceptable viscosity and hardness such as increasing the emulsifier levels and/or using emulsifier blends (KAISER *et al.*, 1998), using fat replacers (BECKETT, 2009), and substituting fat phase with a water-in-oil emulsion (HUGELSHOFER, 2000). Optimizing the particle size distribution is another method of decreasing the fat content (MONGIA and ZIEGLER, 2000; DO *et al.*, 2007). The optimization of the particle size distribution (PSD) method has a significant effect on the rheological and textural properties of chocolate samples such as reduction of the apparent viscosity, decrease of hardness and an increase of melting rate in the mouth (MONGIA and ZIEGLER, 2000). The non-Newtonian flow behavior of molten chocolate is generally studied by some well-known models for shear thinning fluids such as power law, Bingham, Herschel-Bulkley and Casson (SOKMEN and GUNES, 2006). In terms of utilization of the latter model, comparing the rheological methods proposed by International Confectionary Association (ICA, 2000) and Chocolate Manufacturers Association (CMA, 1997) revealed a high correlation between: I) Casson plastic viscosity and apparent viscosity; II) between Casson yield value and yield stress; III) Casson plastic viscosity and Casson yield value and IV) yield stress and apparent viscosity (AFOAKWA *et al.*, 2009). In order to have a quality product, investigating the changes which occur in the product matrix at every manufacturing stage could be very useful (GLICERINA *et al.* 2013). Structurally, chocolate is made from fat phase (cocoa butter and emulsifier), in which solid material (crystal sugar, milk powder and cocoa powder) are spread (BECKETT, 2000). The composition of chocolates in terms of fat and nonfat cocoa solids, and sugar content affect their rheological characteristics (FERNANDES *et al.*, 2013). In addition to cocoa butter, emulsifier also forms one of the constituents of chocolate fat phase. In the chocolate matrix, emulsifiers cover sugar particles to develop the flow in cocoa butter. This assists in the equal distribution of particles in emulsion and prevents agglomeration. Some emulsifiers decrease viscosity and yield stress significantly, so they will be very useful additives in production of chocolates with reduced fat. Lecithin and PGPR are emulsifiers usually used in chocolate (SCHANTZ and RHOM, 2005). Both lecithin and PGPR work synergistically with other emulsifiers, such as ammonium phosphatide and citric acid esters (STIER, 2009). Citric acid ester has the attributes of the lecithin and PGPR combination (BECKETT, 2009). Emulsifiers have ability of changing viscosity in certain foods (WALTER and CORNILLON, 2001). This feature is extremely important in producing chocolate, for example in chocolate coating, pumping and molding, etc. (RECTOR, 2000). Emulsifiers have been used in chocolate to modify and improve the flow characteristics of chocolate since chocolate was first processed. However, the most important of emulsifier applications in chocolate industry is improving flow parameters and minimizing consumption of cocoa butter and its costs of production (SCHANTZ and RHOM, 2005). Achieving desirable functional properties is not only

related to providing a basic level of knowledge about ingredients, but also understanding each ingredient's effect in the combinational form will help the manufacturers to satisfy the consumers expectations (MANZOCCO *et al*., 2014). The purpose of this study was to investigate the possibility of producing reduced fat compound chocolate in ball mill refiner and using some selected emulsifiers in the manufacturing process including lecithin, polyglycerol polyricinoleate and citrem (citric acid ester).

2. MATERIAL AND METHODS

2.1. Materials

Cocoa powder (Guan Chong cocoa manufacture Sdn Bhd, Malaysia), refined sugar, cocoa butter substitute (CBS) (Cargill, Malaysia), Lecithin, Polyglycerol polyricinoleate (PGPR) and citrem (Palsgard, Juelsminde, Denmark).

2.1.1 Preparation of compound chocolate samples

The basic formulation of the dark compound chocolate contained 46.5% cocoa powder, 30% sugar, 23% CBS, and 0.5% lecithin. The method for producing compound chocolate was as follows: first, all raw materials, including cocoa powder, refined sugar, palm kernel oil and lecithin, were weighed and poured into semi-industrial Ball mill device (Sepehr machine company, Tehran, Iran). Eventually, fourteen formulas were produced (twelve formula in addition to two basic formula with 60 and 90 refining times as the control samples). Mixing, refining (in two groups, one for 60 and the other one for 90 minutes) and conching were done simultaneously in this device for 30 min at 60 °C and speed of 100 rpm. Each sample was then divided into seven portions. Afterward, emulsifiers were added to the samples (Lecithin and citrem at two levels of 0.5 and 1 % and PGPR at two levels of 0.25 and 0.5%). The conching process was performed (Heidolph mixer) at a speed of 60 rpm for 30 minutes. Next, the mixture was refrigerated at 4°C for 30 min in silicon containers. Finally the samples were kept in aluminum foils and stored at room temperature for analysis.

2.1.2 Moisture content measurements

The moisture content of chocolate samples was determined using oven method (IOCCC, 1952).

2.1.3 Particle size distribution measurements

Particle size distribution was determined through laser diffraction method by particle analysis machine (SHIMADZU SALD-2101), according to MCFARLANE (1999). Before analysis, the compound chocolate samples dissolved in acetone solvent and stirred vigorously under ultrasonic waves of 50 Hz, 200 W for 5 minutes. Low-intensity ultrasound produced optimal component emission. After the initial preparation, samples were transferred to the laser chamber. Results obtained from the laser chamber of the parameters of the largest particle size (D90), the mean particle volume (D50) and the smallest particle size (D10) in micrometer scale were determined (ALAMPRESE *et al*., 2007) with three replicates.

2.1.4 Hardness measurements

Hardness of samples was measured using a texture analyzer (TA-XT plus, stable micro systems Ltd, Surrey, UK), connected to the computer with the software Texture Expert 1.05. The flow bottom steel probe with 2 mm diameter was utilized for measurements. The maximum force of penetration to samples (45×20×10 mm) was determined with a depth of 5 mm at a speed of 1 mm/s at room temperature. Loading force was set to 0.05 N direction of the sample, and kept constant for all samples. Hardness was taken as the maximum peak force in Newton. Results for hardness are expressed as the mean value of three replicates conducted on each sample.

2.1.5 Rheological measurements

Samples were prepared according to the proposed methods of the International Confectionery Association (ICA, 2000); the compound chocolate sample was and melted in an incubator at a temperature of 50°C for 75 minutes and then, transferred to the rheometer cub. After a pre-shear period of 15 min at 5/s, shear rate was applied from 5 to 50 (ramp up) within 120 s and then shear rate was reduced from 50 to 5 (ramp down), and in each ramp 50 measurements were taken. The temperature was kept constant at 40°C. An Anton Paar rheometer (RheolabQC SN80677512, Austria) was used for all rheological measurements and the data were collected by use of the Rheoplus/32 service V3.10 software. The apparent viscosity of the samples was measured at 40/s and results are reported as the mean value of two replicates. SERVAIS *et al.* (2003) reported that the apparent viscosity can be measured at 30, 40 or 50/s depending on the type of product, but recommended the measurement at 40/s for the chocolate regarding to its repeatability. In this study, a locally designed model for analysis of flow time independent characteristics was utilized to analyze the flow properties of compound chocolate. Due to the decrease in viscosity by increasing the shear rate for all rheological behavior applied and non- Newtonian actions of compound chocolate samples, 4 non-Newtonian models (dependent on shear rate) were fitted on the test data (shear stress – shear rate). These four models include (should be in the sequence that is in the Table 2):

Power law ($\tau = k(\dot{\gamma})^{n}$)، Bingham ($\tau - \tau_0 = \eta_{pl}\dot{\gamma}$), Herschel-Bulkley ($\tau - \tau_0 = \eta_{pl}(\dot{\gamma})^{n}$) and Casson ($\tau^{0.5} = \tau_0 + (\eta_{pl})^{0.5}.(\dot{\gamma})^{0.5}$);

Where,

τ is shear stress, $\tau 0$ is yield stress, ηpl is plastic viscosity, $\gamma^{\cdot}$ is shear rate, n is flow behavior index and K is consistency index. Molten chocolate is a non-Newtonian fluid with a yield stress, which can be characterized using a number of mathematical models, including the Bingham, Herschel-Bulkley and Casson models (ICA, 2000; SERVAIS *et al*., 2003; KONAR, 2013). To select the best model for describing time-independent rheological behavior of compound chocolate samples, three statistical parameters of correlation coefficient (R), Root Mean Square Error (RMSE) and Standard Error (SE), were utilized.

2.1.6 Statistical analysis

The SPSS version 21, Curve expert softwares and analysis of variance (ANOVA) were used for statistical analysis of experimental data. Due to unequal levels of used emulsifiers in the formulae, the significance of difference among samples was examined By Nested following Duncan's multiple range tests for mean comparisons.

3. RESULTS AND DISCUSSIONS

3.1. Moisture content

Moisture content of all samples ranged from 0.39 to 0.52. Moisture contents of all samples were within an acceptable range for chocolate (below 1.5 percent). AFOAKWA *et al*. (2007) reported that a moisture content of the chocolate samples over 1.5 percent would have a negative impact on the rheological properties.

3.2. Particle size distribution

Results for the D90, D50 and D10 of the samples are shown in Table1. Since increase in emulsifier level and conching did not lead in change of particle size, only base formula was studied. The mean particle size in the D90, D50, and D10 was 10%, 50% and 90%; the particles were finer than this size, respectively. In this study, as expected, by increasing the refining time, all parameters in the particle size distribution were reduced. Observations in this study determined the particle size of both samples to be below 30 μm. BECKETT (2009) reported that the size of the largest particle is a key parameter for chocolate production and plays a critical role in the hardness, sensory properties, and other properties of chocolates. The largest particle size (D_{90}) plays an important role in the creation of grittiness and mouth feel, however smaller particles affect the flow properties (BECKETT, 2000; MONGIA and ZIEGLER, 2000). Particle size in chocolate roughly ranges between 1 and 50 μm, whereby particles larger than 30 μm cause a gritty perception in the mouth. KRUGER (1999) reported that minimum D90 size for optimal rheological properties was 6 μm. However, in this study, the minimum size of D90 in both of samples was greater than 6 μm. Particle size and flow properties of chocolate are very important factors in determining the viscosity and also texture of final product (MINIFIE, 2012).

Table 1. D_{90}, D_{50}, and D_{10} values in control and basic formulae.

Sample	D_{90} (μm)	D_{50} (μm)	D_{10} (μm)
CH_{b1}	26.87 ± 0.59^{a}	7.67 ± 0.04^{a}	1.71 ± 0.04^{a}
CH_{b2}	22.21 ± 0.34^{b}	6.91 ± 0.05^{b}	1.59 ± 0.03^{b}

(CH_b: basic formula, 1: refining time in 60 min, 2: refining time in 90 min).

3.3. Hardness

Hardness of samples ranged from 32.09 to 53.25 N. As expected, hardness decreased by increasing the levels of emulsifiers in the samples (Fig. 1). Hardness showed inverse relationships with PS, fat and lecithin contents specially in low fat (25%) chocolate samples (AFOAKWA, 2009). At both 60 and 90 minutes of refining time, citrem 1% was the softest and the PGPR 0.25% was the hardest sample (Fig. 1). There was no significant difference between samples containing 0.25% PGPR at 60 min refining time and 0.5% PGPR and citrem at 90 min refining time ($p<0.05$) and also the results showed that, there was no significant differences between samples containing 0.5% PGPR, 0.5% and 1% lecithin at the first refining time ($p<0.05$). Previously, TISONCIK (2010) claimed that increasing concentrations of lecithin and PGPR led in decrease of hardness characteristics of dark chocolate. By increasing the refining time from 60 to 90 minutes and reducing the particle

size from 26.87 μm to 22.211 μm, the hardness of the samples containing lecithin, PGPR and citrem increased due to the interaction between the particles of the compound chocolate. Reducing the particle size leads to increase in resistance of chocolates to break and gives a harder texture (AFOAKWA *et al.*, 2009). In similar results, AFOAKWA *et al.* (2008) reported that by reducing the particle size from 50 microns to 18 microns, the hardness of chocolate samples increased. Do *et al.* (2007) concluded that by selecting a specific range of particle sizes, hardness of chocolate samples could be reduced and controlled. BECKETT (2009) reported different factors like formulation, production method, tempering, polymorphism and cooling temperatures determine the hardness of the chocolate samples. In this study no tempering was required since CBS had been used. In addition, the cooling temperature and production method of all samples were the same. So it can be concluded that desired hardness was achieved by changing the emulsifier or by combination of emulsifiers. There is direct correlation between sensory properties during consumption and hardness; therefore, measuring the hardness parameter is an important indicator for assessing qualitative changes of chocolates with different formulations.

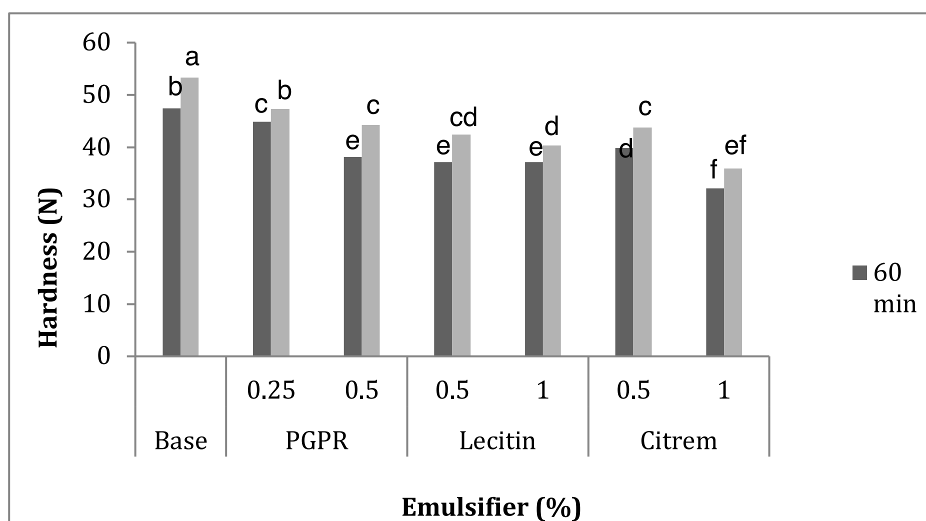

Figure 1. Hardness of the all samples with different emulsifiers at two refining times. Different letters indicate statistically significant differences ($p<0.05$).

3.4. Rheological parameters

3.4.1 Evaluation of fitted models

Table 2 illustrates the results of the three statistical parameters of R, RMSE and SE. By fitting the data of the shear rate - shear stress on the four rheological models of Power Law, Bingham, Herschel-Bulkley and Casson, the Casson model showed the highest R, low RMSE and the lowest SE. Therefore it was the best model to analyze the samples containing lecithin and citrem. The ICA (2000) has proposed Casson model as an appropriate model to analyze the rheological properties of chocolates. In the samples containing PGPR, due to a low yield stress and close to zero, a negative intercept was obtained in the Casson, Bingham and Herschel-Bulkley models. Therefore, the Casson model could not be used for analysis, while the power law model was successful in analyzing the samples containing PGPR.

Table 2. The measured values of the three statistical parameters of R, RMSE and SE.

	Model	R	RMSE	SE
CH_{b1}	Power law	0.996	4.263	18.11
	Bingham	0.989	4.206	30.57
	Herschel-Bulkley	0.998	3.602	12.97
	Casson	0.993	3.575	0.48
CH_{b2}	Power law	0.999	3.627	10.49
	Bingham	0.995	2.854	25.37
	Herschel-Bulkley	0.998	3.754	14.63
	Casson	0.998	2.105	0.29
CH_{l11}	Power law	0.999	3.672	2.53
	Bingham	0.998	1.706	14.03
	Herschel-Bulkley	0.999	2.961	4.24
	Casson	0.999	0.894	0.13
CH_{l12}	Power law	0.999	2.333	8.66
	Bingham	0.993	3.216	11.46
	Herschel-Bulkley	0.999	2.143	7.93
	Casson	0.999	2.274	0.27
CH_{l21}	Power law	0.999	1.575	4.28
	Bingham	0.999	2.057	11.13
	Herschel-Bulkley	0.999	0.998	8.60
	Casson	0.999	1.072	0.19
CH_{l22}	Power law	0.999	3.015	3.60
	Bingham	0.994	2. 706	5.66
	Herschel-Bulkley	0.999	0.589	3.43
	Casson	0.998	1.421	0.23
CH_{p11}	Power law	0.999	3.787	1.96
	Bingham	-	-	-
	Herschel-Bulkley	-	-	-
	Casson	-	-	-
CH_{p12}	Power law	0.999	1.673	7.63
	Bingham	-	-	-
	Herschel-Bulkley	-	-	-
	Casson	-	-	-
CH_{p21}	Power law	0.999	3.212	6.54
	Bingham	-	-	-
	Herschel-Bulkley	-	-	-
	Casson	-	-	-
CH_{p22}	Power law	0.999	2.229	9.18
	Bingham	-	-	-
	Herschel-Bulkley	-	-	-
	Casson	-	-	-
	Casson	0.999	1.050	0.11
CH_{c11}	Power law	0.999	1.469	5.72
	Bingham	0.999	0.464	2.83
	Herschel-Bulkley	0.999	0.712	1.29

	Casson	0.999	0.420	0.06
CH_{c12}	Power law	0.995	2. 370	14.70
	Bingham	0.985	4.451	27.60
	Herschel-Bulkley	0.997	3.303	12.68
	Casson	0.993	3.497	0.40
CH_{c21}	Power law	0.999	0.812	3.33
	Bingham	0.999	1.169	6.03
	Herschel-Bulkley	0.999	1.004	1.53
	Casson	0.999	0.410	0.04
CH_{c22}	Power law	0.999	2.194	5.36
	Bingham	0.999	0.875	5.02
	Herschel-Bulkley	0.999	2.830	0.95
	Casson	0.999	0.395	0.03

(CH_b: The sample containing base formulation, CH_l: The sample containing lecithin, CH_p: The sample containing PGPR, CH_c: The sample containing citrem; the first number: Refining time (1: 60 min, 2: 90 min), the second number: Emulsifier level (1: 0.5% or 0.25% (the sample containing PGPR), 2: 1% or 0.5% (the sample containing PGPR)).

3.4.2 Apparent viscosity

The apparent viscosities of the samples are shown in Table 3.

In this study, no significant differences between samples containing lecithin and basic formula in second refining time ($p<0.05$) were found. A general trend regardless to fat content was seen as consistent decreases in apparent viscosity while increasing particle size (AFOAKWA 2009); increase in particle size from 18 to 50 μ m caused noticeable decrease in apparent viscosity- which was similar with Casson plastic viscosity- specially at low fat (25%). in addition, it was reported that by increasing lecithin from 0.3 to 0.5%, the apparent viscosity decreased regardless to particle size and fat content. This study proves that different refining times have no effect on the specified levels of samples containing citrem, whereas, amount of citrem is effective on apparent viscosity.

Table 3. The measured values of Casson viscosity, Casson yield, and apparent viscosity for different formula.

Sample	Casson viscosity (Pa.s)	Casson yield value (Pa)	Apparent viscosity (Pa.s)
CH_{b1}	18.74[b]	16.92[d]	24.7[b]
CH_{b2}	22.56[a]	22.18[c]	30.4 [a]
CH_{l11}	21.25[a]	18.66 [d]	28[a]
CH_{l12}	14.89[c]	29.37[b]	22.1[c]
CH_{l21}	20.94[a]	31.92[b]	28.9 [a]
CH_{l22}	17.22[b]	66.09[a]	29.6[a]
CH_{c11}	15.13[c]	10.23[e]	21.7[c]
CH_{c12}	13.46[c]	11.15[e]	18.6 [d]
CH_{c21}	14.97[c]	21.23[c]	20.9[c]
CH_{c22}	14.59[c]	15.88 [d]	19.3[d]

(CH_l: The sample containing lecithin, CH_c: The sample containing citrem; the first number: Refining time (1: 60 min, 2: 90 min), the second number: Emulsifier level (1: 0.5%, 2: 1%). Different letters indicate statistically significant differences ($p<0.05$).

Casson model

Casson Viscosity

Values for Casson viscosity and Casson yield value were determined through the Casson model fit on the data (shear stress - shear rate). Casson viscosity values ranged between 13.46 and 22.56 Pa.s (Table 3). In this study, there were no significant differences between samples containing citrem at both refining times and different levels ($p<0.05$). The results showed that different refining time have no effect on samples containing 0.5% lecithin, whereas, in the samples containing 1% lecithin, by increasing refining time, Casson viscosity increased and also by increasing lecithin, Casson viscosity decreased at both refining time. AFOAKWA (2009) reported increase in Casson viscosity while increasing refining time and reducing particle size. Moreover, it was seen that, especially at lower fat and lecithin levels, Casson plastic viscosity, Casson yield value, yield stress and apparent viscosity decreased in higher particle sizes. Fat reduction up to 30% has little effect on the Casson parameters; however, in chocolates with a fat content of less than 30%, by reducing the fat content, the Casson parameters, particularly the Casson viscosity, will increase (BECKETT, 2000).

Casson yield value

The Casson yield values are shown in Table 3. The yield stress or yield value relates to shape retention, pattern holding, feet and tails, inclined surface coating and presence of air bubbles (SEGUINE, 1988). The Casson yield values ranged 10.23 to 66.09. The sample having citrem in the initial refining time and level of 0.5% had the least yield value and lecithin in second refining time and level of 1% had the highest yield stress. In all samples, by increasing refining time and reducing particle size, the Casson yield stress increased. Evaluation of rheological characteristics revealed that increasing particle size, fat percentage (more specifically in low fat samples (25%)) and lecithin concentration play as a reduction agent for Casson yield values (AFOAKWA, 2009). It was observed that Casson yield value of the samples containing lecithin was more than samples with base formulations. The reason is that if the amount of lecithin rises above 0.3%, Casson yield stress increases (FINCKE, 2013). The samples containing citrem at initial refining time was the most effectives in reducing Casson yield value ($p <0.05$). Yield value is affected largely by interparticle contacts and consequently shows a linear dependence on the mean particle size, or more accurately, on the specific surface area (MONGIA, 1997; MONGIA and ZIEGLER, 2000). By decreasing the particle size there are more particles for intermolecular contact, thus the Casson yield value increases. PRENTICE (1984) reported that when particle size decreases, interactions and subsequent friction constants between the particles increase, thus the Casson yield stress increases.

Power law model

As described before, power law model was chosen as an appropriate flow model for PGPR containing samples (Table 4). The shear stress-shear rate tests (in the mentioned range) showed a consistency coefficient range of 17.49 to 26.05 for the four formulations. However, the estimated flow behavior indices showed to be close to $n=1$ for all formulae. It can be concluded that adding PGPR emulsifier to the compound chocolate caused change in consistency index but it did not affect the flow behavior index. It is also worthy to note that, in samples containing 0.25% PGPR, increasing refining time was affective and

led to increased consistency index, whereas, there was no significant deference between samples having 0.5% PGPR ($p<0.05$).

Table 4. The values of consistency index, flow behavior index, and apparent viscosity for the samples containing PGPR.

Sample	Consistency index (Pa.s)	Flow behavior index	Apparent viscosity (Pa.s)
CH_{p11}	17.49^{c}	0.99^{a}	19.5^{c}
CH_{p12}	19.72^{b}	0.99^{a}	22.6^{b}
CH_{p21}	26.05^{a}	0.99^{a}	25.5^{a}
CH_{p22}	20.40^{b}	0.99^{a}	22.4^{b}

(CH_p: The sample containing PGPR, the first number: Refining time (1: 60 min, 2: 90 min), the second number: Emulsifier level (1: 0.25%, 2: 0.5%). Different letters indicate statistically significant differences ($p<0.05$).

4. CONCLUSIONS

To conclude, the refining time as a main factor affecting particle size distribution, emulsifier types and their levels are two important factors in optimization of compound chocolate with reduced fat content. Reduction of particle size increased the Casson yield value, although the rheological properties were related to type of emulsifiers and refining times, too. In addition, the hardness of the samples decreased by increasing emulsifier content and decreasing refining time. The Casson model was selected as an appropriate rheological model to illustrate the rheological parameters of the samples containing the citrem and lecithin as emulsifiers. Nevertheless, chocolate models with reduced fat content containing the PGPR were not in a good agreement with the Casson model. On the contrary, the power law model showed the highest correlation to their flow behavior. Finally, reduction of fat content leads to an increase in the molten compound chocolate viscosity and hardness, therefore, using citrem emulsifier because of significant reduction in the hardness and rheological parameters such as apparent viscosity, Casson viscosity and Casson yield value can be effective and useful for production of reduced fat dark compound chocolate.

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PAPER

HOMEMADE TOMATO SAUCE IN THE MEDITERRANEAN DIET: A RICH SOURCE OF ANTIOXIDANTS

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ABSTRACT

The basic ingredients used to make the Italian *soffritto* were studied in order to define the polyphenol, antioxidant capacity and lycopene content of homemade or commercial tomato sauces, as well as their contribute in whole wheat or refined wheat pasta.
The addition of aromatic herbs to sauces increased polyphenols and antioxidant capacity, with basil providing the biggest boost, whereas ready-made commercial tomato sauces showed the lowest antioxidant values. Cooked whole wheat pasta with homemade tomato sauce offers an enormous amount of antioxidants, which could protect against oxidative stress.

Keywords: antioxidant activity, extra virgin olive oil, lycopene, polyphenols, tomato sauces, vegetables and aromatic herbs

1. INTRODUCTION

The Mediterranean Diet (Med Diet) is based on the daily consumption of fresh vegetables, aromatic herbs, whole grains and extra virgin olive oil (EVOO), cooked quickly to conserve the molecular integrity of their nutrients (BERTUCCIOLI and NINFALI, 2014). Indeed, most of the beneficial effects of the Med Diet stem from the high level of vitamins, carotenoids and in particular polyphenols, found in homemade Mediterranean cuisine (DRAGSTED, 2003).

Soffritto (sauté) is the Italian world that perfectly describes the process of gently cooking vegetables in oil to soften them and release their flavours. During the preparation of a sauté, onions, garlic, celery and carrots are chopped and gently sautéed in extra virgin olive oil (EVOO) for some minutes. The addition of the tomato (*Solanum lycopersicum*) sauce is another important step in the preparation. Forty polyphenols and seven carotenoids have been identified in a typical Med Diet tomato sauce, whose composition has attracted the interest of food professionals for its nutritional and chemo preventive value (MARTÍ *et al*., 2016; SHEN *et al*., 2007; VALLVERDÚ-QUERALT *et al*., 2013).

Tomatoes contain several micronutrients, including vitamin C, vitamin E, folates, phenolic compounds and lycopene (VALLVERDÚ-QUERALT *et al*., 2012).

Beyond differences in their nutritional contents, due to the tomato cultivar and agronomic conditions, the antioxidant compounds and lycopene concentration found in processed tomato products is markedly affected by the average maturity stage of the bulk of processed tomatoes (COOPERSTONE *et al*., 2015; GHASEMZADEH *et al*., 2016; GÓMEZ *et al*., 2001; GUINE and GONCALVES, 2016; RAFFO *et al*., 2002; ZANFINI *et al*., 2016).

In the Med Diet tomato sauce, the addition of aromatic herbs, mainly basil, oregano and marjoram, near the end of the cooking represents an important step able to enhance its taste and flavor (GUINE and GONCALVES, 2016). Basil (*Ocimum basilicum*) contains more than 200 bioactive compounds, including monoterpenes, phenolic acids, steroids, vitamins (A, C, E, K) and flavonoids (GHASEMZADEH *et al*., 2016). Oregano (*Origanum virens*) mainly contains carvacrol, cinnamaldehyde and essential oils and marjoram (*Origanum marjorana*) is rich in phenolic acids, flavonoids and essential oils (GUINE and GONCALVES, 2016).

The Med Diet tomato sauce may play a key role in conferring higher antioxidant activity to the pasta dish. Antioxidant activity is an important parameter in assessing the quality of products, as it measures the global antioxidant system of the product and appears to be closely related to the prevention of oxidative stress linked diseases (GHISELLI *et al*., 2000).

Pasta is the popular worldwide product made with durum wheat semolina. Remarkable total antioxidant capacity is attained when pasta is made with whole grain flour, due to the higher polyphenol content of whole grains compared to refined wheat (ANTONINI *et al*., 2017; LIU, 2007).

The aim of this study was to evaluate the polyphenol, ORAC and lycopene values of four homemade tomato sauces containing EVOO, fresh vegetables, tomato puree, and different types of aromatic herbs. These homemade sauces were compared with their commercially available industrially produced counterparts. Whole wheat pasta was compared with refined wheat pasta, to rank their respective polyphenol and ORAC values.

2. MATERIALS AND METHODS

2.1. Chemicals

Folin-Ciocalteu reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,20-Azobis(2-methylpropionamidine) dihydrochloride), fluorescein (2-(3-hydroxy-6-keto-xanthen-9-yl)benzoic acid) and caffeic acid were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3) was supplied by Carlo Erba Reagents (Milano, MI, Italy). Acetone, ethanol, ethylacetate, methanol, n-hexane were purchased from VWR International Inc. (Radnor, PA, USA).

2.2. Samples

Fresh vegetables, garlic (cv. *Aglio bianco*), celery (cv. *Plein Blanc Pascal*), carrots *(cv. Flakkè 2*), onions (dorata cv. *Dorata di Parma*; tropea cv. *Tropea Rossa Tonda Record*; suasa cv. *Cipolla di Suasa*), aromatic herbs (basil, oregano and marjoram), tomato puree (glass bottle, 750 g) and sunflower seed oil (plastic bottle, 1 l) were purchased from the local supermarket. Artisanal EVOO (cv. *Coratina*) was supplied by a producer from Apulia.

2.3. Chemical quality parameters of EVOO

Regarding the chemical quality parameters of EVOO, free acidity (given as % of oleic acid) and peroxide values (meqO_2/kg of oil) were evaluated according to the European Economic Community Regulation no. 2568/91 and its later modifications (EEC, 1991).

2.4. Homemade tomato sauce preparation
The sauces were prepared as follows (portions for 4 people).

Sauce 1. EVOO (30 g), onion (dorata, 50 g), celery (25 g), carrots (25 g), garlic (2 g). The mixture was heated 10 min at 180°C, tomato puree (180 g) was then added and cooked for an additional 10 min.
Sauce 2. The same steps and ingredients as *sauce 1* with the addition of basil (2 g), which was added at the same time as the tomato puree.
Sauce 3. The same steps and ingredients as *sauce 1* with the addition of oregano (2 g), which was added at the same time as the tomato puree.
Sauce 4. The same steps and ingredients as *sauce 1* with the addition of marjoram (2 g), which was added at the same time as the tomato puree.
The sauces were cooked on electric or gas heating plates and the temperature was controlled by a thermometer. The evaporation of the water reduced the weigh from about 300 g to 200 g, thus allowing the dressing of 4 pasta dishes.

Eight samples of industrially produced sauces where purchased at a local supermarket. Average composition was: tomato pulp (75%), tomato concentrate, onion, olive or seed oil, basil (2%), carrots, sugar, salt, celery, natural basil aromas.
Refined wheat pasta (RWP) was purchased from the local supermarket, whereas whole wheat pasta (WWP) was made by a local producer with whole grain (*Triticum durum*, cv. Odisseo), milled with a stone mill (sifting rate 15%), extruded using bronze dies and dried at 45°C.
Table 1 shows the nutritional data of RWP and WWP, as reported by the manufacture's indications, as well as those of sauce 1 obtained with a nutritional software.

Table 1. Macronutrient composition and fiber in the typical Med Diet pasta dish.

Nutritional data per serving*	Sauce 1 (50 g)	RWP (80 g)	WWP (80 g)	Sauce 1 (50 g) + RWP (80 g)	Sauce 1 (50 g) + WWP (80 g)
Energy (kcal)	55	282	259	337	314
Carbohydrates (g)	1.8	63.3	53.0	65.1	54.8
Proteins (g)	0.6	8.7	10.7	9.3	11.3
Lipids (g)	5.1	1.1	2.0	6.2	7.1
Fiber (g)	0.8	2.2	5.1	3.0	5.9

*Per serving: 50 g of Sauce 1; 80 g of RWP or WWP. RWP, refined wheat pasta; WWP, whole wheat pasta.

2.5. Lycopene extraction and assay

The lycopene analysis was performed as previously described (DAVIS *et al.*, 2003; PERIAGO *et al.*, 2004), with some modifications. Briefly, 2 g of sauce was mixed with 4 mL of distilled water and homogenized for 5 min with a Potter homogenizer. Then 1 g of homogenate was extracted with 20 mL of hexane:acetone:ethanol (2:1:1) mixture. The solution was shaken for 10 min and then centrifuged at 1800 x g at 5°C for 10 min. The absorbance of the supernatant was measured at 472 nm. A calibration curve was prepared with lycopene pure powder (Sigma-Aldrich Inc., St. Louis, MO, USA). Values were expressed as mg of lycopene per 100 g of sauce.

2.6. Extraction of polyphenols from vegetables and sauces

This analysis was performed following the procedure previously described (AGBOR *et al.*, 2014), with some modifications. Each sample (11 g) was homogenized with the Potter homogenizer. Then 0.5 g of homogenate was added to 8 mL of 50% methanol/water containing 1.2 M HCl. The sample was heated for 2h at 95°C and then centrifuged at 1000 x g at 5°C for 10 min. The supernatant (SN1) was brought to 10 mL with distilled water. 2M NaOH (8 mL) was added to the pellet and the mixture was shaken for 2 h at room temperature and centrifuged at 1000 x g at 5°C for 10 min.
The supernatant (SN2) was transferred into a flask and brought to 10 mL with distilled water. Both supernatants were combined (SN1 + SN2), and this solution (20 mL) was used for the total polyphenol and antioxidant capacity determination.

2.7. Extraction of polyphenols from oils

This analysis was performed as previously reported (ANTONINI *et al.*, 2016a). Briefly, the oil sample (3 g) was mixed with 5 mL of 80% methanol. The solution was vortexed for 2 min and then centrifuged at 1000 x g at 5°C for 10 min.
The supernatant was transferred into a Falcon tube at 4°C. The extraction was repeated twice, and the two supernatants were combined and preserved for the polyphenol and antioxidant capacity assay.

2.8. Extraction of polyphenols from raw and cooked pasta

Whole wheat pasta (WWP) and refined wheat pasta (RWP) samples (80 g) were cooked for 10 min in 2 L of boiling water. Raw and cooked pasta was freeze-dried and milled in a ZM 200 ultracentrifugal mill with a 0.5 ring sieve (Retsch, Haan, Germany). Free and bound

polyphenols were extracted from the freeze dried material as previously reported (ANTONINI *et al*., 2016b). The extracts were used for polyphenol and antioxidant capacity assays.

2.9. Polyphenols assay

The phenol compounds were assayed using the Folin-Ciocalteu method, as previously reported (SINGLETON *et al*., 1999). The absorbance of the mixture was measured at 725 nm. A calibration curve was prepared with caffeic acid (Sigma-Aldrich Inc., St. Louis, MO, USA).

For vegetables, polyphenol values were expressed as mg/100 g of fresh weight; for oils, as mg/kg; for sauce, as mg/100g of product. For raw and cooked pasta, total phenol values were obtained by the sum of free + bound phenols and expressed as mg/80 g dry weight (d.w.). Moisture was determined in the raw and cooked pasta using a thermal balance (Sartorius MA 40, Gottingen, Germany) after drying at 120°C to constant weight.

2.10. Oxygen Radical Absorbance Capacity (ORAC) assay

The antioxidant capacity of phenols was determined by the ORAC method (NINFALI *et al*., 2005; PRIOR *et al*., 2003), using a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany) equipped with a temperature-controlled incubation chamber and an automatic injection pump. The incubator temperature was set at 37°C.

The following mix was used for the hydrophilic ORAC (H-ORAC): 200 μl of 0.096 μM fluorescein sodium salt in 0.075 M Na-phosphate buffer (pH 7.0), 20 μl of sample or Trolox. The reaction was initiated with 40 μl of 0.33 M AAPH. The blank was 0.075 M Na-phosphate buffer (pH 7.0). Fluorescence was read at 485 nm ex. and 520 nm em. until complete extinction (NINFALI *et al*., 2005). A calibration curve was made each time with the standard Trolox in 0.075 M Na-phosphate buffer (pH 7.0).

The lipophilic ORAC (L-ORAC) for the antioxidant contribution of lycopene and liposoluble vitamins was measured as follows. The sauce was extracted with hexane (1:5 w/v) twice. After centrifugation at 1800 x g for 10 min, the supernatants were combined and dried under nitrogen flow, then re-suspended in 1 mL of 50% acetone, which was mixed with 7 mL of 7% hydroxypropyl β-cyclodextrin (Kleptose HP oral grade, Roquette, France) in 50% acetone (OU *et al*., 2013). After the incorporation of the lipophylic extract into the β-cyclodextrins by rotating overnight in the dark, the solution was centrifuged and read with the Fluostar Optima plate reader fluorimeter, with the same reaction mixture of the H-ORAC and the blank with 20 μl of 7% β-cyclodextrins in 50% acetone, diluted with phosphate buffer. A calibration curve was made each time with the standard Trolox in β-cyclodextrins.

For vegetables, ORAC values were expressed as μmol Trolox Equivalents (TE)/100 g of fresh weight; for oils, as μmol TE/kg; for sauce, as μmol TE/100g of product. For raw and cooked pasta, total ORAC (free + bound phenols) values were expressed as μmol TE/80 g d.w.

2.11. Statistical analysis

The chemical parameters of oils were detected in triplicate and values were expressed as the mean±SD. Polyphenol and lycopene concentrations were measured in triplicate and results were the mean±SD. ORAC data were obtained by eight independent determinations for each sample and results were the mean±SD. Statistical significance was

tested using Student's t test and one-way ANOVA, with a $p \leq 0.05$ indicating a significant difference between data sets (SPSS® 17.0 software, IBM, Chicago, IL, USA).

3. RESULTS AND DISCUSSIONS

3.1. Antioxidant content and activity of raw and sautéed vegetables

We first investigated the total phenols and antioxidant capacity of the individual vegetables after they had been sautéed for 10 min in EVOO to assess how each vegetable was stable during the sautéing process. Results are reported in Fig. 1.

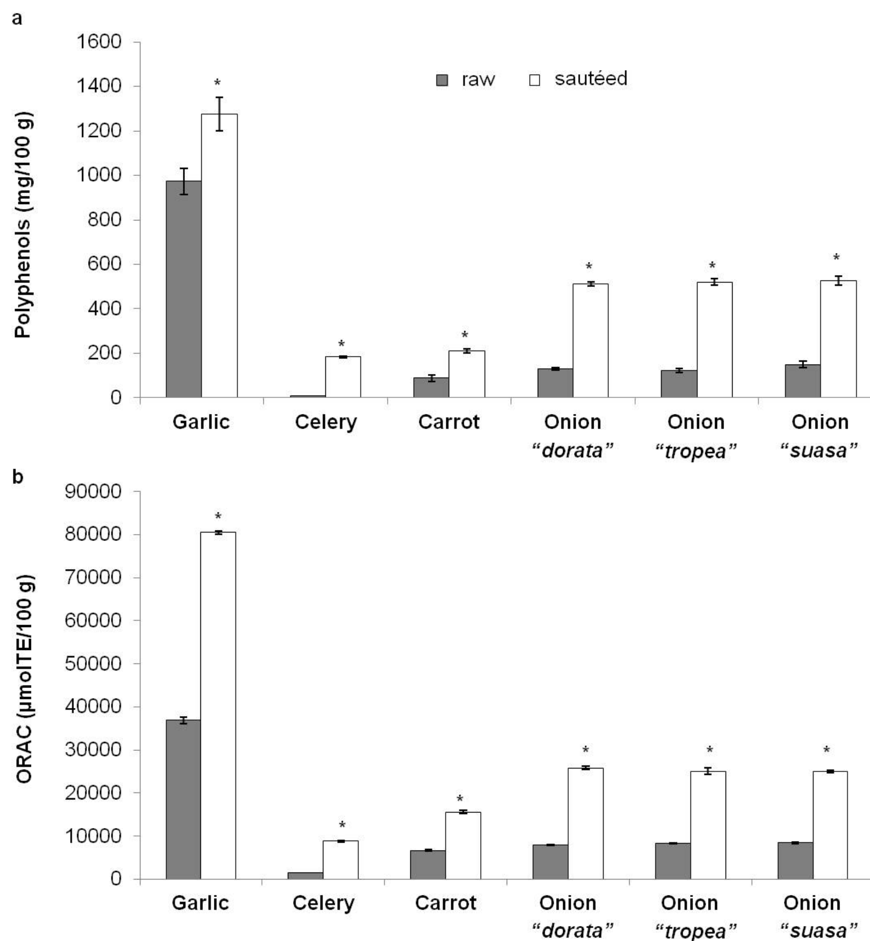

Figure 1. Polyphenols (a) and antioxidant capacity (b) in raw and EVOO sautéed vegetables. The cooking time was 10 min at 180±5°C.
(*) indicates statistically significant differences ($p \leq 0.05$) between raw and sautéed vegetables (Student's t test).

After the vegetables had been sautéed in EVOO and placed on filter paper for few minutes to get rid of excess oil, their total phenols (Fig. 1a), as well as their antioxidant capacity (Fig. 1b), increased remarkably compared to their raw values. The increase was due to the penetration of the oil into the vegetable tissues and the evaporation of the water from

those tissues. Moreover, the polyphenols bound to the fiber were freed by the thermal disruption of chemical bonds and new molecules, including Maillard products, may have been formed (FRATI *et al*., 2016; NICOLI *et al*., 1999; SANTOS *et al*., 2013).
In the three sautéed onion cultivars (dorata, tropea, suasa), there was an average increase of 70% in polyphenols and ORAC values compared to raw values, but no statistically significant difference between the three cultivars was observed (Fig. 1).
As onion is the major vegetable in the soffritto, possibly the study of other genetic varieties would be an important issue, for increasing the antioxidants in the sauté.

3.2. Chemical and antioxidant parameters in EVOO and seed oil after cooking

In line with the best tradition of the Med Diet, we only used EVOO for sautéing the vegetable mixture. However, as many people and many industrial companies use seed oil, we analyzed the chemical modifications of the EVOO in comparison with sunflower seed oil after sautéing in the absence or in the presence of the vegetable mixture.
Table 2 shows the quality parameters of oils: acidity, peroxide number, polyphenols and ORAC.

Table 2. Quality parameters of raw and sautéed EVOO and sunflower seed oil in the presence or in the absence of the vegetable mixture.

	Acidity (% oleic acid)	Peroxide number ($mEqO_2$/kg)	Polyphenols (mg/kg)	ORAC (µmolTE/kg)
		EVOO		
Raw	0.68±0.05 [a]	5.01±0.47 [c]	360±20 [a]	15,600±940 [a]
Sautéed alone†	0.66±0.07 [a]	22.17±1.02 [a]	200±11 [c]	8,580±500 [c]
Sautéed with vegetables‡	0.60±0.05 [a]	10.02±0.98 [b]	250±15 [b]	9,900±600 [b]
		Sunflower seed oil		
Raw	0.11±0.02 [b]	1.02±0.14 [c]	20±0.3 [a]	678±40 [a]
Sautéed alone†	0.22±0.03 [a]	38.47±2.90 [a]	3.0±0.5 [c]	68±4 [c]
Sautéed with vegetables‡	0.18±0.04 [a]	27.61±3.11 [b]	8.0±0.4 [b]	151±14 [b]

[a-c]Different letters indicate, for each quality parameter, statistically significant differences among raw, sautéed alone or sautéed with vegetables EVOO or sunflower seed oil ($p \leq 0.05$; one-way ANOVA). †EVOO or sunflower seed oil were sautéed 15 min at 180±5°C in the absence of vegetables; ‡EVOO or sunflower seed oil were sautéed 15 min at 180±5°C in the presence of the vegetable mixture (the same used to prepare the homemade tomato sauce: onion, celery, carrots and garlic).

The acidity of raw sunflower seed oil was comparatively smaller than that of raw EVOO, due to the neutralization step during refining (CASAL *et al*., 2010). Nevertheless, in the sautéed EVOO, the acidity remained unchanged, whereas it increased in the sautéed sunflower seed oil (Table 2).
Concerning the peroxide number, Table 2 shows that the thermal treatment led to a moderate increase in the EVOO and a marked increase in the sunflower seed oil, due to the high polyunsaturated fatty acid concentration in the latter, highly susceptible to the oxidation (CASAL *et al*., 2010). Moreover, the vegetables protected the oils from oxidation, limiting the increase of the peroxide number, with respect to the oils sautéed alone (Table 2).

A similar trend was obtained for polyphenols and ORAC values, as the vegetables protected the oils from the decay of the two parameters during the thermal treatment (Table 2).
Therefore, the EVOO remains the preferred option for making sauces for three main reasons: 1) Higher concentration of polyphenols (NINFALI *et al*., 2002); 2) Reduced phenol and ORAC losses in the presence of the vegetables; 3) Higher stability of the peroxide index.

3.3. Homemade and commercial tomato sauces and lycopene values
After the addition of the tomato puree to the fried vegetables, we evaluated total ORAC, including both hydrophilic (H-ORAC) and lipophilic (L-ORAC) fraction, the polyphenol and lycopene values of the homemade (sauces 1-4) and industrially produced (commercial) sauces. Results are reported in Table 3.

Table 3. Polyphenols, antioxidant capacity and lycopene in homemade and commercial sauces.

	Polyphenols (mg/100 g)	H-ORAC	L-ORAC (µmolTE/100 g)	Total ORAC	Lycopene (mg/100 g)
Sauce 1	207±9 [c]	10,184±509 [c]	886±44 [c]	11,070±303 [c]	18.72±1.12 [a]
Sauce 2	298±8 [a]	16,216±811 [a]	1,604±80 [a]	17,820±730 [a]	21.22±1.27 [a]
Sauce 3	250±12 [b]	13,800±690 [b]	1,200±110 [b]	15,000±403 [b]	17.92±1.43 [a]
Sauce 4	274±13 [b]	13,431±671 [b]	1,011±85 [b]	14,442±397 [b]	18.45±1.66 [a]
Commercial	134±18 [d]	6,595±330 [d]	573±29 [d]	7,168±353 [d]	13.70±1.20 [b]

[a-d]Different letters indicate statistically significant differences among samples ($p \leq 0.05$; one-way ANOVA). Commercial values are the average of 8 commercial ready-to-eat sauces; H-ORAC, hydrophilic ORAC; L-ORAC, lipophilic ORAC; Total ORAC, given by the sum of H- and L-ORAC.

The addition of tomato puree to the sautéed vegetables confers to the sauces many powerful antioxidants, including flavonoids present in the tomato puree (VALLVERDÚ-QUERALT *et al*., 2012), as well as lycopene, which largely contributes to the lipophilic antioxidant capacity (L-ORAC) (CANO *et al*., 2003).
The H-ORAC, which correlates to the ascorbic acid content (CANO *et al*., 2003), contributed to the highest antioxidant capacity (Table 3), representing more than 90% of total ORAC (WU *et al*., 2004).
Regarding the lycopene content, no statistically significant difference was observed among the homemade sauces ($p > 0.05$) (Table 3).
All of the aromatic herbs, namely basil, oregano, and marjoram, increased the polyphenol and antioxidant capacity values of the tomato sauce, with the sauce 2, with basil, showing the highest polyphenol and total ORAC values (Table 3). Basil leaves were found to yield the highest increase, possibly due to the herb's high phenol and flavonoid contents (GUINE and GONCALVES, 2016; NINFALI *et al*., 2005).
The commercial sauces had significantly lower polyphenol, ORAC and lycopene values than do homemade sauces (Table 3). Although only eight commercial samples were assessed, our data show that homemade sauces have greater nutritional value than do their store-bought counterparts. The lower nutritional value of commercial sauces may be due to the use of seed oil instead of EVOO, non homogeneously ripened fruits, concentrated ingredients stored for long periods, rough processing technologies in the sauce preparation (NINFALI and BACCHIOCCA, 2004).

Hence, the ability to make homemade tomato sauces using fresh vegetables and aromatic herbs is part of the cultural background and has important health implications (HOFFMAN and GERBER, 2015; PRIOR *et al*., 2007; VALUSSI, 2012).
The experiences linked to the Food Literacy Project (https://dining.harvard.edu/food-literacy-project), promoted by the Harvard University and the Lifelong Learning by the European Union Commission, are focused on the increase of the consumers' consciousness in the culinary practices and sustainable nutrition. In this light, Table 4 summarizes the best ways to select, cook and store the vegetables to be used in the homemade sauce.

3.4. Antioxidants in pasta with tomato sauce

Fig. 2 shows the concentration of free and bound polyphenols (Fig. 2a) and ORAC (Fig. 2 b) values in whole wheat pasta (WWP) and refined wheat pasta (RWP), under raw or cooked conditions.

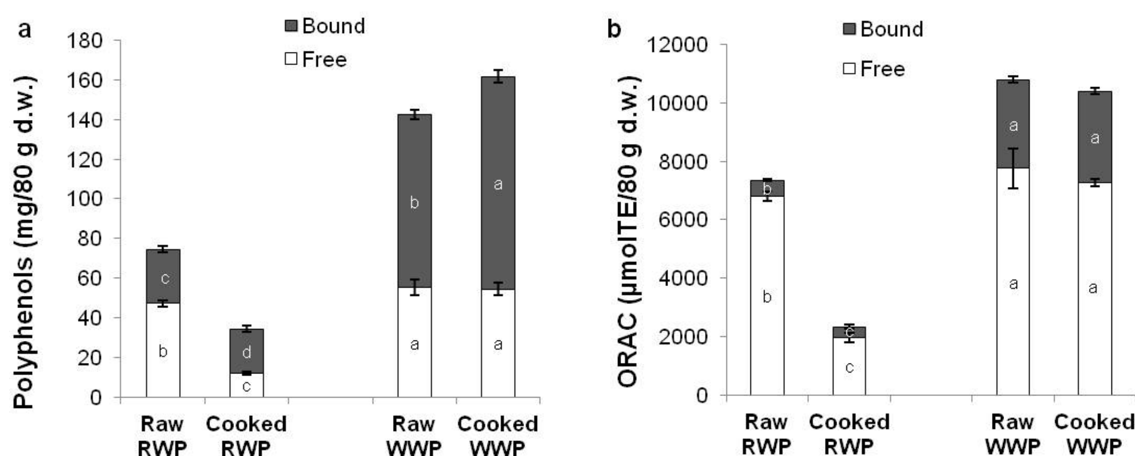

Figure 2. Free and bound polyphenols (a) and antioxidant capacity (b) in raw and cooked whole wheat pasta (WWP) and refined wheat pasta (RWP). Polyphenol values are expressed as mg/80 g of raw and cooked pasta, on dry weight (d.w.). ORAC values are expressed as μmol of Trolox equivalents (TE)/80 g of raw and cooked pasta, on d.w.
a,b Different letters indicate statistically significant differences among raw and cooked RWP and WWP ($p \leq 0.05$, Student's t test), for each component (free and bound).

Given that the average portion per meal, for a healthy adult, is 80 g of pasta (http://www.sinu.it/public/20141111_LARN_Porzioni.pdf), the values are reported per serving of raw and cooked pasta, on dry weight.
Concerning the raw pasta, WWP showed significantly higher total polyphenol (Fig. 2a) and ORAC (Fig. 2b) values than RWP. When the free and bound polyphenols were compared, the bound were predominant in WWP with respect to RWP, due to the loss of polyphenols in the refining process (ANTONINI *et al*., 2017; PANATO *et al*., 2017).
After cooking, total polyphenols and ORAC values remained almost unchanged in the WWP, whereas they were reduced by 50 and 70%, respectively, in the RWP. In the cooked RWP, the free polyphenols and ORAC were markedly reduced, as compared to the bound ones (Fig. 2). This aspect is linked to the presence of the fiber which preserve the polyphenols during cooking (LIU, 2007).

Table 4. Nutritional values of sauce ingredients with guidelines regarding their selection, storage and cooking.

Vegetable/ Aromatic herb	Main antioxidants	Heat resistance	Freshness index	Seasonality	Storage	References
Carrot	Carotenoids, hydroxycinnamic acids, anthocyanidins, vitamin C	β-carotene is heat resistant and becomes more bioavailable through cooking in oil	Roots should be firm, smooth, bright in color	All year	Up to two weeks if stored without leaves in the refrigerator	(LEMMENS *et al.*, 2011) (SELJÅSEN *et al.*, 2013)
Celery	Phenolic acids, flavonoids, stilbenoids, furanocoumarins, phytosterols, vitamin C, β-carotene	Heat for as little time as possible (5-15 min)	Crisp, tight and compact, stalks with pale bright green leaves	All year	Fresh for 5-7 days in the refrigerator	(YAO *et al.*, 2011) (OVODOVA *et al.*, 2009)
Garlic	Allicin (thiosulfinate)	Heat for as little time as possible (5-15 min)	Plump with unbroken skin	All year	Fresh for 1 month stored in a cool, dark place	(MARTINS *et al.*, 2016)
Onion	Quercetin, vitamin C	Heat for as little time as possible (5-15 min)	Well shaped, no opening at the neck, with crisp, dry outer layer of skin	All year	Fresh for four weeks in a dark ventilated space at room temperature	(BYSTRICKÁ *et al.*, 2013)
Tomato	Lycopene, β-carotene, hydroxycinnamic acids, flavonoids, vitamin C and E, folate	Lycopene is heat resistant and-becomes more bioavailable through cooking in oil	Red, well shaped and smooth skinned	From May to September	Up to two weeks at room temperature and out of direct exposure to sunlight, depending on ripeness	(GÓMEZ *et al.*, 2016) (VALLVERDÚ-QUERALT *et al.*, 2012)
Basil	Phenolic acids, flavonoids, β-carotene, vitamins (A,C,E,K) essential oils	Add near the end of the cooking to retain its maximum essence and flavor	The leaves should be deep green in color	In the summer	Fresh, wrapped in a paper towel in the refrigerator; dried, in a sealed glass container in a cool, dark place	(GHASEMZADEH *et al.*, 2016; LEE *et al.*, 2005)
Oregano	Carvacrol, cinnamaldehyde, essential oil	Add fresh near the end of the cooking; at the beginning if dried	Firm stems with fresh leaves, green in color	All year	Fresh, wrapped in a paper towel in the refrigerator; dried, in a sealed glass container in a cool, dark place	(TEIXEIRA *et al.*, 2013) (GUINE and GONCALVES, 2016)
Marjoram	See oregano					

4. CONCLUSIONS

This study focuses on the contribution in terms of phenols, antioxidant capacity and lycopene of homemade tomato sauce, a staple of the Med Diet.
For the sake of transparency, the food industry, which produces ready-made sauces, should rank the quality of the ingredients used in their sauces, as well as the impact of industrial processing technologies, used in their production by measuring polyphenols and antioxidant capacity before and after processing.
The main nutritional suggestions of this study are the followings:

a) Use EVOO instead of seed oil to increase the phenol contribute;
b) Use short cooking time: about 10 min are sufficient to allow the oil to make free the phenols of vegetables;
c) Add fresh vegetables and brilliant red tomato sauce with high lycopene concentration;
d) Add aromatic herbs near to the end of cooking to increase the antioxidant capacity of the basic tomato sauce;
e) Use whole wheat pasta, which has greater polyphenols and antioxidant capacity than refined wheat pasta and dress it with a 1:2 ratio of sauce/pasta.

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PAPER

EFFECT OF DEGREE OF RICE MILLING ON ANTIOXIDANT COMPONENTS AND CAPACITIES

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ABSTRACT

De-husking and milling-induced changes in the content of antioxidant compounds and the antioxidant capacities of rice fractions were investigated in this study. Six fractions - rice husk, brown rice, and milled rice (MR) after four different degrees of milling (MR-3.5, MR-5.3, MR-7.1, and MR-9.9) - were extracted with 70% aqueous ethanol or water. Total phenolic and flavonoid contents decreased significantly ($p < 0.05$) as the degree of milling increased. Rice husk and brown rice fractions showed higher 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities than those of the MR, as well as higher levels of antioxidant components (total phenolics and total flavonoids). Phytochemicals such as phenolic compounds and vitamin E are mainly concentrated in the outer layers of the grains rather than in the endosperm. These findings suggest that consuming rice milled to a lesser degree may have certain health benefits.

Keywords: rice, degree of milling, antioxidant, phenolics, vitamin E

1. INTRODUCTION

Rice (*Oryza sativa*) is one of the world's most important cereal crops and a main staple food in Korea and many other Asian countries. As much as 75% of the daily caloric intake of the population of some Asian countries is derived from rice (HUANG *et al*., 2015). The rice grain has a hard husk that protects the kernel within. Brown rice is obtained by removing the husk but leaving the bran, germ, and endosperm. Milled rice is produced by removing the bran layers of the rough rice kernel by milling (BUTSAT and SIRIAMORNPUN, 2010). Although brown rice and under-milled rice are considered excellent sources of calories and nutrients such as vitamins and minerals (ZHANG *et al*., 2014), white rice is preferred by consumers because of its good eating quality. However, because of growing health consciousness, some consumers in Asia have recently started consuming rice milled to a lesser degree or even brown rice.

Plant-derived phytochemicals, which are potential sources of natural antioxidants, may combat oxidative stress in the human body by maintaining the balance between oxidants and antioxidants (TEMPLE, 2000). Many naturally occurring phytochemicals in plant-derived products contain a complex mixture of phenolic compounds that can exert several biological effects including antioxidant activity (PANDEY and RIZVI, 2009). It has been reported that phenolic compounds may function as free radical scavengers and quenchers of singlet oxygen. Their antioxidant activities have been attributed to their redox properties (PATEL *et al*., 2011). Antioxidant phytochemicals that quench free radicals may play a significant role in human health. A wide variety of biologically active phytochemicals are concentrated mainly in the pericarp and aleurone layers in cereal grains such as rice, wheat, and oats (BUTSAT and SIRIAMORNPUN, 2010; HA *et al*., 2006). Several studies have demonstrated that the aleurone layer has high nutritive value and has beneficial health effects, such as decreasing the incidence of atherosclerotic disease (DANIEL *et al*., 1999; MORTON *et al*., 2000), lowering blood cholesterol (OKARTER and LIU, 2010), and preventing cardiovascular disease (MARTINEZ-VALVERDE *et al*., 2000). It has also been reported to have an anticancer effect (NEWMARK, 1996). Some of these protective effects may be attributed to polyphenols, which comprise several classes of flavonoids and vitamin E as well as other phenolic constituents and are present in the aleurone layer. However, dehusking and milling decreases the content of antioxidant compounds in the grain. Thus, the degree of milling is an important factor in the nutritional value of milled rice.

Previous studies have reported the effect of milling on the physicochemical properties of rice and the cooking and textural properties of milled rice (SINGH *et al*., 2005; LIU *et al*., 2015). Several studies have also reported the content of antioxidant compounds and the antioxidant activity of white or black rice and the contents of various beneficial components (several phenolic compounds, tocopherols, tocotrienols, and γ-oryzanol) of rice bran or husks (ADOM and LIU, 2002; ZHANG *et al*., 2010; BUTSAT *et al*., 2009). However, limited information is available on the correlation between the antioxidant compound content and activity of rice husks, brown rice, and rice milled to different degrees. Therefore, the aim of this study was to determine the antioxidant compound content and antioxidant activity of milled rice fractions and to evaluate husks as a source of natural antioxidants.

2. MATERIALS AND METHODS

2.1. Materials

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), the diammonium salt of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu's (FC) phenol reagent, tocopherol (α-, β-, γ-, and δ-T), tocotrienol (α-, β-, γ-, and δ-T3), ascorbic acid, gallic acid, (+)-catechin, butylated hydroxytoluene (BHT), potassium hydroxide, and sodium chloride were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals and solvents used were of analytical or HPLC-grade.

2.2. Dehusking and milling of rice

The paddy rice sample (*O. sativa* L. Chucheongbyeo, short-grain rice) used in this study was obtained from the Korea Food Research Institute. The paddy sample was harvested in 2014. The rice variety used in the study is commercially available in Korea. Rice was threshed to separate the rice husk (RH) and brown rice (BR) using an automatic rice husker (Kett TR200, Kokyo, Japan). The brown rice samples were polished in a McGrill no. 2 mill (Ricepal32, Yamamoto Co., Ltd., Tendo, Japan) to obtain rice grains milled to different degrees (3.5% [MR-3.5], 5.3% [MR-5.3], 7.1% [MR-7.1], and 9.9% [MR-9.9]). The degree of milling was determined using the following relationship: [1 − (1000 − kernel weight of milled rice)/(1000 − kernel weight of brown rice)] × 100. All fractions, except the bran, were stored in double-sealed polythene bags at -25°C prior to analysis.

2.3. Sample preparation

All fractions were ground using a blender. Samples of approximately 200 g were extracted using 1 L of 70% aqueous ethanol or distilled water in a shaker (JSSI-100T, JS Research Inc., Gongju, Korea) at 25±3°C for 24 h. The extracts were filtered using Whatman no. 2 filter paper, and the residues were discarded. The filtrate was concentrated at 40°C using a vacuum rotary evaporator (R-205, Büchi, Flawil, Switzerland), lyophilized, and then stored at -20°C until further analysis.

2.4. Determination of total phenolic and flavonoid contents

The concentrations of total phenolic and flavonoid compounds in the extracts were measured spectrophotometrically. Total phenolic content (TPC) was determined using the FC-method (DEWANTO *et al*., 2002) with some modifications. Standard solution or sample extracts were mixed with 2 mL of 2% sodium carbonate solution and 100 μL of 1 N Folin-Ciocalteu reagent. After incubating for 30 min at 25±3°C, the absorbance at 750 nm was measured using a spectrophotometer. The results are expressed as mg of gallic acid equivalents per 100 g of sample (the equation of the standard curve was $y = 0.0024x - 0.0897$, $R^2 = 0.998$).
Total flavonoid content (TFC) in the samples was determined by a colourimetric method (TIAN *et al*., 2011). Briefly, either standard solution or sample extracts were mixed with 1.25 mL of distilled water and 75 μL of 5% $NaNO_2$. After incubating for 30 min at 25±3°C, 150 μL of 10% $AlCl_3 \cdot 6H_2O$ was added, and the mixture was allowed to stand for 5 min before the addition of 0.5 mL of 1 M NaOH. The absorbance at 510 nm was then measured. The results are expressed as (+)-catechin equivalents per 100 g of sample (the equation of the standard curve was $y = 0.0024x + 0.0275$, $R^2 = 0.998$).

2.5. Determination of the vitamin E composition

Total vitamin E content and the content of each of its isomers in the samples were determined using HPLC with a fluorescence detector (WIE *et al.*, 2009). A 10 mL aliquot of ethanol containing pyrogallol (6%, w/v) was added to 1 g of sample in a saponification vessel. After sonication for 5 min, 5 mL of 60% potassium hydroxide was added, and the vessel was flushed with nitrogen gas for 1 min. An air condenser was attached to the vessel, and the contents were digested at 70°C for 50 min in a shaking water bath. The contents were cooled in an ice bath, 20 mL of 2% sodium chloride was added, and the mixture was extracted three times with 20 mL of hexane/ethyl acetate (85:15, v/v) containing 0.001% BHT. The extracts were pooled and diluted to 50 mL and filtered through a 0.45 μm nylon membrane filter. An aliquot of the filtered extract was analysed using a normal-phase HPLC system (PU-1580; Jasco, Tokyo, Japan). Analysis of tocopherol and tocotrienol isomers was performed on a LiChrospher® Diol column (250 × 4 mm, 5 μm; Merck, Darmstadt, Germany) using a mobile phase of hexane/isopropanol (98.7:1.3, v/v) at a flow rate of 1.0 mL/min. Peaks were detected by fluorescence (FP-1520 l Jasco) using an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Tocopherol and tocotrienol peaks were identified by comparing their retention times to those of standards (the standard curve equations and R^2 values were α-T, $y = 0.000001x + 0.021928$, $R^2 = 0.998$; α-T3, $y = 0.000001x + 0.018294$, $R^2 = 0.997$; β-T, $y = 0.000001x + 0.005295$, $R^2 = 0.997$; γ-T, $y = 0.000001x + 0.024276$, $R^2 = 0.998$; γ-T3, $y = 0.000001x - 0.000236$, $R^2 = 0.998$; δ-T, $y = 0.0000005x + 0.0036275$, $R^2 = 0.998$; δ-T3, $y = 0.0000003 + 0.0110694$, $R^2 = 0.999$).
All analyses, except for that of vitamin E, were conducted in triplicate.

2.6. Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was assayed by the method described by Choi and Lee (2009) with some modifications. Briefly, 0.1 mL of 0.2 mM DPPH radical solution (1 mL) was added to 20 μL of sample extract or a standard solution of ascorbic acid, and the mixture was allowed to stand for 30 min. The absorbance of the mixture at 520 nm was measured against a blank of distilled water and an ascorbic acid standard calibration curve was constructed. The DPPH radical scavenging activity was expressed as ascorbic acid equivalent antioxidant activity (AEAC) and defined as mg of ascorbic acid equivalents per 100 g of sample (the equation of the standard curve was $y = -0.0268x + 1.4586$, $R^2 = 0.991$).

2.7. Determination of ABTS radical scavenging activity

Total antioxidant capacity of each sample extract was determined with an improved cationic ABTS radical method using a spectrophotometer (GONG *et al.*, 2013). The ABTS radical was generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and letting the mixture stand overnight in the dark at 25±3 °C. The solution of positively charged ABTS radicals was diluted with distilled water to obtain an absorbance of 1.0 at 734 nm. Diluted ATBS radical solution (1 mL) was then added to 50 of μL sample extract or ascorbic acid standard solution. After 90 min, the absorbance of the mixture was measured at 734 nm. The antioxidant activity of the extracts was expressed as mg of AEAC in 100 g of sample (the equation of the standard curve was $y = -0.0055x + 1.1115$, $R^2 = 0.995$). All analyses were performed in triplicate.

2.8. Statistical analysis

Data from the analyses of each of the samples in triplicate were reported as the mean±SD. Different samples were compared using one-way analysis of variance using SAS, version 8.1 (SAS Institute, Cary, USA). A value of $p < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSIONS

Antioxidants can be classified into two groups according to their solubility; hydrophilic antioxidants, such as phenolic and flavonoid compounds, and lipophilic antioxidants (fat-soluble), such as vitamin E (DHIBI *et al.*, 2012). These compounds exhibit antioxidant properties in many in vitro model systems and have the potential to reduce the risk of chronic diseases associated with oxidative stress (LIU, 2003).

3.1. Total phenolic content

Phenols are one of the most effective antioxidative components in plant-derived foods, including fruits, vegetables, and grains (CHOI and LEE, 2009). These compounds are effective antioxidants because of their ability to donate a hydrogen atom or an electron to form stable radical intermediates. Therefore, it is important to quantify the TPCs to determine their contribution to the antioxidant activities of the test samples. The results of TPC analysis are expressed as mg of gallic acid equivalent/100 g of sample (Table 1).

Table 1. Total phenolic and flavonoid contents of milled fractions obtained from paddy rice. Means with different letters in a column are significantly different ($p < 0.05$).

Solvent	Rice fraction	Total phenolic content [1)]	Total flavonoid content [2)]
70% ethanol	RH	166.02±4.48 [a3)]	18.42±0.84 [a]
	BR	45.94±0.51 [b]	9.53±0.91 [b]
	MR-3.5	20.23±0.13 [c]	5.84±0.34 [c]
	MR-5.3	16.94±0.14 [c]	4.84±0.20 [d]
	MR-7.1	10.61±0.11 [d]	2.77±0.33 [e]
	MR-9.9	7.64±0.08 [d]	1.57±0.05 [f]
Water	RH	32.39±1.95 [a]	5.71±0.23 [a]
	BR	26.15±0.26 [b]	5.44±0.54 [a]
	MR-3.5	17.61±1.06 [c]	4.77±0.42 [b]
	MR-5.3	10.42±0.21 [d]	2.67±0.29 [c]
	MR-7.1	10.38±0.07 [d]	2.45±0.10 [cd]
	MR-9.9	6.69±0.15 [e]	1.89±0.04 [d]

[1)] Means±standard deviation (SD) for triplicate determinations expressed as mg of gallic acid equivalents/100 g of sample.

[2)] Means±SD for triplicate determinations expressed as mg of catechin equivalents/100 g of sample.

TPCs in the 70% ethanol and water extracts were 7.64-166.02 and 6.69-32.39 mg/100 g of sample, respectively. The determination of TPC was affected by the extraction solvent; 70% ethanol was more effective than water. Solvent extraction is frequently used to isolate antioxidants, and both the yield and antioxidant activity of the extracts are strongly dependent on the solvent; this is because compounds with different polarities often have different antioxidant potentials (MARINOVA and YANISHLIEVA, 1997). Many studies have indicated that using 70% ethanol in water as the extraction solvent affords significantly higher quantities of phenolic compounds than other solvents, such as methanol and water (AJILA *et al*., 2011). The ethanol extracts show the highest antioxidant activities, which is consistent with our results. Ethanol-water extraction systems were used in the present study since they are the most widely employed solvents for reasons of chemical hygiene and ease of availability. More importantly, these solvents are compatible with the production of food-grade materials (SOONG and BARLOW, 2004). TPCs in the rice fractions for all samples were found to be in the following order: RH > BR > MR-3.5 > MR-5.3 > MR-7.1 > MR-9.9. The ethanol extract of the husk fraction exhibited the highest TPC (166.02±4.48 mg/100 g of sample), and the TPC decreased significantly ($p < 0.01$) as the degree of milling increased. Phenolic compounds are located predominantly in the bran layer, which is progressively removed during the milling process (BUTSAT *et al*., 2009). Rice husk and rice bran are also rich in TPC (VIJAYALAXMI *et al*., 2015). The outer layers of the cereal grains, such as husk, pericarp, testa and aleurone cells, contain the highest concentrations of TPC, whereas its concentration is considerably lower in the endosperm (KAHKONEN *et al*., 1999). The effect of dehusking and milling on TPC could be due to the variable distribution of phenolic compounds in the husk and bran.

3.2. Total flavonoid content

Flavonoids are a group of phenolic compounds that contain two aromatic rings linked by three carbons that are usually part of an oxygenated heterocycle. These compounds have potent antioxidant and anticancer activities (SHEN *et al*., 2009). The TFC, expressed as mg of catechin equivalents per 100 g of sample, was lower than the TPC in all the samples tested (Table 1). The TFCs in the milled fractions were 1.57-18.42 and 1.89-5.71 mg/100 g of sample in the 70% ethanol and water extracts, respectively. RH had the highest TFC among the six rice fractions, followed by BR, MR-3.5, MR-5.3, MR-7.1, and MR-9.9 in that order. The order of abundance of TFC was similar to that of TPC in all samples. This result indicates that the concentration of flavonoids increases from the endosperm to the aleurone layer.

3.3. Total content of vitamin E and its isomers

Vitamin E is another antioxidant present in grains that protects polyunsaturated fatty acids in cell membranes from oxidative damage (SLAVIN *et al*., 1999). The antioxidant activity of the tocopherols and tocotrienols (collectively known as chromanols) is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals. Vitamin E is synthesized only by plants. Therefore, it is a vital nutrient for humans and animals that can be obtained only from dietary sources (KAHKONEN *et al*., 1999). The individual concentrations of eight vitamin E isomers (α, β, γ, and δ-T and α, β, γ, and δ-T3) and their total contents in different parts of the rice grain are presented in Table 2. Four tocopherol isomers, α, β, γ, and δ-tocopherol, and three tocotrienol isomers, α, γ, and δ-tocotrienol, were identified. β-Tocotrienol was not detected.

Table 2. Composition of eight vitamin E isomers in the milled fractions obtained from paddy rice. Means with different letters in the columns for each fraction are significantly different ($p < 0.05$).

Rice fractions	Tocopherol (T)				Tocotrienol (T3)				Total vitamin E
	α-T	β-T	γ-T	δ-T	α-T3	β-T3	γ-T3	δ-T3	
RH	1.15±0.05^{b}	0.04±0.00^{bc}	0.18±0.00^{c}	0.51±0.00^{c}	0.35±0.01^{b}	ND	0.51±0.00^{c}	0.02±0.00^{d}	2.27±0.08^{c}
BR	1.25±0.09^{b}	0.04±0.01^{b}	0.21±0.01^{b}	1.19±0.02^{bc}	0.76±0.05^{a}	ND	1.19±0.02^{a}	0.05±0.00^{a}	3.53±0.15^{b}
MR-3.5	1.61±0.03^{a}	0.06±0.00^{a}	0.28±0.01^{a}	1.23±0.05^{a}	0.70±0.04^{a}	ND	1.23±0.05^{a}	0.05±0.00^{a}	3.98±0.11^{a}
MR-5.3	0.88±0.04^{c}	0.03±0.00^{c}	0.16±0.02^{c}	0.66±0.03^{bc}	0.32±0.01^{b}	ND	0.66±0.03^{b}	0.04±0.00^{b}	2.12±0.11^{cd}
MR-7.1	0.72±0.14^{c}	0.02±0.00^{d}	0.13±0.02^{d}	0.56±0.06^{bc}	0.26±0.04^{b}	ND	0.56±0.06^{bc}	0.03±0.00^{bc}	1.74±0.26^{d}
MR-9.9	0.30±0.06^{d}	0.01±0.00^{e}	0.06±0.00^{e}	0.47±0.10^{b}	0.15±0.03^{c}	ND	0.47±0.10^{c}	0.03±0.00^{c}	1.06±0.20^{e}

Means±standard deviation (SD) of duplicates (mg/100 g of sample).
ND, not detected.

The vitamin E level was 0.86-4.09 mg/100 g of sample in all the extracts of the six fractions. MR-3.5 was the best source of vitamin E (3.98±0.11 mg/100 g), followed by BR (3.53±0.15 mg/100 g), RB (2.27±0.08 mg/100 g), MR-5.3(2.12±0.11 mg/100 g), MR-7.1(1.74±0.26 mg/100 g), and finally MR-9.9 (1.06±0.20 mg/100 g). Tocopherols are known to be the principal antioxidants present in rice bran. The average content of total vitamin E in BR was higher than that in RH (HUANG and NG, 2011). The levels of vitamin E detected in the BR and RH fractions in our study were similar to previously reported values of 1.04-3.25 mg/100 g of sample (KAHKONEN *et al.*, 1999), 3.62–3.82 mg/100 g of sample and 1.81-4.08 mg/100 g of BR, and 0.41–5.50 μg/100 g of RH (SOONG and BARLOW, 2004; OKARTER and LIU, 2010). The major forms of vitamin E were α-T and γ-T3, whereas β-T and δ-T3 were present in trace amounts and β-T3 was not detected. These results were in agreement with the finding that the major isomers of vitamin E in rice samples were γ-T3 and α-T, whereas the δ-T and β-T3 contents were the lowest (HA *et al.*, 2006). Similar to the present study, previous studies have shown that vitamin E was more concentrated in regions close to the bran and husk layers than in the endosperm (SOONG and BARLOW, 2004). The removal of the husk, the aleurone layer, and the germ during milling reduces the vitamin E content of the final milled products (OKARTER and LIU, 2010). Lipid fractions are distributed mainly in the outer layers of rice grains (ADOM and LIU, 2002). These findings are in good agreement with our data in which the vitamin E content decreased as the degree of milling increased. Therefore, our results indicate that antioxidant components comprising total phenolics, total flavonoids, and vitamin E are primarily concentrated in the outer layers rather than in the endosperm.

3.4. DPPH radical scavenging activity

The DPPH and ABTS radical scavenging activities are related to the nature of the phenolic compounds as that affects their electron transfer/hydrogen donating ability (WETTASINGHE and SHAHIDI, 2000; CHOI *et al.*, 2007). The increase in free radicals can accelerate the oxidation of foods and decrease their quality. The radical scavenging activity is very important due to the deleterious role of free radicals in foods and in biological systems. Therefore, this study investigated the free radical scavenging activity of the 70% ethanol and water extracts of different rice fractions using DPPH and ABTS

assays. Both these radicals are commonly used for in vitro assessment of antioxidant activity.

The DPPH radical is stable and is widely used to evaluate the free radical scavenging activities of many plant extracts. The capacity of the six rice fractions to act as hydrogen donors in the transformation of the DPPH radical to its reduced form was examined. AEAC values are often used to rank the antioxidant activity of unknown mixtures (KONG and LEE, 2010). The AEAC of a sample determines its antioxidant activity relative to that of ascorbic acid. The DPPH radical scavenging activities of the milled rice fractions, expressed as mg of AEAC per 100 g, are shown in Fig. 1. The colour of the DPPH reagent changes from purple to yellow as a result of the antioxidant activity of the test sample. Similar to the antioxidant content, the free radical scavenging activity was affected by the extraction solvent; 70% ethanol was more effective than water. The DPPH radical scavenging activities of the extracts of the six samples were in the order RH > BR > MR-3.5 > MR-5.3 > MR-7.1 > MR-9.9 for both solvents. The results show that the antioxidant capacity of all extracts decreased during the milling process. The high TPC and TFC in the husks may account for the strong DPPH radical scavenging activity. This finding is consistent with the finding of a previous study that found that the antioxidant activity was dependent on the composition of the milled fractions (SHEN *et al.*, 2009; BUTSAT and SIRIAMORNPUN, 2010). The DPPH radical scavenging activity correlated directly with the TPC and TFC in the extracts of the six samples with correlation coefficients (R2) of 0.880 and 0.942, respectively. However, there was no correlation between DPPH radical scavenging activity and total vitamin E content (R2 = 0.274). Thus, there is a strong association between the TPC and TFC of different milled fractions and their respective DPPH radical scavenging activities.

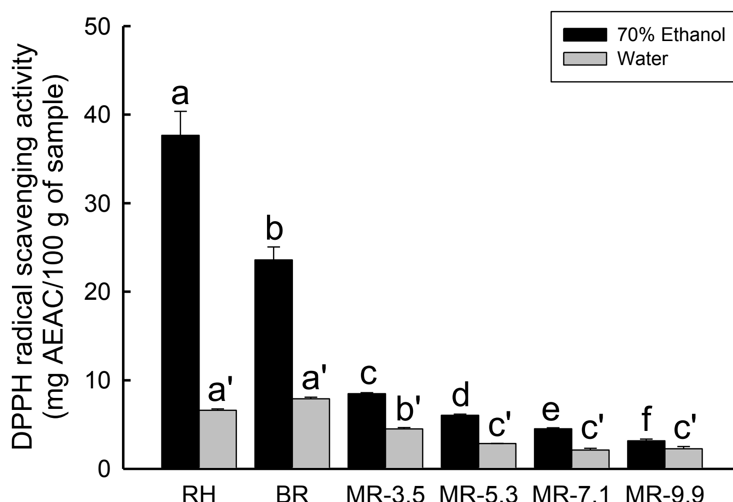

Figure 1. DPPH radical scavenging activity of milled fractions obtained from paddy rice. Data are expressed as the mean±SD of the results for the six rice fractions. [a–f, a′–c′] Means with different letters are significantly different ($p < 0.05$) by one-way analysis of variance.

3.5. ABTS radical scavenging activity

Total antioxidant content (TAC) was measured using the ABTS assay. The ABTS radical scavenging test is widely used to determine the antioxidant activity of both hydrophilic and lipophilic compounds and to measure the relative radical scavenging activity of hydrogen-donating and chain-breaking antioxidants in many plant extracts (APAK *et al.*, 2007). The cationic ABTS radical assay can be used over a wide pH range, is inexpensive, and is more rapid than the DPPH radical assay (SLAVIN *et al.*, 1999). Fig. 2 shows the antioxidant activities of the extracts expressed as mg of AEAC per 100 g of sample. The extraction solvent influenced the antioxidant activity of the extracts of all the fractions. Of the two methods used in this study, extraction with 70% ethanol afforded higher antioxidant activities. TAC varied among the different fractions obtained from different degrees of milling. The TAC of 70% ethanol and water extracts ranged from 5.20-48.74 and 1.88-4.13 mg of AEAC per 100 g sample, respectively. The TAC of the samples tested in decreasing order was RH > BR > MR-3.5 > MR-5.3 > MR-7.1 > MR-9.9. Thus, the TAC in rice decreased significantly ($p < 0.01$) as the degree of milling increased. Many bioactive compounds including phenolic antioxidants, tocopherol, and tocotrienol were present in higher quantities in the removed husk and bran than in the remaining endosperm (IQBAL *et al.*, 2005; HA *et al.*, 2006). The highest TAC was detected in RH, which was consistent with the results of the analyses of TPC, TFC, vitamin E and DPPH radical scavenging ability. The lowest TAC was observed for MR-9.9, which exhibited markedly lower TPC, TFC, and vitamin E levels, and lower DPPH radical scavenging capacity than those of the other samples. There was a positive correlation (R2 = 0.996) between ABTS and DPPH radical cation scavenging activities. The decrease in TAC might have been the result of decreased levels of antioxidant compounds. TAC was positively correlated with TPC and TFC. The correlation coefficients (R2) between the ABTS assay and TPC and between the ABTS assay and TFC were 0.800 and 0.873, respectively. There was no correlation between the vitamin E and TAC. These results confirm that the phenolic and flavonoid compounds may be the major contributors to the antioxidant activity of the grains, which is consistent with the findings of earlier studies (BUTSAT and SIRIAMORNPUN, 2010; ADOM and LIU, 2002).

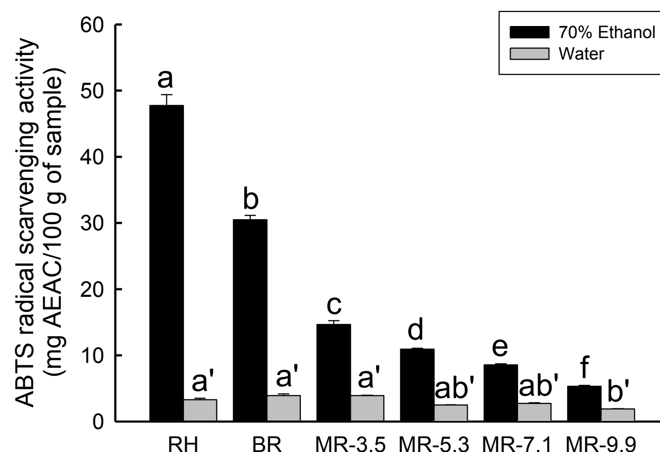

Figure 2. ABTS radical scavenging activity of milled fractions obtained from paddy rice. Data are expressed as the mean±SD of the results for the six rice fractions. a-f, a'-b' Means with different letters are significantly different ($p < 0.05$) by one-way analysis of variance.

4. CONCLUSIONS

This study shows that the antioxidant strength of rice depends on the degree of milling. BR contains more components that have health benefits than MR, such as polyphenolics, flavonoids, and isomers of vitamin E. These results demonstrate that brown rice is a good dietary source of antioxidants and has health benefits. Thus, it is important to modulate the milling process to preserve bioactive compounds.

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PAPER

EFFECT OF GELATIN-BASED EDIBLE COATINGS INCORPORATED WITH *ALOE VERA* AND GREEN TEA EXTRACTS ON THE SHELF-LIFE OF FRESH-CUT APPLE

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ABSTRACT

The objective of the present study was to evaluate the combined effect of edible coatings (gelatin, citric acid, ascorbic acid, and calcium chloride) incorporated with *Aloe vera* (50, 100, and 150%) and green tea (5, 10, and 15%) extracts on physicochemical, microbial, and sensorial properties of fresh-cut apples at 4°C for 16 days. Significant differences in terms of quality parameters were observed between the control and coated apple slices. The highest variation in quality parameters was observed in the control, while the least variations were observed in coated slices with 150% *Aloe vera*. Also, the softening trend was slowed down by edible coatings. Furthermore, in basic coatings, the microbial growth inhibition was a function of the *Aloe vera* and green tea extract concentrations. Generally, the higher concentrations of *Aloe vera* and green tea extracts were found to maintain the quality parameters of apple slices for a longer time during the storage period.

Keywords: apple slice, edible coating, *Aloe vera*, green tea extract

1. INTRODUCTION

In order to preserve freshness and to control spoilage and pathogenic bacteria growth, it is recommended to use edible coatings on fresh-cut fruit to extend their shelf life. For this purpose, natural polysaccharides, proteins, and antioxidants are used as raw materials for edible coatings and films (DHALL, 2013). Edible coatings can also be used as carriers of antimicrobials, antioxidants, anti-browning, flavouring, and colouring agents that improve the nutritional, sensorial, and microbiological properties of fresh-cut fruit (OMS-OLIU *et al*., 2010; VALENCIA-CHAMORRO *et al*., 2011). A dip treatment of fresh-cut fruit in organic acids (such as citric acid and ascorbic acids) and calcium salts as an alternative to sulphites were used to prevent enzymatic browning after fruits peeling and/or cutting (OMS-OLIU *et al*., 2010). Also, calcium treatments can maintain or improve the tissue firmness and crispness (OMS-OLIU *et al*., 2010). In this regards, edible coatings containing *Aloe vera* and green tea extracts are well documented in the literature. *Aloe vera* gel and gelatin have been used as edible coatings in fruit storage technology (ANDRADE *et al*., 2014; DANG *et al*., 2008). The barrier properties of Aloe gel coatings towards respiratory gases (CHAUHAN *et al*., 2011), as well as its antimicrobial functions (MARTÍNEZ-ROMERO *et al*., 2006) in coated fruit and fresh-cut fruit are reported. Besides, gelatin coatings show good barrier characteristics against oxygen and aroma transfers at low and intermediate relative humidity. However, gelatin has poor barrier properties against water vapour transfer due to its hydrophilic nature (ANDRADE *et al*., 2014). In recent years, the *Aloe vera* gel has been used as an edible coating for sweet cherries (MARTÍNEZ-ROMERO *et al*., 2006), mangoes (DANG *et al*., 2008), apples (CHAUHAN *et al*., 2011; SONG *et al*., 2013), papayas (MARPUDI *et al*., 2011), fresh-cut kiwifruit (BENÍTEZ *et al*., 2015; BENÍTEZ *et al*., 2013), and fresh-cut orange (RADI *et al*., 2017). Besides, the effect of *Aloe vera* coating, containing anti-browning solution, on apples slices (SONG *et al*., 2013) has been published in literature.
Furthermore, tea (*Camellia sinensis*), is a good source of polyphenolic compounds, which have strong antioxidant properties. The high antioxidant capacity and overall antimicrobial activity of green tea have been attributed to catechins and their oxidized condensation products (MARTÍN-DIANA *et al*., 2008; MATAN *et al*., 2015). Coating with gelatin incorporated with green tea extract successfully retarded the microbial growth and therefore extended the shelf life of fresh-cut orange during cold storage (RADI et al., 2017). Such properties made us use green tea as our coating alternative.
The aim of the present study was to investigate the combined effects of edible coatings containing gelatin, calcium chloride, ascorbic acid, and citric acid as well as various concentrations of *Aloe vera* and green tea extracts on physicochemical and microbial characteristics of fresh-cut apples during storage.

2. MATERIALS AND METHODS

2.1. Materials

Gelatin, sodium hydroxide, calcium chloride, citric acid, ascorbic acid, and plate count agars (PCA) were purchased from Merck (Darmstadt, Germany). *Aloe vera* leaves and green tea were purchased from a local wholesale market (Yasooj, Iran). Red apples (*Red Delicious*) grown at Semirom Orchard (Isfahan, Iran) were freshly harvested at a commercially mature stage, sorted to eliminate the damaged ones, and selected for uniform size and colour.

2.2. Preparation of the film-forming solutions for coating the apple slices

Aloe vera extract was obtained from fresh *Aloe vera* leaves according to the method described by NAVARRO *et al*. (2011). The extract was used intact (for *Aloe vera* 100% treatment) or was diluted 50:50 with distilled water (for *Aloe vera* 50% treatment). Moreover, the *Aloe vera* gel was concentrated to 150% using a rotary evaporator (Heidolph, Germany) at 45°C. Moreover, green tea extract was prepared, based on the SIRIPATRAWAN and HARTE (2010) method. The total solid content (TSC) of tea extract was determined by the air oven method at 105°C. According to the TSC of tea, the final concentration of extracts was adjusted at 5, 10, and 15% TSC using a Rotary evaporator (Heidolph, Germany) at 45°C. Gelatin powder was dissolved in distilled water or concentrated-adjusted *Aloe vera* and tea extracts by stirring and heating to 50°C under nitrogen gas atmosphere to form 1% gelatin solution.
The following coating solutions were assigned: (A) basic formula 1 (BF1): gelatin (1%), citric acid (0.1%), and calcium chloride (0.5%); (B) basic formula 2 (BF2): gelatin (1.0%), citric acid (0.1%), calcium chloride (0.5%), and ascorbic acid (0.5%); (C) the basic formula 1 and 2 with *Aloe vera* extract at three levels (50, 100, and 150%) that was abbreviated as BF1 or BF2+50, 100, and 150% Aloe; (D) the basic formula 1 and 2 with green tea extract at three levels (5, 10, and 15%) that was abbreviated as BF1 or BF2+5, 10, and 15% GT; and (E) coated with water which served as control.

2.3. Coating the apple slices

Apples of uniform size and shape, and without any signs of mechanical damage, were selected, washed with chlorinated water (50 mg Cl_2/kg H_2O) and manually sliced in chilled water (5–6°C). Apple slices were dipped in the above-mentioned coating solutions for 1 min. and then drained for 30 min. The prepared apple slices were placed in polyethylene terephthalate (PET) clamshells (140 × 128 × 30 mm^3) (Pars Plastic Khuzestan, Ahwaz, Iran), and stored at 4°C for 16 days.

2.4. Measurement of titratable acidity (TA) and total soluble solids (TSS)

The apple slices were homogenized in a blender (Moulinex, Barcelona, Spain) and centrifuged at 2000 rpm for 1 min. to obtain a clear juice. The titratable acidity and total soluble solid of clear juice were measured (RADI *et al*., 2010).

2.5. Weight loss determination

The weight loss in the samples was calculated as loss in weight of the apple slices in each container during storage and the values were reported on a percentage basis (RADI *et al*., 2010).

2.6. Firmness measurement

The firmness of the apple slices was measured using a Texture Analyzer (CT3, Brookfield Engineering Laboratories, Stoughton, MA, USA) with a uniaxial penetration test. A stainless steel flat-end probe of 4 mm diameter was used to evaluate the firmness of the apple slices. The test conditions used for the measurement were pre-test speed 2mm/s; test speed 1 mm/s; post-test speed 10 mm/s; penetrating distance of 10 mm into the fruit, and a trigger force of 5 g (BENÍTEZ *et al*., 2013).

2.7. Measurement of colour

The surface colour of the samples was measured using a Hunter colorimeter (Colorflex, Virginia, USA). Hunter CIE L* for lightness, a* for redness, and b* for yellowness were determined (RADI *et al*., 2017).

2.7. Microbiological evaluation

The microbiological analysis of the apple slices was carried out for standard plate counts in accordance with CHAUHAN *et al*. (2011) procedures. The results were expressed as log CFU/g of sample.

2.8. Sensory analysis

Sensory evaluation was performed immediately after the apple slices were prepared at storage times of 0, 8, and 16 days. Twelve panellists were asked about the different quality attributes (colour, aroma and flavour, texture or firmness, and overall acceptance) of the apple slices using a scale with anchors at 0 and 5 as follows: colour, ranging from dark (0) to colour normal (5); aroma and flavour of apple, from weak (0) to strong (5), texture from soft (0) to hard (5). A final, overall preference test was also performed with a hedonic scale from dislike extremely (0) to like extremely (5). Scores from 2.5 to 5 were considered acceptable.

2.9. Statistical analysis

All the experiments were run in triplicate. Statistics on a completely randomized design were performed with the analysis of variance (ANOVA) procedure in SAS (Release 9.1, SAS Institute Inc., Cary, NC) software and mean comparisons were carried out by Duncan's multiple range test ($p<0.05$).

3. RESULTS AND DISCUSSIONS

3.1. Titratable acidity (TA) and total soluble solids (TSS)

The effects of coating treatments on the TA and TSS parameters during cold storage are shown in Tables 1 and 2. The TA levels in the control and coated samples gradually decreased during the storage period, and the difference was significant in the control sample only on day 16. But, the decreasing trends of TA in coated samples were not significant during the storage period (data not shown for apple slices coated with BF1 and BF2 containing *Aloe vera* and green tea extracts).

A further reduction of acidity in the control sample in comparison with trehalose/NaCl/sucrose-coated apple slices on the eighth day (ALBANESE *et al*., 2007) and gel-coated apple slices with cysteine, citric acid, ascorbic acid, and *Aloe vera* during the 16th day (SONG *et al*., 2013) were also reported. This phenomenon was linked to the malic acid decrease due to an increase in the respiration rate following peeling and cutting (ALBANESE *et al*., 2007). The higher acidity of coated apple slices could be attributed to the barrier properties of Aloe gel coatings towards respiratory gases (CHAUHAN *et al*., 2011; RADI *et al*., 2017). It seems that during storage, organic acids are used as substrates in respiration metabolism, thereby decreasing the TA and increasing the TSS (BENÍTEZ *et al*., 2013).

Table 1. Titratable acidity changes in the control and coated apple slices with basic formulas (BF1 and BF2) during the 16 days of storage at 4°C.

Treatment	Storage time (day)				
	0	4	8	12	16
Control	0.37±0.02Aa*	0.35±0.03Aa	0.34±0.03Aa	0.35±0.03Aa	0.27±0.02Bb
BF1	0.37±0.05Aa	0.36±0.02Aa	0.33±0.04Aa	0.31±0.01Aa	0.31±0.03Aa
BF2	0.34±0.04Aa	0.38±0.05Aa	0.35±0.03Aa	0.35±0.03Aa	0.32±0.02Aa

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column are statistically different (p<0.05).

Table 2. TSS changes in the control and coated apple slices with basic formulas (BF1 and BF2) incorporated *Aloe vera* and green tea extracts during the 16 days of storage at 4°C.

Group	Treatment	Storage time (day)				
		0	4	8	12	16
1	Control	16.03±0.02Ae*	16.08±0.01Ad	16.18±0.02Ac	16.31±0.02Ab	16.36±0.02Aa
	BF1	16.01±0.02Ad	16.03±0.01Bd	16.08±0.02Bc	16.17±0.02Bb	16.22±0.02Ba
	BF2	16.02±0.01Ad	16.02±0.02Bd	16.08±0.01Bc	16.15±0.02Bb	16.23±0.02Ba
2	BF1	16.01±0.02Ad	16.03±0.01Ad	16.08±0.02Ac	16.17±0.02Ab	16.22±0.02Aa
	BF1+50% Aloe	16.01±0.01Ae	16.04±0.01Ad	16.07±0.02Ac	16.15±0.02Ab	16.23±0.02ABa
	BF1+100% Aloe	16.02±0.01Ad	16.04±0.01Ad	16.06±0.01Ac	16.13±0.01Bb	16.19±0.02BCa
	BF1+150% Aloe	16.02±0.02Ad	16.03±0.02Ad	16.05±0.01Ac	16.11±0.01Cb	16.17±0.02Ca
3	BF2	16.02±0.01Ad	16.02±0.02Ad	16.08±0.01ABc	16.15±0.02ABb	16.23±0.02Aa
	BF2+50% Aloe	16.02±0.01Ad	16.03±0.02Ad	16.09±0.02Ac	16.16±0.02Ab	16.20±0.01Aa
	BF2+100% Aloe	16.01±0.01Ad	16.03±0.01Ad	16.06±0.02Bc	16.13±0.01BCb	16.18±0.02Ba
	BF2+150% Aloe	16.01±0.01Ae	16.04±0.01Ad	16.05±0.01Bc	16.11±0.02Cb	16.15±0.01Ca
4	BF1	16.01±0.02Ad	16.03±0.01Ad	16.08±0.02Ac	16.17±0.02Ab	16.22±0.02Aa
	BF1+5% GT	15.99±0.02Ae	16.03±0.02Ad	16.08±0.02Ac	16.18±0.01Ab	16.21±0.01ABa
	BF1+10% GT	16.02±0.01Ad	16.04±0.01Ad	16.07±0.02ABc	16.16±0.02ABb	16.20±0.02ABa
	BF1+15% GT	16.00±0.05Ad	16.02±0.01Adc	16.05±0.01Bc	16.14±0.02Bb	16.19±0.01Aa
5	BF2	16.02±0.01Ad	16.02±0.02Ad	16.08±0.01ABc	16.15±0.02ABb	16.23±0.02Aa
	BF2+5% GT	15.99±0.02Ae	16.03±0.01Ad	16.08±0.01Ac	16.15±0.02Ab	16.20±0.01Ba
	BF2+10% GT	15.98±0.03Ad	16.03±0.02Ac	16.06±0.01Bc	16.13±0.02Ab	16.18±0.01Ca
	BF2+15% GT	15.99±0.03Ad	16.01±0.01Ad	16.05±0.02Bc	16.12±0.02Ab	16.18±0.01Ca

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column of each group are statistically different (p<0.05).

The TSS of the control and coated samples significantly increased with storage time, while the coated samples showed a slight increase compared to the control sample (Table 2). In this regard, there was a significant difference between the control and the coated samples with the basic formulas (BF1 and BF2 without *Aloe vera* and green tea extracts) only after four days of storage. But, no difference was observed between the BF1 and BF2, which indicated the same effect of BF1 and BF2 treatments on apple slices during storage time.
Increasing the concentration of *Aloe vera* and green tea extracts in the basic formulas (BF1 and BF2) increased the TSS significantly only after eight days of storage, and especially at

the end of the storage period. Furthermore, no significant differences were found between BF1+Aloe and BF2+Aloe treatments in similar concentrations of the *Aloe vera* extract (50, 100, and 150%). Without considering the BF1 and BF2 coatings, samples coated with higher concentrations of *Aloe vera* and green tea extracts showed a lower increase in TSS at the end of the storage periods. The highest increase of TSS was observed in the control (~ 2.5%, TSS increased from 16.0 to 16.4 after 16 days of storage at 4°C), while the lowest increase was observed in samples coated with BF2+150% Aloe (~ 1.3%, TSS increased from 16.0 to 16.2).

The findings of this study were similar to the results of AHMED *et al*. (2009), MARPUDI *et al*. (2011), and RADI *et al*. (2017), who reported that TA decreased and TSS increased with increasing storage time in nectarines, papaya and fresh-cut orange, respectively. During ripening, organic acids are used as substrates in respiration metabolism, thereby resulting in an increase in TA and decrease in TSS. In general, it seems that the total soluble solid content tends to increase over the storage period as a consequence of the ripening process (BENÍTEZ *et al*., 2013). A reduction in the respiration rate has been observed in sweet cherries (MARTÍNEZ-ROMERO *et al*., 2006) and kiwifruits (BENÍTEZ *et al*., 2013) coated with *Aloe vera* gel.

Furthermore, softening occurs primarily because of an enzymatic degradation (pectin methylesterase and polygalacturonase) of the cell wall, which is mainly composed of cellulose, hemicelluloses, and pectins (OMS-OLIU *et al*., 2010). This may affect some physicochemical characteristics such as pH, TA, TSS, etc., of fresh-cut fruit. In this regard, the increasing trend of TSS in apple slices can be attributed to the softening and may, therefore, be associated with ripening (BENÍTEZ *et al*., 2013).

3.2. Weight loss

The weight loss is mainly associated with moisture evaporation through the surface of fruit slices (OLIVAS *et al*., 2007). All samples demonstrated a gradual weight loss during storage (Table 3). The weight loss of uncoated fruit (25.10 %) was significantly greater than those of coated fruits during storage time. The weight loss of apple slices coated with BF1 and BF2 treatments was significantly lower than the control ($p<0.05$). In this regard, no significant difference was observed between BF1 and BF2 treatments until the eighth day, but the difference was significant on the 12th and the 16th days, and also the weight loss of BF1was significantly lower than BF2 at the end of the storage time.

The least rate of weight loss (11.18 % and 11.52 %) was observed, respectively, in the samples coated with BF1+150% Aloe and BF2+150% Aloe treatments. Consequently, the weight loss of apple slices coated with BF+Aloe was significantly lower than other samples ($p<0.05$). Accordingly, the BF+Aloe coating was more effective than the BF+Green tea coatings. In the case of BF+15% GT weight loss was ~18.5%. Furthermore, weight loss of samples coated with both basic coatings (BF1 and BF2) containing 150% Aloe and 15% GT was significantly lower than of those coated with a lower level of extracts at the end of the storage periods.

Similar results were obtained by SONG *et al*. (2013) and RDAI *et al*. (2017). These authors reported that the weight loss increased during storage, but the weight loss of the *Aloe vera* gel-coated apple slices was significantly ($p<0.05$) reduced compared to the control during storage. The binding of *Aloe vera* gel molecules to the surface of apple slices may have reduced the porosity of apple slices, resulting in lower water loss (SONG *et al*., 2013). It is reported that *Aloe vera* gel reduces the respiration rate, ethylene production, weight loss and, therefore, the softening of fresh-cut fruit textures (BENÍTEZ *et al*., 2013).

Table 3. Weight loss changes in the control and coated apple slices with basic formulas (BF1 and BF2) incorporated *Aloe vera* and green tea extracts during the 16 days of storage at 4°C.

Group	Treatment	Storage time (day)				
		0	4	8	12	16
1	Control	0.5±0.2Ae*	12.13±0.26Ad	19.68±0.14Ac	23.32±0.26Ab	25.10±0.37Aa
	BF1	0.3±0.1Ae	8.34±0.36Bd	12.19±0.29Bc	16.5±0.7Bb	18.41±0.19Ba
	BF2	0.3±0.2Ae	7.7±0.43 Bd	12.63±0.23Bc	15.59±0.26Cb	19.55±0.3Ca
2	BF1	0.3±0.1Ae	8.34±0.36Ad	12.19±0.29Ac	16.5±0.7Ab	18.41±0.19Aa
	BF1+50% Aloe	0.43±0.15Ae	7.9±0.58ABd	11.69±0.17Ac	14.90±0.28Bb	17.20±0.2Ba
	BF1+100% Aloe	0.23±0.15Ae	7.23±0.32Bd	10.3±0.35Bc	13.09±0.24Cb	15.41±0.2Ca
	BF1+150% Aloe	0.2±0.10Ae	6.2±0.2Cd	8.15±0.39Cc	9.50±0.22Db	11.18±0.19Da
3	BF2	0.3±0.2Ae	7.7±0.43Ad	12.63±0.23Ac	15.59±0.26Ab	19.55±0.3Aa
	BF2+50% Aloe	0.27±0.15Ae	7.78±0.14Ad	12.40±0.25Ac	14.59±0.27Bb	17.51±0.29Ba
	BF2+100% Aloe	0.23±0.15Ae	7.24±0.39Ad	10.69±0.17Bc	12.50±0.17Cb	15.51±0.34Ca
	BF2+150% Aloe	0.37±0.21Ae	5.99±0.32Bd	8.72±0.21Cc	10.20±0.17Db	11.52±0.23Da
4	BF1	0.3±0.1Ae	8.34±0.36BCd	12.19±29Bc	16.5±0.7Ab	18.41±0.19Aa
	BF1+5% GT	0.27±0.15Ae	8.10±0.14Cd	11.72±0.20Cc	14.87±0.21Bb	17.23±0.25Ba
	BF1+10% GT	0.37±0.15Ae	8.84±0.33ABd	11.81±0.25BCc	13.27±0.36Cb	17.50±0.32Ba
	BF1+15% GT	0.17±0.12Ae	9.2±0.36Ad	12.68±0.18Ac	14.70±0.23Bb	18.4±0.22Aa
5	BF2	0.3±0.2Ae	7.7±0.43Bd	12.63±0.23Bc	15.59±0.26Bb	19.55±0.3Aa
	BF2+5% GT	0.23±0.15Ae	10.09±0.29Ad	12.19±0.4Bc	14.56±0.21Cb	17.51±0.40Ca
	BF2+10% GT	0.17±0.12Ae	9.65±0.34Ad	12.69±0.18Bc	15.84±0.24ABb	18.11±0.21Ba
	BF2+15% GT	0.27±0.15Ae	9.81±0.28Ad	13.31±0.28Ac	16.09±0.24Ab	18.50±0.20Ba

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column of each group are statistically different (p<0.05).

3.3. Texture evaluation

The texture degradation and softening trend continued through the storage time, but its rate was slowed down by the BF1 and BF2 coating compared to the control sample (Table 4).

The maximum firmness was observed in BF1+150% Aloe (firmness decreased from 10.51 to 9.82 N after 16 days of storage at 4°C), while the least firmness was observed in control (firmness decreased from 10.44 to 5.62 N after 16 days of storage at 4°C). In addition, similar concentrations of *Aloe vera* (50, 100, and 150%) and green tea (5, 10, and 15%) extracts used in the BF1 and BF2 coatings had no significant effect on firmness, which indicated the same effects of BF coatings on apple slices during storage. Regardless of the BF1 and BF2 coatings, samples coated with the higher concentration of *Aloe vera* and green tea extracts had higher firmness. There was also no significant difference between coated samples with green tea and *Aloe vera* extracts.

The lower firmness of the control sample than the coated samples was probably due to the growth of spoilage microorganisms in the sliced apple, but which was limited in coated samples due to the antimicrobial properties of *Aloe vera* and green tea extracts (BENÍTEZ *et al*., 2013; MATAN *et al*., 2015). Softening occurred primarily due to the enzymatic degradation (pectin methylesterase and polygalacturonase) of the cell wall. Calcium is reported to maintain firmness by cross-linking with pectins to form insoluble calcium pectates, which strengthen the structure of the cell wall (OMS-OLIU *et al*., 2010). In this

regard, the fresh-cut apples, treated with calcium, showed no significant differences throughout the three weeks of storage (ALANDES *et al*., 2006).

Table 4. Apple slices firmness changes (N) in the control and coated apple slices with basic formulas (BF1 and BF2) incorporated *Aloe vera* and green tea extracts during the 16 days of storage at 4°C.

Group	Treatment	Storage time (day)				
		0	4	8	12	16
1	Control	10.44±0.25Aa*	8.70±0.13Cb	6.34±0.18Cc	6.02±0.08Bd	5.62±0.11Be
	BF1	10.48±0.24Ab	10.88±0.09Aa	9.68±0.20Ac	9.13±0.08Ad	8.87±0.06Ad
	BF2	10.24±0.08Aa	10.23±0.06Ba	9.33±0.11Bb	9.10±0.07Ac	8.86±0.11Ad
2	BF1	10.48±0.24Ab	10.88±0.09Aa	9.68±0.20Bc	9.13±0.08Cd	8.87±0.06Cd
	BF1+50% Aloe	10.43±0.09Aa	10.50±0.12Ba	9.91±0.08ABb	9.52±0.11Bc	8.98±0.08Cd
	BF1+100% Aloe	10.34±0.11Aa	10.26±0.06Ca	9.96±0.06Ab	9.73±0.09Ac	9.25±0.11Bd
	BF1+150% Aloe	10.51±0.13Aa	10.41±0.08BCa	10.05±0.10Ab	9.86±0.06Ac	9.82±0.10Ac
3	BF2	10.24±0.08Ca	10.23±0.06Ba	9.33±0.11Cb	9.10±0.07Cc	8.86±0.11Cd
	BF2+50% Aloe	10.35±0.13BCa	10.16±0.05Bb	9.87±0.08Bc	9.45±0.10Bd	9.09±0.14BCe
	BF2+100% Aloe	10.52±0.09ABa	10.27±0.09ABb	10.06±0.09Ab	9.71±0.17Ac	9.32±0.12ABd
	BF2+150% Aloe	10.55±0.08Aa	10.43±0.12Aa	10.04±0.07Ab	9.87±0.06Ab	9.47±0.14Ac
4	BF1	10.48±0.24Ab	10.88±0.09Aa	9.68±0.20Bc	9.13±0.08Cd	8.87±0.06Dd
	BF1+5% GT	10.27±0.08Aa	10.21±0.02Ba	9.99±0.04Ab	9.46±0.12Bc	9.05±0.10Cd
	BF1+10% GT	10.34±0.14Aa	10.22±0.04Bab	10.03±0.15Ab	9.63±0.12Bc	9.23±0.08Bd
	BF1+15% GT	10.47±0.03Aa	10.34±0.03Ba	10.13±0.06Ab	9.83±0.07Ac	9.50±0.07Ad
5	BF2	10.24±0.08Ba	10.23±0.06Aa	9.33±0.11Bb	9.10±0.07Cc	8.86±0.11Cd
	BF2+5% GT	10.33±0.09ABa	10.27±0.05Aa	9.93±0.07Ab	9.64±0.12Bc	9.34±0.07Bd
	BF2+10% GT	10.34±0.08ABa	10.23±0.08Aa	9.96±0.09Ab	9.85±0.10Ab	9.46±0.11ABc
	BF2+15% GT	10.44±0.06Aa	10.24±0.14Aab	10.08±0.14Ab	9.86±0.12Ac	9.53±0.10Ad

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column of each group are statistically different ($p<0.05$).

The results of studies have indicated that *Aloe vera* reduces the respiration rate and ethylene production, weight loss, and softening (BENÍTEZ *et al*., 2013). In this regard, CHAUHAN *et al*. (2011) showed that Aloe gel coating alone or in combination with shellac, preserves the firmness in apple slices. Further, the *Aloe vera* edible coating application generally resulted in harder kiwifruit slices (BENÍTEZ *et al*., 2013).
In addition to the antimicrobial effects, the improvement in mechanical properties of the films incorporating green tea extracts may be responsible for the interaction between polymeric matrix and polyphenolic compounds from green tea extracts (SIRIPATRAWAN and HARTE, 2010).

3.4. Colour change

Colour is an important factor in the perception of the quality of fresh-cut fruit during their shelf-life. The colour indices (L^*, a^*, and b^*) of apple slices stored at 4°C for 16 days were measured and only L^* is reported in Table 5. Statistical analysis showed that the L^*, a^*, and

b^* colour indices significantly changed during storage. A significant increase in colorimetric a^* and b^* values, and a significant decrease in the L^* value were observed in apple slices during storage time. The colour indices of apple slices showed a significant difference ($p<0.05$) between the uncoated and coated samples.

Table 5. L^* changes in the control and coated apple slices with basic formulas (BF1 and BF2) incorporated *Aloe vera* and green tea extracts during the 16 days of storage at 4°C.

Group	Treatment	Storage time (day)				
		0	4	8	12	16
1	Control	76.00±2.00Aa*	72.33±0.58Ab	69.00±1.00Ac	66.33±1.53Cd	64.67±1.53Bd
	BF1	74.00±1.00Aa	71.66±0.58Ab	70.00±1.00Ac	68.66±0.58Bcd	68.00±1.00Ad
	BF2	75.66±1.15Aa	73.00±1.00Ab	71.00±1.00Abc	71.33±1.14Abc	70.00±1.00Ac
2	BF1	74.00±1.00Aa	71.66±0.58Ab	70.00±1.00Bc	68.66±0.58Acd	68.00±1.00Cd
	BF1+50% Aloe	74.33±1.53Aa	71.66±0.58Ab	70.33±0.56ABbc	68.66±0.55Acd	68.00±1.00Cd
	BF1+100% Aloe	74.66±1.53Aa	72.33±1.50Aab	71.33±0.57ABcb	70.33±1.00Aa	70.33±0.58Ba
	BF1+150% Aloe	74.67±0.56Aa	72.67±1.53Aab	72.00±1.00Acb	70.33±1.53Ac	70.67±0.55Ac
3	BF2	75.66±1.15Aa	73.00±1.00Ab	71.00±1.00Abc	71.33±1.14Abc	70.00±1.00Ac
	BF2+50% Aloe	75.33±1.50Aa	73.00±2.00Aab	70.67±1.15Acb	69.66±1.12Ac	69.00±1.00ABc
	BF2+100% Aloe	75.34±1.14Aa	73.33±1.50Ab	72.33±0.54Ab	70.33±0.59Ac	70.00±1.00ABc
	BF2+150%Aloe	76.67±1.55Aa	74.66±1.12Aa	72.00±1.00Ab	71.67.00±1.50Ab	71.33±1.10Bb
4	BF1	74.00±1.00Aa	71.66±0.58Bb	70.00±1.00Bc	68.66±0.58Ccd	68.00±1.00Bd
	BF1+5% GT	75.00±0.95Aa	72.00±1.00Bb	71.00±1.00ABb	69.00±1.00BCc	68.66±1.12ABc
	BF1+10% GT	75.33±1.49Aa	74.33±1.50Aa	71.67±1.45ABb	70.66±0.52ABb	69.33±1.50ABb
	BF1+15% GT	76.33±1.44Aa	74.67±1.10Aab	73.00±1.00Abc	71.66±1.50Acd	70.66±0.51Ad
5	BF2	75.66±1.15Ba	73.00±1.00Bb	71.00±1.00Abc	71.33±1.14ABbc	70.00±1.00Ac
	BF2+5% GT	75.00±1.00ABa	72.67±1.60Bb	71.00±1.05Acb	69.66±0.62Bc	69.33±0.60Ac
	BF2+10% GT	76.33±1.61ABa	75.33±1.55Aa	72.66±1.49Ab	71.00±1.00ABb	70.66±1.09Ab
	BF2+15% GT	77.66±1.55Aa	76.33±0.63Aa	73.34±1.60Ab	72.33±1.17Abc	71.00±1.00Ac

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column of each group are statistically different ($p<0.05$).

The reduction trend of L^* values in coated and uncoated samples occurred at different rates during the storage ($p<0.05$), showing a darkening tendency in the surface colour of the apple slices. The least reduction trend for L^* was observed in the coated samples with both basic coatings (BF1 and BF2) containing 150% Aloe and 15% GT (~ 7.0%), in contrast to the control, which had the highest L^* reduction (14.9%). The L^* reduction during storage may be related to the occurrence of browning (MARTÍN-DIANA *et al*., 2008).

The least increasing trend for a^* and b^* was observed in the coated samples with both basic coatings (BF1 and BF2) containing 150% Aloe and 15% GT in contrast to control, which had the highest a^* and b^* increasing (a^* and b^* respectively increased from -5.33 and 22.0 to 4.33 and 33.67 after 16 days of storage at 4°C).

The variations of L^*, a^*, and b^* in the coated samples were significantly low at the highest concentrations of *Aloe vera* and green tea extracts at the end of the storage periods, confirming the effect of these coatings in preventing the darkening and browning of apple slices. But at the same concentrations (50, 100, and 150%) of *Aloe vera* and green tea (5, 10,

and 15%) extracts, there was no significant difference between BF1 and BF2 treatments in any of the measured colour parameters.
Colour is a critical quality property of fresh-cut fruit, since the slicing of fruit may often lead to enzymatic browning by polyphenol oxidases and peroxidases, which react with phenolic compounds and cause surface browning (ALBANESE *et al*., 2007; OMS-OLIU *et al*., 2010). Furthermore, the oxidative degradation of ascorbic acid and non-enzymatic browning are reported to be a major deteriorative reactions occurring during storage (WIBOWO *et al*., 2015). Thus, anti-browning agents such as ascorbic acid, thiol-containing substances, carboxylic acids, and certain phenolic acids have been studied (OMS-OLIU *et al*., 2010). In this study, citric and ascorbic acids were used as anti-browning agents to inhibit enzymatic browning. Cut-surface colour of apple slices that had been treated with ascorbic acid (in BF2) was well maintained than BF1-coated samples, but this effect was not significant.
An increase in the browning reactions in fresh-cut apples during storage was observed after increases in Hunter a^* and b^* values and a decrease in L^* (PEREZ-GAGO *et al*., 2006; SONG *et al*., 2013). The findings of this study indicated that the variations of colour in Aloe-treated apple slices, especially at higher concentration (150%), were significantly lower than BF1- and BF2-coated apple slices. Similarly, CHAUHAN *et al*. (2011) reported that the L^*, a^*, and b^* values of the *Aloe vera* gel-coated apple slices showed fewer changes compared to the control during storage for 30 days at 6°C, suggesting the anti-browning functionality of the *Aloe vera* coating.
It is reported that the application of *Aloe vera* gel coating is an effective method for maintaining the colour of fresh-cut apple slices, as the *Aloe vera* gel coating can act as an oxygen barrier film, thus reducing enzymatic browning. However, the coating does not completely prevent oxidative browning (SONG *et al*., 2013). Therefore, to inhibit enzymatic browning, anti-browning agents were added to the *Aloe vera* gel coating solution (SONG *et al*., 2013). In this regard, SONG *et al*. (2013) reported that *Aloe vera* gel, containing 0.5% cysteine, was most effective in delaying the browning of apple slices during storage.
The L^* levels of coated apple slices increased with green tea extract concentrations. But the increase was not significant, indicating the anti-browning effects of green tea extract. Conversely, MARTÍN-DIANA *et al*. (2008) reported that an increase in green tea concentrations decreased the L^* values of coated lettuce.

3.5. Microbial analysis

Fresh-cut fruit is highly susceptible to pathogenic and spoilage microorganisms during the preparatory steps as a consequence of cross-contamination, the presence of a large area of cut surfaces, and juice and sugar leakage from damaged tissues (OMS-OLIU *et al*., 2010). The microbial growth on the surface of coated apple slices showed significant differences between the coated and uncoated samples (Table 6).
A significant difference was found between the BF1- and BF2-coated samples after 12 days of storage. The total viable counts gradually and significantly increased with storage time in all treatments (Table 6). The microbial population of the control sample was higher than in the other treatments (2.63 and 6.78 log CFU/g at 0 and 16 days of storage, respectively), while samples coated with BF1+15% GT had the lowest microbial count compared to other treatments (2.42 and 3.17 log CFU/g at day 0 and 16 of storage period, respectively). The microbial counts in the *Aloe vera*- and green tea-coated samples did not exceeded 4.0 log CFU/g during storage time and were significantly lower than the coated samples with BF1 and BF2 ($p<0.05$). Therefore, the addition of *Aloe vera* and green tea extracts in BF1 and BF2 coatings reduced the microbial population significantly, and this effect was

enhanced at higher concentrations of these extracts. In both the BF1 and BF2 coatings, the inhibition of microbial growth was a function of the *Aloe vera* and green tea extracts. Significant differences were also found between BF1 and BF2 treatments in similar concentrations of *Aloe vera* (100 and 150%) and also green tea extract (10% and 15%). The results showed that the antimicrobial effect of green tea was more than that of *Aloe vera*, especially in the higher concentrations of these extracts.

Table 6. Microbial growth (log CFU/g) in the control and coated apple slices with basic formulas (BF1 and BF2) incorporated *Aloe vera* and green tea extracts during the 16 days of storage at 4°C.

Group	Treatment	Storage time (day)				
		0	4	8	12	16
1	Control	2.48±0.06Ae*	3.16±0.07Ad	5.25±0.13Ac	6.10±0.04Ab	6.78±0.04Aa
	BF1	2.63±0.09Ad	2.73±0.09Cd	3.22±0.05Bc	4.02±0.04Bb	4.94±0.07Ba
	BF2	2.52±0.11Ae	2.92±0.05Bd	3.17±0.06Bc	3.73±0.08Cb	4.65±0.11Ca
2	BF1	2.63±0.09Ad	2.73±0.09Ad	3.22±0.05Ac	4.02±0.04Ab	4.94±0.07Aa
	BF1+50% Aloe	2.47±0.06Bd	2.56±0.05Bd	3.03±0.08Bc	3.32±0.07Bb	3.91±0.09Aa
	BF1+100% Aloe	2.45±0.09Bd	2.56±0.07Bd	2.96±0.07Bc	3.22±0.01BCb	3.92±0.05Aa
	BF1+150% Aloe	2.43±0.05Be	2.56±0.08Bd	2.92±0.06Bc	3.17±0.08Cb	3.95±0.07Aa
3	BF2	2.52±0.11Ae	2.92±0.05Ad	3.17±0.06Ac	3.73±0.08Ab	4.65±0.11Aa
	BF2+50% Aloe	2.47±0.06Ae	2.62±0.06Bd	2.95±0.09Bc	3.51±0.09Bb	3.99±0.06Ba
	BF2+100% Aloe	2.49±0.06Ad	2.59±0.02Bd	2.86±0.11Bc	3.19±0.07Cb	3.65±0.10Ca
	BF2+150% Aloe	2.43±0.05Ad	2.54±0.05Bd	2.80±0.12Bc	3.14±0.11Cb	3.56±0.08Ca
4	BF1	2.63±0.09Ad	2.73±0.09Ad	3.22±0.05Ac	4.02±0.04Ab	4.94±0.07Aa
	BF1+5% GT	2.47±0.04ABd	2.57±0.04Bd	2.96±0.06Bc	3.29±0.09Bb	4.14±0.10Ba
	BF1+10% GT	2.43±0.09Bd	2.49±0.02BCcd	2.62±0.04Cc	3.08±0.13Cb	3.64±0.09Ca
	BF1+15% GT	2.42±0.12ABc	2.47±0.03Cc	2.55±0.06Cc	2.85±0.06Db	3.17±0.06Da
5	BF2	2.52±0.11Ae	2.92±0.05Ad	3.17±0.06Ac	3.73±0.08Ab	4.65±0.11Aa
	BF2+5% GT	2.51±0.05Ad	2.59±0.05Bd	2.89±0.08Bc	3.26±0.10Bb	4.10±0.08Ba
	BF2+10% GT	2.42±0.03Ad	2.46±0.06Cd	2.58±0.02Cc	3.11±0.09Bb	3.85±0.10Ca
	BF2+15% GT	2.48±0.03Ac	2.44±0.03Cc	2.52±0.03Cc	2.86±0.09Cb	3.30±0.07Da

*Mean ± standard deviation (n = 3); Means followed by the different small letter within the same row or by the different capital letter within the same column of each group are statistically different ($p<0.05$).

According to Table 6, lower microbial populations in samples coated with *Aloe vera* and green tea extracts can be attributed to antimicrobial properties of the coated compounds (BENÍTEZ *et al.*, 2013; MATAN *et al.*, 2015; RADI *et al.*, 2017)). *Aloe vera* extract was reported to have antimicrobial functions, significantly reducing mesophilic bacteria, and especially showing antifungal activity (MARTÍNEZ-ROMERO *et al.*, 2006; VALVERDE *et al.*, 2005). Some individual components found in *Aloe vera* gel, such as saponins, acemannan, and anthraquinone derivatives, are known to have antibiotic activity and could be responsible for its antibacterial activity (VALVERDE *et al.*, 2005). Green tea, too, is a rich source of polyphenols (mainly catechins and catechin derivatives) and the antimicrobial activity of green tea has been attributed to these compounds (MARTÍN-DIANA *et al.*, 2008; MATAN *et al.*, 2015). MATAN *et al.* (2015) and RADI *et al.* (2017)

confirmed the antimicrobial activity in green tea extracts on fresh-cut dragon and fresh-cut orange, respectively.
The reduction of microbial populations presented in this study was in good agreement with the antimicrobial effects of *Aloe vera* coating on table grape ((VALVERDE *et al*., 2005), sweet cherry (MARTÍNEZ-ROMERO *et al*., 2006), apple slices (CHAUHAN *et al*., 2011; SONG *et al*., 2013), kiwifruit slices (BENÍTEZ *et al*., 2015), raspberry fruit (HASSANPOUR, 2015), and fresh-cut orange (RADI *et al*., 2017) which reduced the aerobic bacteria, as well as yeast and mould counts during storage.

3.6. Sensory analysis

The quality attribute scores (colour, aroma and flavour, texture or firmness, and overall acceptance) for the control and coated samples were studied on days 0, 8, and 16 (Fig. 1).

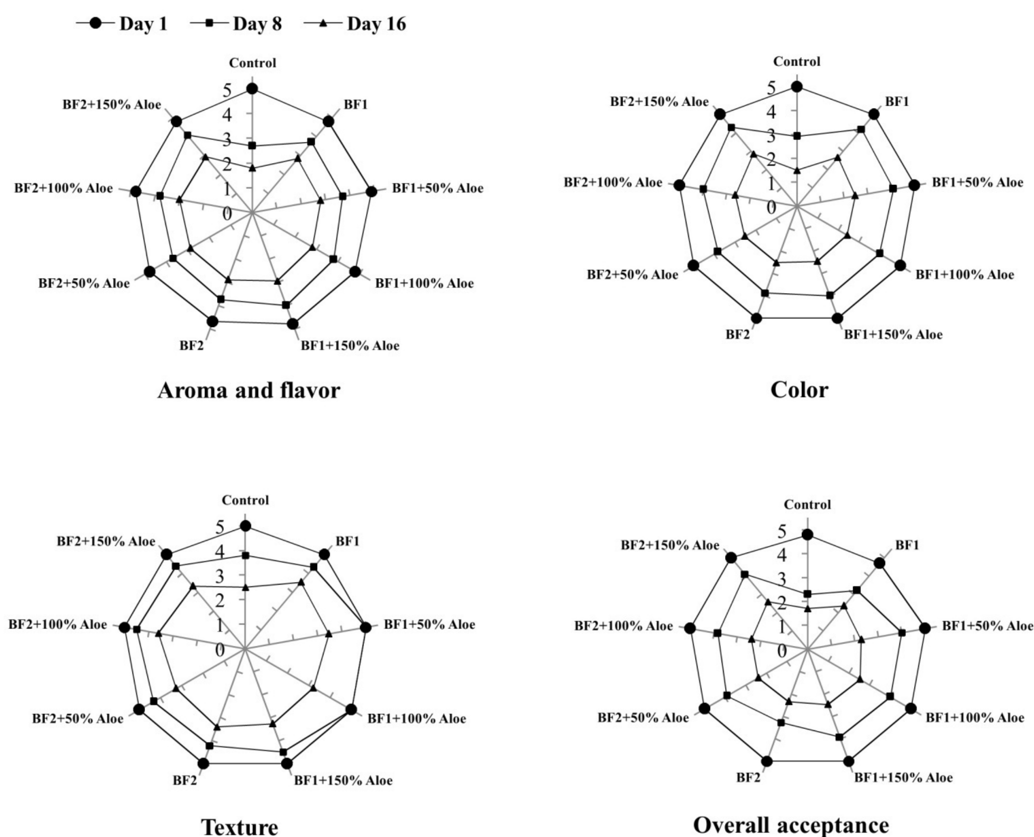

Figure 1: Sensory attributes of apple slices coated with basic formulas (BF1 and BF2) incorporated with *Aloe vera* extracts during the 16 days of storage at 4ºC.

The panellists gave greater sensory scores to coated slices than uncoated slices at the three stages of the experiment (Days 0, 8, and 16). Thus, the sensory analyses revealed the beneficial effects of coating in terms of delaying browning and maintaining the sensory quality of the apple slices. All edible coating treatments resulted in higher sensory scores than uncoated apple slices for all quality factors tested. But, except for colour, the other sensory characteristics were not significantly different in control and BF1 and BF2. In this

regard, the colour score of the BF1 was significantly higher than those of BF2 and control samples. Although increasing the concentration of *Aloe vera* and green tea extract in the basic formulas led to higher sensory scores, no significant difference was observed between the apple slices coated with different concentrations of *Aloe vera* and green tea extracts. But, at the end of the storage, the panellists gave greater sensory scores (colour and overall acceptance) to BF2+150% Aloe-coated slices than the other treatments. Unexpectedly, Aloe gel-coated samples showed the lowest firmness at the end of storage even at high concentrations. The higher scores of coated slices compared to the uncoated ones were reported in the *Aloe vera* gel-coated apple slices (CHAUHAN *et al*., 2011; SONG *et al*., 2013) and Aloe-coated orange slices (RADI *et al*., 2017).

4. CONCLUSIONS

In this study, an attempt was made to use *Aloe vera* and green tea extracts in gelatin-based coating to maintain the freshness of apple slices. Although gelatin-based coatings obtained higher quality attributes than those of control during storage time, the coatings did not completely prevent chemical and biochemical reactions. The addition of *Aloe vera* and green tea extracts in the gelatin-based coatings maintained the quality parameters of apple slices for a longer time during the storage period. In this regard, the least increasing trend for a^* and b^* was observed in samples coated with both gelatin-based coatings (BF1 and BF2) containing 150% *Aloe vera* and 15% green tea extracts. The samples coated with higher concentrations of *Aloe vera* and green tea extracts had lower increases in TSS at the end of storage periods. In terms of microbial count, the total count gradually and significantly increased with storage time in all treatments. The antimicrobial compounds *Aloe vera* and green tea extracts contributed to the lower microbial populations in samples coated with them. Slices coated with 150% *Aloe vera* and 15% green tea extracts obtained higher values for firmness. Moreover, the panellists gave greater sensory scores to coated apple slices than uncoated samples during the storage period. In this regard, the BF2+150% *Aloe vera* sample achieved higher sensory attributes than those of other treatments at the end of storage time.

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PAPER

INFLUENCE OF WATER ACTIVITY ON *LISTERIA MONOCYTOGENES* GROWTH IN "SALSICCIA SARDA" FERMENTED SAUSAGE

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ABSTRACT

"Salsiccia Sarda" is catalogued as a ready-to-eat food (RTE) for which actual European Legislation proposes microbiological criteria for *L. monocytogenes.* This study evaluates the influence of water activity (a_w) on *L. monocytogenes* growth in 180 "Salsiccia Sarda" samples. A challenge test was performed to determine the *L. monocytogenes* growth potential (δ). The highest values of δ were detected in samples with limited values of a_w, showing that this product is frequently around the limits for the growth of this pathogen. Our results provide some critical information about process parameter combinations that could lead to greater safety of this product and better *L. monocytogenes* control.

Keywords: Challenge test, growth potential, legislation, *Listeria monocytogenes*, ready to eat meat products, "Salsiccia Sarda", water activity

1. INTRODUCTION

The implementation of HACCP (Hazard Analysis and Critical Control Points) system is fundamental to minimize the percentage of microbiological risk associated with food consumption. *Listeria monocytogenes* is a pathogen that may contaminate different foods and many areas of the food processing environment (BARZA, 1985; SCHLECH, 2000; GAULIN *et al*., 2003; MACDONALD *et al*., 2005; VARMA *et al*., 2007; JACKSON *et al*., 2011; AISSANI *et al*., 2012). This pathogen causes listeriosis in humans and animals; human listeriosis can cause serious illness in immunocompromised individuals, pregnant women, newborns and elderly people. There has been a gradual increase in cases of listeriosis over the past 5 years in European Union countries (EFSA, 2014). In Italy, between 1993 and 2000, the number of cases of listeriosis increased (PETRUZZELLI *et al*., 2010). *L.monocitogenes* was mostly found in ready-to-eat foods (RTE) and was responsible for many outbreaks associated with the consumption of RTE meat, poultry, dairy, fish and vegetable products (LIU, 2006; LIANOU and SOFOS, 2007; Chan and WIEDMANN, 2009; EFSA, 2014; CORONEO *et al*., 2016). The possible presence of pathogens is a critical issue when dealing with a wide variety of fermented, dried and semi-dried sausages. These products are classified as ready-to-eat foods (RTE) in which the presence of *L. monocytogenes* can pose a health risk to consumers. With this regard, some recent listeriosis cases are linked to the consumption of RTE meat products (PHAC, 2009). *L. monocytogenes* contamination tends to increase during the production process of meat products because their production requires different handling steps and exposure to contaminated surfaces in the processing environment. Raw meat is an important contamination source and may be contaminated by *L. monocytogenes* from the slaughterhouse environment or during the meat processing. Once the production plant has been contaminated, *L. monocytogenes* can survive on work surfaces and equipment and grow on the meat products due to its high ability to tolerate environmental stress factors (wide ranges of pH and a_w, high salt concentration, presence of nitrite and nitrate, and refrigeration temperature) (PETRUZZELLI *et al*., 2010; MUREDDU *ET AL*., 2014; MELONI *et al*., 2014). Sardinia has a long tradition of quality meat-products and "Salsiccia Sarda" is considered the Sardinian salami par excellence. It is a fermented RTE meat product included on the list of Italian traditional food products. It is made from minced lean pork mixed with different ingredients (salt, pepper, fennel and herbs). The mixture is introduced into a natural pork casing and, subsequently, the products are first heated to 20-22°C for 4-6 hours and then dried for six days in a fermentation chamber. During the first day of drying, the products are stored at 20-22°C and 60% relative humidity. In the subsequent five days of drying, the temperature is gradually reduced to 15°C and the relative humidity is gradually increased to 70%. The product is then dried and finally subjected to ripening for a period ranging from 8 to 25 days. The "Salsiccia Sarda" marketed shows a pH value of 5.28 and a_w ranging from 0.90 to 0.95 (GRECO *et al*., 2005).

Previous studies of traditional fermented meat products showed that the prevalence of *L. monocytogenes* was 10% in France (THÉVENOT *et al*., 2005), 10.6% in Chile (CORDANO and ROCOURT, 2001) and between 13% and 42% in Italy (DE CESARE *et al*., 2007; MELONI *ET AL*., 2009). PETRUZZELLI *et al*. (2010) reported a *L. monocytogenes* prevalence of 45.7% in traditional salami samples from the Marche region (central Italy).

"Salsiccia Sarda" is catalogued as a ready-to-eat food (RTE) for which actual European Legislation, Regulation (EC) 2073/2005 as amended by Regulation (EC) 1441/2007 (European Commission, 2005; European Commission, 2007), specifies microbiological criteria for *L. monocytogenes.* According to these regulations, the *L. monocytogenes* growth is not supported in RTE products with pH <4.4 or $a_w \leq 0.92$ or with pH ≤ 5.5 and $a_w \leq 0.94$. For RTE products that meet these conditions, a criterion of risk acceptability was established

of 100 CFU (Colony-Forming Unit)/g during the shelf-life. Although several studies (MELONI *et al*., 2009; MELONI *et al*., 2012) have shown the presence of *L. monocytogenes* in 42% of fermented "Salsiccia Sarda", the contamination levels are always lower than 100 CFU/g. Many factors affect the growth capacity of *L. monocytogenes* in foods and the intrinsic and extrinsic properties (i.e. pH, NaCl content, a_w, food composition, competing microflora, antimicrobial constituents naturally present, growth temperature, atmospheric gases) are certainly the most important (BEAUFORT *et al*., 2008).

During the production process of fermented meat, *L. monocytogenes* can survive due to its ability to tolerate low pH conditions and high salt concentrations (FARBER and PETERKIN, 1991). Its survival is also linked to the absence of specific procedures in the production process. One of the very important factors that influences the growth/survival of *L. monocytogenes* is the a_w. Its variation inhibits part of the aerobic flora and a selection of the lactic flora (LAB) causing a decrease in pH during production. Thus, careful monitoring of the a_w parameter, together with the proper choice of the ripening time, are essential for the microbial safety and stability of fermented sausages which, if marketed with a low maturation level, may be microbiologically unsafe. The aim of the present study was to assess the presence of *L. monocytogenes* in 84 naturally contaminated samples of "Salsiccia Sarda" over a seven month period. Moreover the growth of *Listeria monocytogenes* in 180 experimentally spiked samples of "Salsiccia sarda" was evaluated using a full factorial experimental design. This design evaluated the impact of storage times and temperatures, level of ripening, packaging conditions and type of sausage.

2. MATERIALS AND METHODS

During the period between December 2014 and June 2015, a total of 84 "Salsiccia Sarda" samples, collected from local manufacturing plants in Sardinia, were examined. All of the samples were transported under a controlled temperature (4°C) and were subjected to analysis at the Laboratory of Food Hygiene at the University of Cagliari which operates in conformity with European standard UNI CEI EN ISO/IEC 17025:2005.

2.1. Microbiological analyses

The presence/absence of *L. monocytogenes* was investigated using the international standard method UNI EN ISO 11290-1:2005. Twenty-five grams of "Salsiccia Sarda" samples were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici, Uta, Cagliari, Italy) incubated at 30°C ± 1 °C for 24h ± 2 h (primary enrichment). Afterwards, 0.1 mL of the primary enrichment was transferred into a 10 mL tube containing Fraser broth (Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37°C ± 1 °C for 48h ± 2 h (secondary enrichment). After incubation, primary and secondary enrichment broths were streaked onto Agar Listeria Ottaviani Agosti (ALOA, Microbiol Diagnostici, Cagliari, Italy) and Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) Agar (Microbiol Diagnostici, Cagliari, Italy) plates and incubated at 37°C, respectively. From the positive sample plates, up to 5 presumptive colonies were subcultured on Tryptone Soy Yeast Extract Agar (TSYEA, Microbiol Diagnostici, Cagliari, Italy) and incubated at 37°C for 24 h. Species confirmation was obtained with the following tests: Gram staining, catalase and oxydase test (Microbiol Diagnostici, Cagliari, Italy), haemolytic activity, CAMP tests on sheep blood agar (Microbiol Diagnostici, Cagliari, Italy) and the biochemical test API Listeria® (BioMérieux, Marcy-l´Etoile, France). In all biochemical reactions the reference strain *L. monocytogenes* ATCC 35152 was used as positive.

2.2. Molecular investigation

Detection of *L. monocytogenes* was performed according to the previously published PCR protocols described by CORONEO *et al*. (2016).
The DNA extraction was performed using the DNeasy Merikon food kit (Qiagen, Hilden, Germany). Following the manual indications, twenty-five gram samples of "Salsiccia sarda" were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici, Cagliari, Italy), and incubated at 30°C ±1°C for 24h ± 2 h. After the pre-enrichment step, 1mL of each sample was taken and centrifugated for three minutes at 11,000 x g. The *L. monocytogenes* DNA was detected using the Merikon *L. monocytogenes* kit (Qiagen, Hilden, Germany). *L. monocytogenes* ATCC 35152 was used as PCR-positive control in all amplifications and molecular grade water as negative control. The reaction was carried out with the StratageneTM Mx3005PqPCR (Stratagene, La Jolla, CA, USA) as it follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60 °C for 23 s and extension at 72°C for 10 s.

2.3. Challenge tests

The present study used 180 "Salsiccia Sarda" samples which were produced in Sardinia. The samples were characterized by two different ripening times (i.e., 12 days and 20 days) because these are the levels of ripening most commonly used to meet the market demands. The 180 samples consisted of 90 "Salsiccia Sarda" samples of pure pork (n.45 at 12 days and n.45 at 20 days of ripening) and 90 myrtle flavored "Salsiccia Sarda" samples (n.45 at 12 days and n.45 at 20 days of ripening). The ingredients of each product are shown in Table 1.

Table 1. Ingedients of "Salsiccia Sarda"samples.

Ingredients	"Salsiccia sarda" (%)	"Salsiccia sarda" myrtle flavored (%)
Meat and fat		
Minced lean pork	87.0	87.0
Pork back fat	8.0	8.0
Additive		
Salt	3	3
Dextrose and sucrose	0.736	0.736
Potassio nitrate (E252)	0.024	0.024
Sodium ascorbate (E301)	0.040	0.040
garlic	0.15	0.15
Ground pepper	0.25	0.25
Other spice	0.8	0.8
Myrtle flavor		1
Starter	p	p

p: presence.

In this study the 45 samples tested at each ripening time belonged to three different batches (i.e., 15 samples/batch). "Salsiccia Sarda" samples were artificially contaminated with *L. monocytogenes.* The samples not inoculated were defined as Blank Samples (B_s) and

used to evaluate the natural contamination of "Salsiccia Sarda" with *L. monocytogenes*. The testing points were: T_0 which was the time of inoculation, and T_1, T_2, T_3, T_4, T_5, which were respectively the examination points carried out every 45 days for a total of seven and a half months after inoculation. This storage time has been adopted in order to achieve an extreme condition for the purposes of the research.

2.4. Inoculation of "Salsiccia Sarda", packaging and storage conditions

The challenge test was carried out according to the Technical Guidance Document prepared by EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort *et al*., 2008). Three strains of *L. monocytogenes* were used in the study. The inoculum was composed by: *L. monocytogenes* reference strain ATCC 35152 obtained from the American Type Culture Collection and two were wild type strains (serovar 1/2a and 1/2c) previously recovered from the "Salsiccia Sarda" samples. The preparation of inoculum has been previously described (CORONEO *et al*., 2016). The level of contamination was approximately 10-100 CFU/g, which was obtained contaminating 10g of Salsiccia slices with 100 µL of inoculum at a concentration of 10^3 CFU/mL. Colony counts were confirmed by Plate Count Agar (PCA, Microbiol, Ca, It).
The inoculated "Salsiccia Sarda" samples (pure pork and myrtle flavored) were packaged under air (n=180) or Modified Atmosphere (MAP) (i.e., 30% CO_2 and 70% N_2) (n=180) and then stored at three different temperatures, 4°C, 8°C and 25°C. The challenge tests were carried out in independent trials for each batch (A, B and C) performed one week apart. A full factorial design of the variables (i) temperature, (ii) time of analysis, (iii) ripening time, (iv) type of packaging and (v) type of salsiccia was accomplished. The related experimental design is reported in Table 2, leading to 144 different combinations of the variables with three replicates.

Table 2. Experimental design for challenge studies.

Variables						
Temperature	4°C		8°C		25°C	
Time of analysis	T_0	T_1	T_2	T_3	T_4	T_5
Level of ripening		12 d			20 d	
Type of packaging	Normal			Modified Atmosphere Packaging (MAP)		
Type of sausage		Normal			Myrtle flavored	

The detection and enumeration of *L. monocytogenes* was conducted according to International Standard methods UNI EN ISO 11290-1:2005 and UNI EN ISO 11290-2:2005. The enumeration of *L. monocytogenes* was performed on an aliquot of the sample homogenized 1/10 with base Fraser Broth (Microbiol Diagnostici, Cagliari, Italy) and incubated at 20°C ± 2°C for 1 h ± 5 min. A volume of 1mL from each suspension was streaked onto three ALOA plates and incubated at 37°C for 24 and 48 hours. Presumptive colonies of *L. monocytogenes* were counted. The final results were expressed as Log_{10}CFU/g.

2.5. Intrinsic properties

For all the samples of "Salsiccia Sarda" intrinsic properties, pH and a_w, were determined. The measurement of pH and a_w was carried out using pH meter Eutech Instruments pH 510 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and AquaLab4TE (Decagon,

Pullman, WA, USA), respectively. The a_w measurement was performed at different points of the product according to the diagram in Fig. 1 and to International Standard methods UNI 11302: 2009.

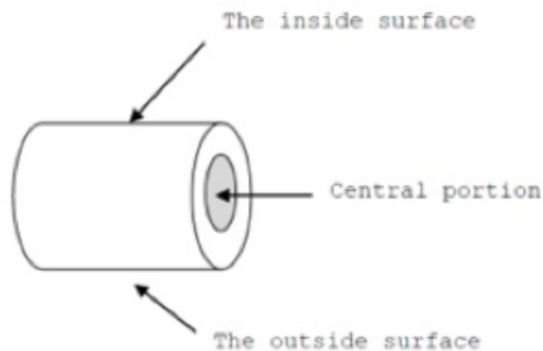

Figure 1. Operation chart used for the a_w measurement in Salsiccia Sarda samples.

2.6. Growth potential

The growth potential (δ) of *L. monocytogenes* was determined by the difference between the counts at the end (T_5) (log_{10}CFU/g) and at the beginning (Time "0") (log_{10}CFU/g) of shelf-life. The RTE product was considered as supporting growth of the *L. monocytogenes* when δ was higher than 0.5 log_{10}. The pathogen was considered not able to grow in a RTE product when δ values were negative or lower than 0.5 log_{10} (BEAUFORT *et al.*, 2008).

2.7. Statistical analysis

All tests of the assessment of *L. monocytogenes* growth were run in triplicate and averaged. Means($\bar{x}$) and standard deviations (s) were computed for each experimental condition. The confidence interval is calculated as

$$\mu = \bar{x} \pm t_{0.05,n} SE(\bar{x}) = \bar{x} \pm t_{0.05,n} \sqrt{s^2/n}$$

where n= 3 is the number of replicates. Analyses were performed using Microsoft Excel XP 2010 and Matlab® 2015 equipped with the toolbox Statistics. Correlation amongst the variables has BEEN estimated by resorting to Spearman's rank correlation coefficient (GIBBONS and Wolfe, 2003). This statistic seems to be the most reasonable choice for our data since it is a non-parametric statistic that reveals to be more robust when dealing with non-linear relationships.

A multiway analysis of variance (*N*-way ANOVA) test is accomplished for testing the effects of the factors: i) storage temperature, which assumes three different levels; ii) measuring time, which is available at 5 different levels; iii) time of ripening (data available at two different levels); iv) absence/presence of myrtle (2 levels); v) type of packaging (2 levels); vi) measured pH and vii) measured a_w. With regards to the latter two variables, the measured values of pH and a_w are divided into classes of width 0.1 and 0.05 respectively.

This allows a finite number of levels of such factors (11 for the pH and 27 for the a_w) to be considered for the statistical test.

3. RESULTS AND DISCUSSIONS

3.1. Conventional microbiological analysis

In the present work, natural contamination of the salsiccia samples analyzed was never detected along the seven month observation period. Our result is consistent with some literature in which several Mediterranean-style dried fermented sausages could be included in the category of RTE products that do not favor *L. monocytogenes* growth. There is, however, a great variability according to local traditions that influence fermentation and ripening (HOSPITAL *et al*., 2012; MELONI, 2015). In fact, as reported by PETRUZZELLI *et al*. (2010), a high frequency of isolation of *L. monocytogenes* was found in Ciauscolo salami manufactured in the Marche region. This type of salami is particularly exposed to the risk of contamination because of its peculiarities (short maturation period, high a_w, rare use of additives and starter cultures).
The microbiological results were confirmed by molecular analysis.

3.2. Challenge test

The challenge tests conducted in this study on the sausage samples subjected to different storage and packaging conditions show that *L. monocytogenes* was unable to survive and grow until the end of shelf life in both situations regarding packaging and refrigeration (with a significance level $P<0.05$). However, we also observed an increase of the pathogen concentration in a specific time of the shelf life in samples with a particular level of ripening (12 days), stored in certain packaging conditions (under air) and with a_w values around 0.92.
In fact, the *L. monocytogenes* concentration in "Salsiccia sarda" samples at 12 days of ripening and stored at 4°C increased from 1.66 log_{10}CFU/g at T_0 to 3.9 log_{10}CFU/g at T_2 when a_w values were equal to 0,922± 0.001 (Table 3).

Table 3. Results of spiked samples packaged under air and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.66	5,2±0.22	0.924±0.007	1.6	5.4±0.40	0.924±0.017	1.4	5.3±0.12	0.921±0.005
	T_1	1.48	5.3±0.12	0.931±0.005	1.6	5.7±0.50	0.931±0.000	2.17	5.7±0.07	0.925±0.015
	T_2	3.9	5,5±0.07	0,922±0.001	3.3	5.8±0.12	0.925±0.005	3.3	5.8±0.05	0.923±0.002
	T_3	3.07	5.7±0.42	0.930±0.001	1	5.8.0±0.25	0.909±0.000	<1	5.7±0.30	0.905±0.001
	T_4	<1	5.8±0.32	0.917±0.012	1	5.7±0.40	0.903±0.015	<1	5.9±0.27	0.890±0.015
	T_5	<1	5.8±0.27	0.912±0.010	<1	5.7±0.32	0.901±0.010	<1	5.8±0.25	0.881±0.004
	δ	-1.35			-1.6			-0.70		

	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
20 days	T_0	1.48	5.7±0.03	0.881±0.012	1.48	5.6±0.35	0.874±0.010	1.3	5.77±0.30	0.868±0.005
	T_1	1.84	5.7±0.04	0.903±0.224	<1	5.8±0.01	0.872±0,248	<1	5.7±0.01	0.901±0.298
	T_2	<1	5.9±0.27	0.908±0.010	<1	5.9±0.02	0.891±0.298	<1	5.8±0.01	0.901±0.248
	T_3	<1	5.8±0,30	0.884±0.005	<1	5.7±0.27	0.881±0.010	<1	5.9±0.07	0.893±0.005
	T_4	<1	5.6±0.22	0.863±0.124	<1	5.8±0.22	0.865±0.020	<1	5.8±0.010	0.872±0.010
	T_5	<1	5.8±0.27	0.852±0.012	<1	5.8±0.02	0.862±0.007	<1	5.9±0.27	0.861±0.007
	δ	-1.17			-1.00			-0.99		

L.m: *L.monocytogenes* concentration expressed as median log_{10}CFU/g **;** **δ**: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

The *L. monocytogenes* concentration decreased in later observations (T_4 and T_5) due to a decrease in a_w and an increase in pH values (Table 3). The same results were observed in samples at 12 days of ripening stored at 8°C and 25°C. In the salsiccia samples with 20 days of ripening, *L. monocytogenes was* able to survive only in samples stored at 4°C at time T_1, whereas at higher temperatures (8°C and 25°C) it was not detected (Table 3).
In both MAP packed salsiccia samples, at 12 and 20 days of ripening, stored at 4°C, 8°C and 25°C, significant *L. monocytogenes* growth was not observed (Tables 4).

Table 4. Results of spiked samples MAP packaged and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				**8°C**			**25°C**		
	Time	**L.m**	**pH**	**a_w**	**L.m**	**pH**	**a_w**	**L.m**	**pH**	**a_w**
12 days	T_0	1.7	5.3±0.45	0.926±0.022	1.00	5.0±0.42	0.914±0.005	1.4	5.2±0.30	0.912±0.002
	T_1	<1	5.24±0.02	0.924±0.005	<1	5.3±0,05	0.906±0.001	<1	5.0±0.32	0.907±0.002
	T_2	<1	5.53±0.12	0.916±0.010	<1	5.6±0.12	0.910±0.001	<1	5.4±0.27	0.901±0.001
	T_3	<1	5.5±0.15	0.910±0.002	<1	5.8±0.17	0.887±0.005	<1	5.5±0.05	0.893±0.050
	T_4	<1	5.6±0.22	0.909±0.124	<1	5.7±0.22	0.899±0.015	<1	5.6±0.30	0.882±0.012
	T_5	<1	5.4±0.15	0.892±0.007	<1	5.6±0.10	0.880±0.007	<1	5.5±0.27	0.871±0.007
	δ	-1.7			-0.7			-1.1		
20 days	T_0	1.48	5.6±0.32	0.854±0.010	1.48	5.6±0.22	0.882±0.020	1.48	5.7±0.22	0.891±0.020
	T_1	<1	5.7±0.30	0.852±0.010	1	5.7±0.30	0.853±0.010	1	5.7±0.30	0.870±0.010
	T_2	<1	5.6±0.25	0.863±0.348	<1	5.5±0.35	0.864±0.007	<1	5.5±0.32	0.885±0.010
	T_3	<1	5.7±0.22	0.872±0.010	<1	5.8±0.30	0.852±0.010	<1	5.8±0.27	0.874±0.007
	T_4	<1	5.8±0.2	0.864±0.007	<1	5.9±0.17	0.851±0.005	<1	5.7±0.22	0.882±0.010
	T_5	<1	5.8±0.00	0.853±0.149	<1	5.8±0.17	0.850±0.0149	<1	5.8±0.10	0.861±0.224
	δ	-1.25			-0.8			-1.13		

L.m: *L.monocytogenes* concentration expressed as median log_{10}CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

In myrtle flavored "Salsiccia sarda" samples at 12 days of ripening and stored at 4°C, the *L. monocytogenes* concentration increased from 1.6 log_{10}CFU/g at T_0 to 2.07 log_{10}CFU/g at T_5. These results show that bacterial survival was greater as evidenced by δ = 0.47 log_{10}CFU/g (Table 5).

Table 5. Results of spiked samples myrtle flavored packaged under air and tested at 12 and 20 days of ripening.

		Storage temperature								
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.6	5.4±0.12	0.923±0.005	1.6	5.5±0.45	0.927±0.017	1.74	5.4±0.27	0.916±0.007
	T_1	1.48	5.3±0.47	0.920±0.020	2.5	5.8±0.15	0.921±0.012	<1	5.6±0.50	0.908±0.124
	T_2	3.17	5.7±0.12	0.931±0.005	3.9	5.9±0.07	0.929±0.005	<1	5.6±0.05	0.907±0.050
	T_3	2.25	5.6±0.15	0.930±0.001	1	5.7±0.17	0.908±0.001	<1	5.8±0.17	0.896±0.007
	T_4	2.3	5.5± 0.30	0.922±0.015	1	5.8±0.15	0.905±0.017	<1	5.7± 0.15	0.891±0.020
	T_5	2.07	5.6±0.22	0.917±0.010	<1	5.7±0.02	0.891±0.010	<1	5.8±0.12	0.883±0.124
	δ	0.47			-1.1			-1.26		
20 days	T_0	1.48	5.4±0.02	0.903±0.017	1.48	5.4±0.30	0.920±0.348	1.3	5.3±0.22	0.920±0.015
	T_1	<1	5.7±0.02	0.901±0.007	<1	5.4±0.22	0.901±0.273	<1	5.9±0.27	0.902±0.010
	T_2	<1	5.6±0.22	0.891±0.422	<1	5.2±0.25	0.885±0.248	<1	5.7±0.42	0.894±0.005
	T_3	<1	5.8±0.27	0.882±0.012	<1	5.4±0.22	0.863±0.075	<1	5.8±0.27	0.882±0.012
	T_4	<1	5.7±0.30	0.844±0.005	<1	5.5±0.30	0.857±0.012	<1	5.7±0.22	0.821±0.015
	T_5	<1	5.6±0.22	0.825±0.012	<1	5.7±0.20	0.842±0.007	<1	5.8±0.12	0.790±0.007
	δ	-1.00			-0.70			-1.05		

L.m: *L.monocytogenes* concentration expressed as median log_{10}CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

In the spiked samples myrtle flavored MAP packaged and tested at 12 days of ripening, the *L. monocytogenes* concentration increased from 1.78 log_{10}CFU/g at T_0 to 3.58 log_{10}CFU/g at T_1, then decreased to 1.78 log_{10}CFU/g at T_2. In all other types of samples *L. monocytogenes* survival and growth was not observed (Tables 6).

3.3. Statistical analysis

The previous considerations can be ascertained in a more rigorous manner by resorting to a multiway ANOVA test. This test has been carried out on the data in order to assess which factors significantly affect the *L. monocytogenes* growth. The results are reported in Table 7 where the statistically significant factors are highlighted with an asterisk. It appears that the growth is strongly influenced by the factors (i) time of analysis (*P*-value = 1.8555e-7) and (ii) a_w (*P*-value = 4.9e-3). It should be noted that the factor "Level of ripening" shows a p-value = 0.0622.

Table 6. Results of spiked myrtle flavored samples MAP packaged and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.78	5.4±0.35	0.927±0.012	1.4	5.2±0.07	0.916±0.002	1.84	5.4±0.01	0.918±0.004
	T_1	3.58	5.8±0.02	0.929±0.015	1	5.5±0.22	0.897±0.002	<1	5.8±0.17	0.909±0.006
	T_2	1.95	5.8±0.12	0.926±0.001	<1	5.6±0.01	0.889±0.001	<1	5.7±0.15	0.900±0.0002
	T_3	<1	5.7±0.17	0.914±0.001	<1	5.7±0.17	0.862±0.015	<1	5.8±0.5	0.770±0.05
	T_4	<1	5.8±0.15	0.906±0.020	<1	5.6±0.20	0.875±0.010	<1	5.6±0.25	0.821±0.006
	T_5	<1	5.7±0.10	0.895±0.005	<1	5.8±0.22	0.868±0.007	<1	5.7±0.22	0.810±0.03
	δ	-1.17			-1.30			-1.26		
20 days	T_0	1.3	5.2±0.35	0.921±0.002	1.48	5.3±0.22	0.916±0.012	1.3	5.3±0.22	0.918±0.005
	T_1	<1	5.3±0.27	0.901±0.010	<1	5.4±0.25	0.915±0.000	<1	5.8±0.32	0.902±0.012
	T_2	<1	5.8±0.25	0.896±0.015	<1	5.6±0.20	0.880±0.002	<1	5.7±0.27	0.894±0.012
	T_3	<1	5.7±0.35	0.886±0.017	<1	5.8±0.22	0.878±0.010	<1	5.6±0.32	0.883±0.015
	T_4	<1	5.9±0.30	0.884±0.012	<1	5.7±0.25	0.874±0.007	<1	5.7±0.27	0.876±0.012
	T_5	<1	5.7±0.25	0.867±0.012	<1	5.6±0.17	0.863±0.124	<1	5.8 ±0.01	0.862±0.075
	δ	-0.5			-1.30			-0.7		

L.m: *L.monocytogenes* concentration expressed as median $\log_{10}$CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications

Table 7. Effect of the variables on the *L. monocytogenes* concentration in "Salsiccia Sarda".

Source	Sum of Squares	Degree of freedom	Mean Square	F-test	Prob>F[a]
Temperature	1.2143e+06	2	6.0717e+05	0.7063	0.4943
Time of analysis	3.6114e+07	5	7.2228e+06	8.4016	1.8555e-7*
pH	6.9740e+06	10	6.9740e+05	0.8112	0.6180
a_w	4.3245e+07	26	1.6633e+06	1.9347	0.0049*
Type of sausage	3.6356e+03	1	3.6356e+03	0.0042	0.9482
Level of Ripening	3.0113e+06	1	3.0113e+06	3.5026	0.0622
Type of packaging	1.0974e+06	1	1.0974e+06	1.2765	0.2594
Error	2.6049e+08	303	8.5969e+05		
Total	3.8097e+08	349			

[a] To determine a significant influence of these variables on the *L. monocytogenes* growth we use N-way ANOVA tests with a significance level $P<0.05$.

Thus, although it cannot be considered as relevant for a significance level of the test a=0.05, we cannot exclude, at least for the data here investigated that it might have some impact on the process. On the other hand, the other variables (i.e. packaging, pH, temperature and absence/presence of myrtle) do not seem to significantly affect the *L. monocytogenes* concentration.

Fig. 2 reports the *L. monocytogenes* concentration with respect to the a_w for the 5 different levels of time. It appears that the microbial concentration reveals a sudden increase for a_w values close to 0.92 at each level of time. The region close to the critical value is zoomed in on in the insets. Incidentally, it was found that the microbial growth may take place even at values slightly less than 0.92 (see insets on Fig. 2C and 2E).

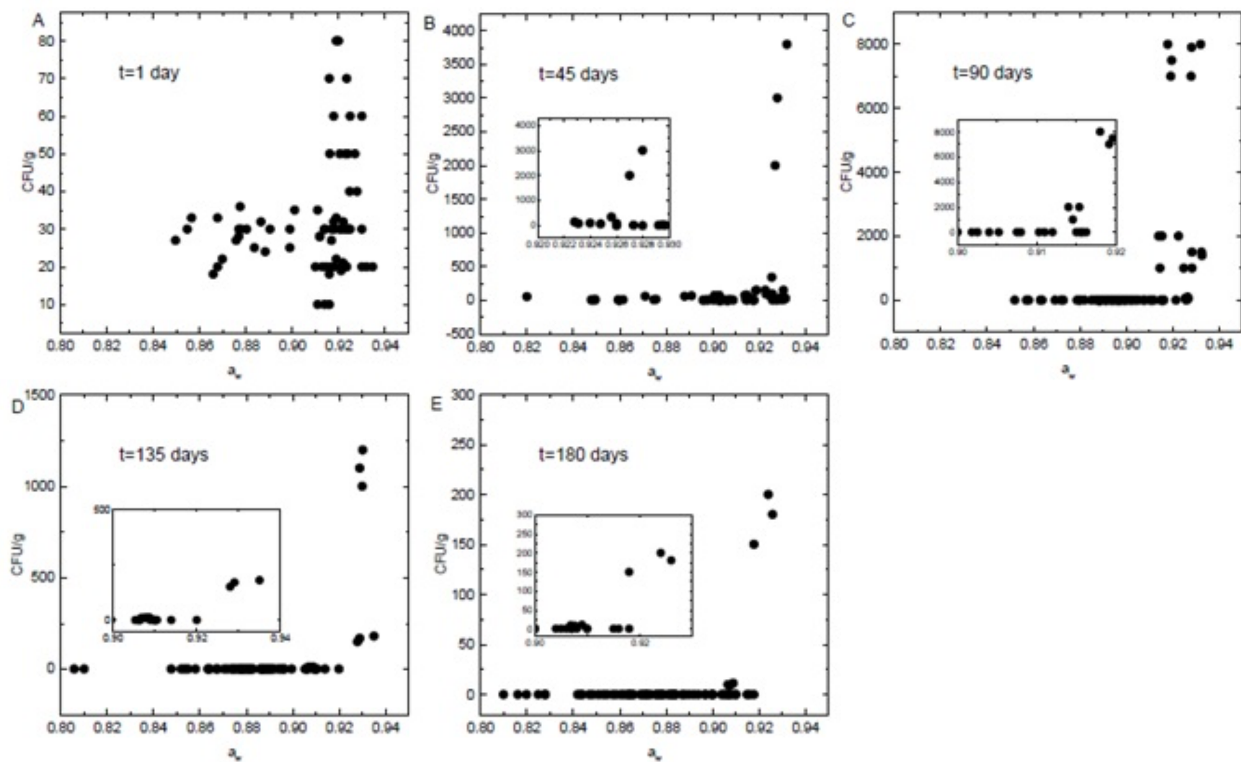

Figure 2. *L. monocytogenes* concentration with respect to the a_w for the different levels of time.

The Spearman correlation coefficient between *L. monocytogenes* concentration and a_w is computed at each different time and the corresponding point estimations are reported in Table 8 together with their P-values. For comparison, the corresponding Pearson correlation coefficients and the related P-values are reported in Table 8.

Table 8. The Spearman correlation coefficient values between *L. monocytogenes* concentration and a_w at each different time of analysis.

	Spearman coefficient		Pearson coefficient	
time	**r**	**p-value**	**r**	**p-value**
1	0.1683	0.1575	0.1844	0.1211
45	0.3716	0.0013	0.2322	0.0497
90	0.6977	9.7e-12	0.4559	5.71e-5
135	0.5919	5.43e-8	0.3434	0.0034
180	0.4272	1.82e-4	0.3447	0.0030

Calculation of the correlation coefficient at time T_6 is meaningless since all the measurements of concentration are zero. It was found that the correlation coefficient is always significantly greater than zero except for the initial time. This further confirms that the a_w is the main factor affecting the growth process. As a final remark, *L. monocytogenes* was never detected in Blank samples (BS) during the challenge test.
These results are consistent with previous studies that have shown a *L. monocytogenes* growth in samples of fermented sausages contaminated with about 5 log_{10}CFU/g (SPERANDII *et al*., 2015). Some manufacturers tend to reduce the ripening period to respond to market needs (HOSPITAL *et al*., 2012; MELONI, 2015). These products with early ripening, as highlighted by our study, may have a_w levels close to 0.92-0.94. This can increase risks associated with the *L. monocytogenes* growth during shelf-life, even in the presence of competitive microflora. Our study showed that, in the presence of an improper ripening time, initial low levels of contamination in the product could however lead to a concentration that is potentially harmful to human health during the first 45 days of storage.

3.4. Intrinsic properties

Among the intrinsic properties, our results showed that the evolution of a_w values is the same of that of other typical Italian fermented meat products (GRECO *et al*., 2005; PETRUZZELLI *et al*. 2010; MELONI *et al*., 2012; MATARAGAS *et al*. 2015a, b). The a_w decreased constantly for both situations regarding packaging and storage temperatures. Its initial values at T_0 were 0.924±0.003 for salsiccia samples at 12 days of ripening when packaged under air and stored at 4°C and at 8°C and 0.921±0.002 for those stored at 25°C. These values decrease to the time T_5 with values equal to 0.912±0.010, 0.901±0.010, 0.881±0.004 for samples stored at 4°C, 8°C and 25°C respectively (Table 3). In general, the sausages with 12 days of ripening showed the average a_w levels typical of products able to support the *L. monocytogenes* growth in all storage conditions (at 4°C, 8°C and 25°C) (Table 3,5)
In salsiccia samples with 20 days of ripening the a_w values are uniform in the products in both situations regarding packaging and storage temperatures. The initial values at T_0 show a decrease of about 0.03-0.08 units at T_5 time ((Table 3, 4, 5, 6). In accordance with other authors (MELONI *et al*., 2014) our results confirm, with reasonable certainty, the safety of products with a longer ripening time.
As far as pH is concerned, our results have showed that the analyzed samples have similar values to those found in most Mediterranean-style fermented sausages (MELONI, 2015) and were close to 5.4-5.8 for both situations regarding packaging and storage temperatures. As reported in other studies (VERMEULEN *et al*., 2007; VERMEULEN *et al*., 2009; MATARAGAS *et al*., 2015b;) the pH level showed a slight increase (0.3-0.5 units), during the experiment, in all sausages analyzed and for both situations regarding packaging and storage conditions. (Tables 3, 4, 5, 6). In general, the a_w and pH values were always within the limits of growth for *L.monocytogenes*.

4. CONCLUSIONS

Previous studies have been carried out to evaluate the effect on *L. monocytogenes* growth and survival during the production of fermented sausages to evaluate the safety of the process (MATARAGAS *et al*., 2015b). The realization, in this study, of an experimental protocol for a challenge test, specific for this traditional product, "Salsiccia Sarda", has

allowed us to obtain usable results for the definition of adequate product security during the shelf-life. In our study, by resorting to statistical tools, the a_w value measured in these fermented sausages was demonstrated as a critical control point.
Through the challenge test, in conjunction with the a_w measurements at each storage time, it has been possible to show that *L. monocytogenes* is able to replicate in a sausage with 12 days of ripening, only when the values of a_w are not less than 0.92. The use of the correct drying process is necessary to lower the a_w which can minimize the potential for *L. monocytogenes* growth. An intermediate time of ripening from 12 to 20 days could be assumed in the production specifications provided that the critical limit is strictly verified for the measurement of water activity (<0.92).

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PAPER

OPTIMISATION OF THE EXTRACTION OF FLAVONOIDS FROM APPLES USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The ultrasound-assisted extraction of flavonoids from apple samples was modelled using response surface methodology. A three-level-three-factor central composite design using the response surface methodology (RSM) was employed to optimise three extraction variables, including temperature, extraction time and ultrasonic power, for the achievement of the highest extraction yield of the flavonoids from lyophilised apple samples. The optimised extraction conditions were 44.61ºC, an extraction time of 26.90 min, and ultrasonic power 480 W. The experimental yield of flavonoids was 6.58 mg g-1 expressed as rutin equivalent, which was close to the predicted yield (6.69 mg g-1). Optimised extraction conditions were applied for the analysis of apple samples of six cultivars.

Keywords: apple, central composite design, flavonoids, HPLC, response surface methodology, ultrasonic extraction

1. INTRODUCTION

Apples play an important role in the human diet. They are one of the most consumed fruits in the whole world (WU *et al*., 2007, CEYMANN *et al*., 2012). Based on data from the year 2014, approximately 84.63 million tonnes of apples are grown per annum. Countries that grow the most apples are China (approximately 40.92 million tonnes per annum), the USA (approximately 5.19 million tonnes per annum) and Poland (3.20 million tonnes per annum) (FAO Statistical Database, 2017). Apples are widely used in the food industry to produce various products and drinks (juice, wine, cider); they are also used unprocessed (MARKS *et al*., 2007, PRICE *et al*., 1999).

Some of the most important biologically active substances in apples are phenolic compounds, which are attributed to natural antioxidants. Oxidative stress causes changes in cell metabolism related to DNA and protein damage as well as lipid peroxidation (COOKE et al., 2003; PIZZIMENTI *et al*., 2010). It can cause inflammatory processes, cardiac, vascular and other diseases (MADAMANCHI *et al*., 2010). Phenolic compounds neutralise reactive forms of oxygen and nitrogen (PANDEY and RIZVI, 2009) and therefore are valuable for the treatment and prophylaxis of various diseases. Qualitative and quantitative composition analyses of raw materials that accumulate phenolic compounds are important and relevant.

The selection of extraction conditions is an important analytical step in developing the qualitative and quantitative analysis methodologies of phenolic compounds in multi-component matrices. Our developed and validated method of flavonoid and phenolic acid determination in apples is published in the paper by LIAUDANSKAS *et al*. (2014). In developing this method, the extraction parameter selection was empirical. It is relevant to compare flavonoid extraction yield when the samples are extracted using empirical extraction conditions and when conditions are selected based on statistical modelling.

2. MATERIALS AND METHODS

2.1. Plant material

Apple samples of the Ligol cultivar were chosen for the extract optimisation analysis. The Ligol cultivar (winter cultivar, bred in Poland) is one of the main cultivars in commercial apple orchards in Lithuania. Optimised extraction conditions were applied for the analysis of the apple samples of different cultivars. The following apple cultivars were included in the comparable researches: Aldas (early winter cultivar, bred in Lithuania, recommended for ecological orchards), Auksis (early winter cultivar, bred in Lithuania,), Connel Red (winter cultivar, bred in USA), Ligol, Lodel (early winter cultivar, bred in Lithuania) and Rajka (early winter cultivar, bred in Czech Republic). The apple trees were grown in the experimental orchard (block 2, row 4, trees 21-40) of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Babtai, Lithuania (55°60′ N, 23°48′ E). The altitude of Babtai town is 57 m above sea level. Trees were trained as a slender spindle, and pest and disease management was carried out according to the rules of the integrated plant protection. The experimental orchard was not irrigated. Tree fertilisation was performed according to soil and leaf analysis. In addition, nitrogen was applied before flowering at the rate of 80 kg ha^{-1}, and potassium was applied after harvest at the rate of 90 kg ha^{-1}. Soil conditions of the experimental orchard were the following: clay loam, pH - 7.3, humus - 2.8%, P_2O_5 - 255 mg kg^{-1} and K_2O - 230 mg kg^{-1}.

2.2. Sample preparation

Apples were cut into slices of equal size (up to 1 cm in thickness), and the stalks and the seeds were removed. The apple slices were immediately frozen in a freezer (at -35°C) with air circulation. Apple samples were lyophilised with a ZIRBUS sublimator 3× 4×5/20 (ZIRBUS technology, Bad Grund, Germany) at a pressure of 0.01 mbar (condenser temperature, -85°C). The lyophilised apple slices were ground to a fine powder (about 100 μm) by using the knife mill Grindomix GM 200 (Retsch, Haan, Germany).
Loss on drying before analysis was determined by drying the apple lyophilisate in a laboratory drying oven to complete the evaporation of free water and volatile compounds (temperature 105°C) and by calculating the difference in raw material weight before and after drying (European Pharmacopoeia, 2010). The data were recalculated for the absolute dry lyophilisate weight.

2.3. Chemicals

All solvents, reagents, and standards used were of analytical grade. Acetonitrile, aluminium trichloride hexahydrate, hexamethylenetetramine and acetic acid were obtained from Sigma-Aldrich GmbH (Buchs, Switzerland), and ethanol from Stumbras AB (Kaunas, Lithuania). Hyperoside, rutin, quercitrin, phloridzin, procyanidin B1 and procyanidin B2, and chlorogenic acid standards were purchased from Extrasynthese (Genay, France); reynoutrin, (+)-catechin and (−)-epicatechin were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland); and avicularin, procyanidin C1 and isoquercitrin were purchased from Chromadex (Santa Ana, USA). In the study, we used deionised water that the Crystal E HPLC (Adrona SIA, Riga, Latvia) water purification system produced.

2.4. Extraction

A total of 2.5 g of lyophilised apple powder (exact weight) was weighed, added to 30 mL of ethanol (70%, v/v) and extracted in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). A total of 480 W is the maximum ultrasonic power that can be achieved by using the Sonorex Digital 10 P ultrasonic bath. The obtained extract was filtered through a paper filter, and the apple lyophilisate on the filter was washed twice with 10 mL of ethanol (70%, v/v) in a 50mL flask. Then, the extract was filtered through a PVDF syringe filter with a pore size of 0.22 μm (Carl Roth GmbH, Karlsruhe, Germany).

2.5. Determination of the total flavonoid content

The total flavonoid content in the extracts of lyophilised apple samples was determined by applying the technique that URBONAVIČIŪTĖ *et al*. (2006) described. It was calculated using the rutin calibration curve and was expressed as its equivalent (mg RE/g) for absolute dry weight (DW).

2.6. High-performance liquid chromatography

The qualitative and quantitative analyses of phenolic compounds were performed according to the previously validated and described high-performance liquid chromatography (HPLC) method (LIAUDANSKAS *et al*., 2014).

2.7. Experimental design and statistical analyses

Before the development of the study through the response surface methodology (RSM), the flavonoid yield from the apples of three extractants (ethanol, methanol, and acetone) of different concentrations were compared. It was determined that the highest yield of flavonoids after four hours of extraction was achieved by macerating apple samples with ethanol 70% (v/v); therefore, this extractant was chosen for further analyses.
Selection of extraction method: The efficacy levels of maceration and extraction in ultrasonic bath methods were compared, and it was determined that the yield was higher when sonification method was applied. The results for the extractant and extraction method selections are discussed more in depth in the paper by LIAUDANSKAS *et al.* (2014).
In general, such multiple parameters as liquid/solid ratio, temperature, time, solvent polarity and ultrasonic power influence the efficiency of the extraction of a compound, and these are the primary extraction parameters that many other authors have referred to (TIAN *et al.*, 2013; RADOJKOVIC *et al.*, 2012; CHEN *et al.*, 2012) In this study, three factors (or independent variables) were selected: temperature (20-60°C), extraction time (5-95 min) and ultrasonic power (48-480 W) (Table 1).

Table 1. Factors and levels for RSM, and central composite experimental design with the independent variables.

Run	Coded and non-coded variable levels						Total flavonoid content, mg RE g^{-1}
	X_1	Temperature, °C	X_2	Extraction time, min	X_3	Ultrasonic power, W	
1	0	40	0	50	0	264	6.889
2	0	40	0	50	-1	48	6.58
3	0	40	-1	5	0	264	4.199
4	0	40	1	95	0	264	6.312
5	-1	20	-1	5	-1	48	3.154
6	1	60	0	50	0	264	6.315
7	0	40	0	50	0	264	7.087
8	1	60	1	95	-1	48	5.618
9	1	60	1	95	1	480	5.963
10	-1	20	1	95	-1	48	4.682
11	1	60	-1	5	-1	48	4.102
12	-1	20	-1	5	1	480	3.555
13	0	40	0	50	0	264	6.794
14	0	40	0	50	0	264	7.175
15	0	40	0	50	0	264	6.821
16	-1	20	0	50	0	264	4.966
17	0	40	0	50	0	264	6.982
18	-1	20	1	95	1	480	5.406
19	1	60	-1	5	1	480	4.665
20	0	40	0	50	1	480	7.465

A three-level-three-factor central composite design was employed to determine the optimal combination of flavonoid extraction variables from apple samples. Table 1 represents the coded and non-coded values of the experimental variables and 20 experimental points. Six replicates (1, 7, 13, 14, 15, 17) were used to evaluate the pure error. Experimental data showed that response variables were fitted to a quadratic polynomial model. The general form of the quadratic polynomial model is presented in Fig. 1, where Y is the dependent variable; β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction respectively. X_i and X_j are the independent variables.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i$$

Figure 1. The general form of the quadratic polynomial model.

Design-Expert® 6.0.8 software (Stat-Ease Inc., Minneapolis, Minnesota, USA) was used to analyse the data, develop models and optimise the extraction conditions. The fitness of the quadratic polynomial model was inspected with the regression coefficient of R^2. The p-value was used to check the significance of the regression coefficient.
All of the experiments (except extraction optimisation researches) were carried out in triplicate. Means and standards errors were calculated with SPSS 20.0 software (Chicago, USA). A single factor analysis of variance (ANOVA) along with the post hoc Tukey's HSD test was employed for statistical analysis. Differences were considered to be significant at the $p<0.05$ level.

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of extraction conditions of total flavonoids in apples

The design matrix and the corresponding results of RSM experiments to determine the effects of the three independent variables, including temperature (X_1), extraction time (X_2) and ultrasonic power (X_3), are shown in Table 2. Through multiple regression analysis of the experimental data, the model for predicted response Y could be expressed with the following quadratic polynomial equation (in the form of coded values), presented in Fig. 2.

$$Y\,(TFC) = 6.86 + 0.49X_1 + 0.83X_2 + 0.29X_3 - 1.08X_1^2 - 1.47X_2^2 + 0.30X_3^2 - 0.071X_1X_2 - 0.027X_1X_3 + 0.013X_2X_3$$

Figure 2. The model for the predicted response Y expressed by the quadratic polynomial equation (in the form of coded values).

Statistical testing of the model was performed in the form of analysis of variance (ANOVA). The ANOVA for the fitted quadratic polynomial model of extraction of polysaccharides is shown in Table 2.

Table 2. Analysis of variance for fitted quadratic model of extraction of phenolic compounds.

Source	Sum of squares	Degree of freedom	p-value
Model	32.32	9	<0.0001 Significant
Residual	0.60	10	
Lack of fit	0.49	5	0.0698 Not significant
Pure error	0.11	5	
Cor. total	32.92	19	

R^2 = 0.9818; R^2_{adj} = 0.9674; C.V. = 4.27%; adequate precision = 26.232.

The results of the analysis of variance for the fitted quadratic model of extraction of phenolic compounds are presented in Table 2. They indicated a high degree of correlation between the observed and predicted values. The lack of fit test determines whether a selected model is adequate for explaining the experimental data or whether another model should be reselected. The value of the lack of fit test indicated that the fitting model was adequate. An adequate precision is a measure of the signal-to-noise ratio, which when greater than 4 is considered to be adequate (CANETTIERI *et al.*, 2007). In addition, the value of adequate precision demonstrates an adequate signal. At the same time, a relatively low value of the coefficient of variation indicates a better precision and reliability of the experimental values. Therefore, the model is adequate for prediction in the range of experimental variables.
Residual analysis of the response surface design was performed. A normal probability plot was applied to the residuals. The data points fell along a straight line, indicating that they were distributed normally. In addition, residual runs for analysis were used. It was determined that the order of observations did not influence the results. Residuals versus predicted responses were plotted. The data points fell on both sides of the zero line, so no pattern could be concluded. The relationship between the actual and predicted values was evaluated. The data points fell along a straight line indicating close similarity between the two data points and the adequacy of the model.

Table 3. Regression coefficients estimate and their significance test for quadratic model.

Source	Sum of squares	Degree of freedom	p-Value
X_1	2.40	1	<0.0001
X_2	6.90	1	<0.0001
X_3	0.85	1	0.0037
X_1^2	3.23	1	<0.0001
X_2^2	5.94	1	<0.0001
X_3^2	0.24	1	0.0717
X_1X_2	0.040	1	0.4337
X_1X_3	5.886×10^{-3}	1	0.7605
X_2X_3	1.378×10^{-3}	1	0.8825

The significance of each coefficient measured using the p-value is listed in Table 3. A smaller p-value means the corresponding variables are more significant. The p-value of the model is less than 0.0001, which indicates that the model is significant and can be used to optimise the extraction variables. The three independent variables (X_1, X_2, X_3) and two quadratic terms (X_1^2 and X_2^2) significantly affect the extraction yield of flavonoids. The interaction among temperature (X_1), extraction time (X_2) and ultrasonic power (X_3) did not affect the extraction yield of flavonoids significantly.

The three-dimensional response surface is the graphical representation of the regression equation and is very useful for judging the relationship between independent and dependent variables. Different shapes of the contour plots indicate whether the mutual interactions among the variables are significant or not. A circular contour plot means the interactions between the corresponding variables are negligible, whereas an elliptical contour suggests that the interactions between the corresponding variables are significant (MURALIDHAR *et al.*, 2001). The three-dimensional representation of the response surfaces generated via the model is shown in Fig. 3–5. With these three variables, when two variables are depicted in three-dimensional surface plots, the third variable is fixed at the zero level.

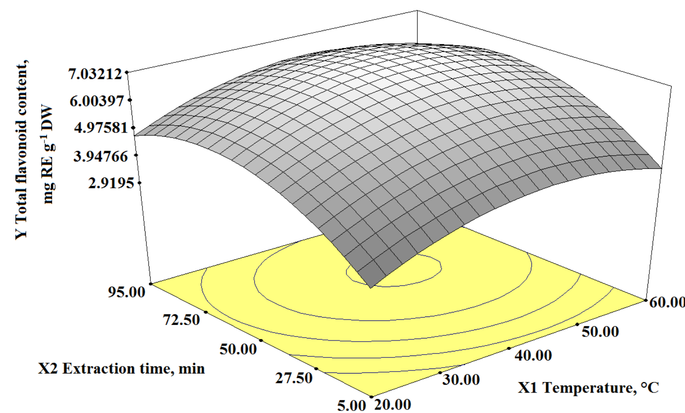

Figure 3. Response surface plot showing the effect of temperature (X_1) and extraction time (X_2). X_3 (Ultrasonic power) = 264 W.

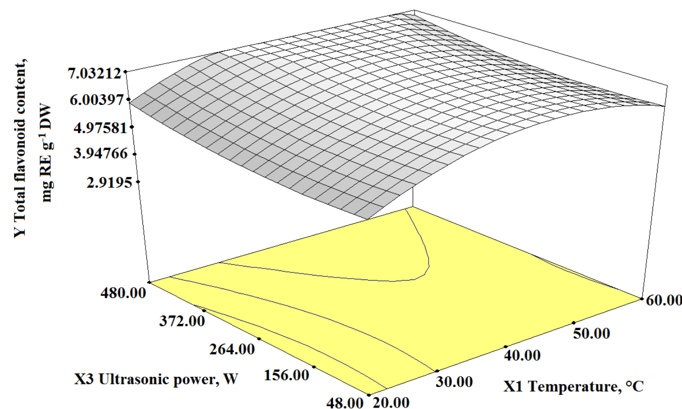

Figure 4. Response surface plot showing the effect of temperature (X_1) and ultrasonic power (X_3). X_2 (Extraction time) = 50 min.

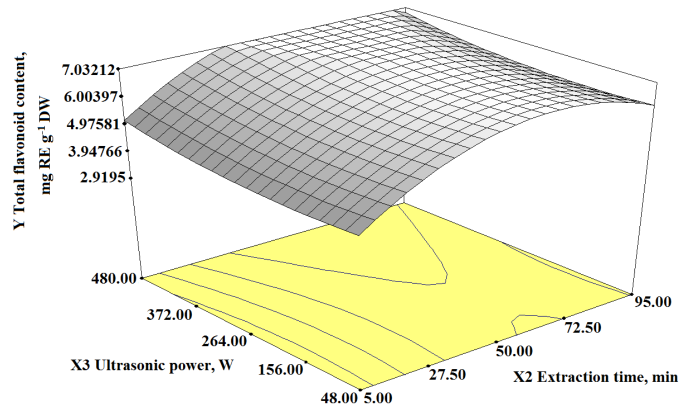

Figure 5. Response surface plot showing the effect of extraction time (X_1) and ultrasonic power (X_3). X_2 (Temperature) = 40°C.

It is found in Figs. 3-5 that all of the three response surfaces are convex in shape, which indicates that the ranges of variables were chosen properly. As shown in Fig. 3, the yield of extraction increases when extraction time and temperature are increased. Based on the chosen model, the projected highest amount of phenolic compounds (6.69 mg g^{-1}) is achieved when samples are extracted at 44.61ºC for 26.90 min. According to the applied model, a further increase of extraction time and temperature decreases the extraction yield.

Flavonoid extraction yield dependency on temperature and ultrasonic power is presented in Fig. 4. The highest extraction yield is achieved when apple samples are extracted at highest ultrasound power (480 W), at 44.61ºC. Extraction time and ultrasound power influence on flavonoid extraction yield is presented in Fig. 5. The highest yield is achieved when the ultrasound power is maximal (480 W), and extraction time is 50 min.

3.2. Optimization of extraction parameters and validation of the model

Through these three-dimensional plots, the suitability of the model equation for predicting the optimal response values was tested using the selected optimal conditions. The results (Table 3) showed that the optimized conditions were ultrasonic temperature of 44.61ºC, extraction time of 26.90 min, and ultrasonic power 480 W. Under these conditions, the predicted extraction yield of flavonoids was 6.69 mg g^{-1}. However, considering the operability in actual production, the optimal conditions can be modified as follows: temperature of 45ºC, extraction time of 27 min, and ultrasonic power 480 W. Under the modified conditions, the experimental yield of flavonoids was 6.58 mg RE g^{-1} (n = 3), which was close to the predicted value. Extraction yield was 4.1 mg RE g^{-1} when extraction conditions were selected empirically (LIAUDANSKAS *et al.*, 2014). When extraction conditions were optimized by statistical modelling method the yield was 37.69% higher than the yield when extraction conditions were selected empirically.

3.3. Analysis of ethanol extracts of apple samples of different cultivars

The chemical composition in fruits of different apple cultivars may vary significantly (CEYMANN *et al.*, 2012; ŁATA *et al.*, 2005; WOJDYŁO *et al.*, 2008), therefore it is very important to determine the qualitative and quantitative composition of individual phenolic compounds in apples that are grown under Lithuanian climatic conditions. Optimized extraction conditions were applied for the HPLC analysis of ethanol extracts of apple samples of six cultivars grown in Lithuania: Aldas, Auksis, Connel Red, Ligol, Lodel and Rajka using previously developed and validated HPLC method (LIAUDANSKAS *et al.*, 2014). These phenolic compounds of various groups were identified and quantified in analysed extracts: procyanidin B1, (+)-catechin, chlorogenic acid, procyanidin B2, (−)-epicatechin, procyanidin C1, rutin, hyperoside, isoquercitrin, reynoutrin, avicularin, quercitrin and phloridzin (synonym phlorizin). Apple sample chromatogram (cultivar Lodel) is presented in Fig. 6. Total amount of phenolic compound in analysed apple sample extracts varied from 2.521 mg g^{-1} (cultivar Connel Red) to 6.430 mg g^{-1} (cultivar Aldas).

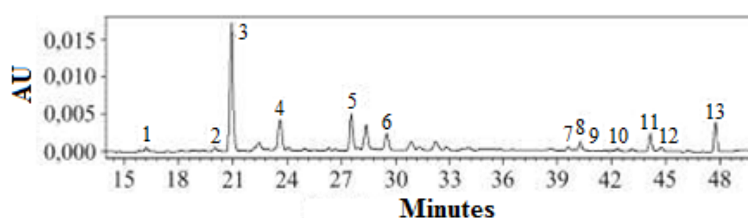

Figure 6. Chromatogram of the ethanol extract of apple sample (λ=280 nm, cultivar Lodel).
1 - Procyanidin B1, 2 - (+)-catechin, 3 - chlorogenic acid, 4 - procyanidin B2, 5 - (–)-epicatechin, 6 - procyanidin C1, 7 - rutin, 8 - hyperoside, 9 - isoquercitrin, 10 - reynoutrin, 11 - avicularin, 12 - quercitrin, 13 - phloridzin.

The highest total amount of quercetin glycoside 0.811 mg g^{-1} was determined in apple samples of cultivar Aldas. It was 1.93 times higher than the lowest total amount of quercetin glycoside (0.420 mg g^{-1}), determined in apple samples of cultivar Auksis. Quantitative composition of quercetin glycoside group compounds determined in apple samples is presented in Table 4.

Hyperoside was a predominant quercetin glycoside group compound in apple samples of cultivars Aldas, Auksis, Connel Red, Ligol and Lodel. It amounted to 25.14-35.16% of the total amount of identified and quantified quercetin glycoside group compounds. VAN DER SLUIS *et al*. indicated similar tendencies of a hyperoside quantitative composition variation. Hyperoside amounted to 23-33% of the total identified quercetin glycosides in samples of cultivars that these authors analysed (VAN DER SLUIS *et al*., 2001). The composition of apple samples of cultivar Rajka stood out among the analysed cultivars, as the predominant compound in this cultivar was quercitrin. Its amount was 1.66 times higher than the amount of hyperoside determined in the apple samples of this cultivar. Quercetin glycoside rutin-hyperoside-isoquercitrin triplet (where the predominant compound is always hyperoside, and levels of rutin are the lowest) was characteristic in apple sample extracts of all analysed cultivars. This pattern was also established in studies of other scientists (MARKS *et al*., 2007; PRICE *et al*., 1999; SCHIEBER *et al*., 2001). The ratio of rutin-hyperoside-isoquercitrin amount varies in the apple samples of different cultivars. It varied from 1:5.1:1.6 (cultivar Connel Red) to 1:11.9:1.2 (cultivar Lodel).

Monomeric ((+)-catechin and (−)-epicatechin) and oligomeric (procyanidin B1, procyanidin B2, and procyanidin C1) flavan-3-ols were determined in the apple samples. The highest total amount of identified and quantified flavan-3-ols (2.919 mg g^{-1}) was determined in apple samples of the cultivar Lodel. It was 2.55 times higher than the lowest amount (1.143 mg g^{-1}), determined in the apple samples of cultivar Connel Red.

In the scientific literature, it was noted that in apples, the amount of (−)-epicatechin is higher than (+)-catechin (WU *et al*., 2007; DUDA-CHODAK *et al*., 2010; KAHLE *et al*., 2005; PANZELLA *et al*., 2013). The results of our analysis also confirm this. The ratio of (+)-catechin and (−)-epicatechin in the samples of different cultivars varied from 1:4.3 (cultivar Rajka) to 1:21.9 (cultivar Lodel). In the paper by WOJDYŁO *et al*., it is specified that in samples of apples grown in Poland, the amount of (+)-catechin varies from 0.010 to 0.720 mg g^{-1}, and (−)-epicatechin – from 0.066 to 2.760 mg g^{-1} (WOJDYŁO *et al*., 2008)

The amounts of procyanidin B2 and C1 were higher than the amount of procyanidin B1 was in the samples of all analysed cultivars. This predisposition of the quantitative composition variation of these compounds in apples was also presented by other authors (DUDA-CHODAK *et al*., 2011). The ratio of procyanidin B1, B2 and C1 amounts in samples of different cultivars varied from 1:5.4:3.4 (cultivar Aldas) to 1:13.8:7.9 (cultivar Auksis). The quantitative composition of flavan-3-ol group compounds identified in apple samples is presented in Table 5.

Qualitative and quantitative analyses of dihydrochalcone group compounds are extremely important because the compounds of this group may be selected as chemotaxonomic indicators in apple cultivar taxonomy, for apple product identification and for the determination of apple juice and cider quality (SCHIEBER *et al*., 2001; ALONSO-SALCES *et al*., 2004; GOSCH *et al*., 2010). The amount of dihydrochalcone phloridzin in the apple samples of the analysed cultivars varied from 0.101 mg g^{-1} (cultivar Rajka) to 0.268 mg g^{-1} (cultivar Lodel) (Table 5). Similar results were presented by other authors as well-the amount of phloridzin in apple fruit comprises 2-6% of the total amount of quantified phenolic compounds (SANONER *et al*., 1999)

The highest amount of chlorogenic acid (3.074 mg g^{-1}) was determined in the apple samples of the cultivar Aldas. It was 4.99 times higher than the lowest determined amount of this acid (0.616 mg g^{-1}), determined in the apple samples of the cultivar Rajka (Table 6). WOJDYŁO *et al*. analysed the apple samples of apples grown in Poland and indicated similar amounts (0.015-2.960 mg g^{-1}) of this acid (WOJDYŁO *et al*., 2008).

Table 4. Quercetin glycoside quantitative variation in apple samples.

Substance	Quercetin glycoside amount (mg g^{-1}, dry raw material)						Coefficient of variation, %
	Aldas	Auksis	Connel Red	Ligol	Lodel	Rajka	
Hyperoside	0.274 ± 0.005^{a}	$0.138 \pm 0.002^{c,d}$	0.152 ± 0.003^{c}	0.129 ± 0.002^{d}	0.154 ± 0.003^{c}	0.184 ± 0.004^{b}	31.10
Isoquercitrin	0.053 ± 0.002^{a}	$0.020 \pm 0.001^{c,d}$	$0.047 \pm 0.002^{a,b}$	0.025 ± 0.001^{c}	0.016 ± 0.001^{d}	0.044 ± 0.002^{b}	45.92
Rutin	0.036 ± 0.001^{a}	$0.015 \pm 0.001^{b,c}$	0.030 ± 0.002^{a}	0.022 ± 0.001^{b}	0.013 ± 0.001^{c}	0.035 ± 0.002^{a}	39.69
Avicularin	0.226 ± 0.005^{a}	0.120 ± 0.002^{c}	$0.099 \pm 0.001^{d,e}$	0.087 ± 0.001^{e}	$0.114 \pm 0.002^{c,d}$	0.139 ± 0.003^{b}	38.15
Reynoutrin	0.080 ± 0.003^{a}	0.053 ± 0.002^{b}	0.039 ± 0.001^{c}	0.037 ± 0.001^{c}	0.043 ± 0.001^{c}	0.025 ± 0.001^{d}	40.92
Quercitrin	0.142 ± 0.003^{b}	0.082 ± 0.001^{e}	$0.103 \pm 0.002^{c,d}$	0.120 ± 0.003^{c}	$0.098 \pm 0.001^{d,e}$	0.305 ± 0.007^{a}	58.30

Different letters in the same row indicate statistically significant differences of individual substance amounts in apple samples of analysed cultivars ($p<0.05$).

Table 5. Flavan-3-ols, chlorogenic acid and phloridzin quantitative variation in apple samples.

Substance	Flavan-3-ols, chlorogenic acid and phloridzin amount (mg g^{-1}, dry raw material)						Coefficient of variation, %
	Aldas	Auksis	Connel Red	Ligol	Lodel	Rajka	
(+)-Catechin	0.092 ± 0.002^{b}	0.077 ± 0.002^{c}	0.034 ± 0.001^{d}	0.033 ± 0.001^{d}	0.042 ± 0.001^{d}	0.121 ± 0.003^{a}	54.26
(–)-Epicatechin	0.720 ± 0.015^{b}	0.448 ± 0.009^{d}	0.315 ± 0.006^{e}	0.311 ± 0.004^{e}	0.928 ± 0.019^{a}	0.525 ± 0.010^{c}	44.86
Procyanidin B1	0.157 ± 0.003^{a}	0.170 ± 0.004^{a}	0.035 ± 0.001^{d}	0.064 ± 0.001^{c}	0.094 ± 0.002^{b}	0.097 ± 0.002^{b}	50.84
Procyanidin B2	0.854 ± 0.017^{c}	0.990 ± 0.021^{b}	0.484 ± 0.007^{e}	0.676 ± 0.014^{d}	1.279 ± 0.023^{a}	0.834 ± 0.015^{c}	31.81
Procyanidin C1	0.534 ± 0.020^{a}	0.366 ± 0.012^{b}	0.275 ± 0.008^{c}	0.258 ± 0.011^{c}	0.576 ± 0.021^{a}	0.249 ± 0.007^{c}	38.56
Phloridzin	0.188 ± 0.004^{b}	0.124 ± 0.002^{d}	0.135 ± 0.002^{d}	0.157 ± 0.003^{c}	0.268 ± 0.005^{a}	0.101 ± 0.002^{e}	36.82
Chlorogenic acid	3.074 ± 0.068^{a}	2.498 ± 0.052^{b}	0.773 ± 0.013^{e}	1.249 ± 0.020^{d}	1.629 ± 0.035^{c}	0.616 ± 0.011^{e}	59.41

Different letters in the same row indicate statistically significant differences of individual substance amounts in apple samples of analysed cultivars ($p<0.05$).

The study results confirmed the hypothesis of CEYMANN *et al*., that apple cultivars can be classified based on what types of compounds phenolic acids or flavan-3-ols are predominant in the apple samples (CEYMANN *et al*., 2012). The predominant compound in the apple samples of the cultivars Aldas and Auksis was chlorogenic acid, and in the apple samples of other cultivars, it was flavan-3-ol group compounds. The coefficient of variation, which reflects the variation amplitude of every compound, was calculated to evaluate the variation of the quantitative composition of the phenolic compounds in the apple samples of different cultivars. It varied from 31.10% to 59.41% (Tables 5 and 6). The highest calculated coefficient of variation was for chlorogenic acid, and the lowest was for hyperoside.

4. CONCLUSIONS

In this study, we applied RSM for the extraction of flavonoids from lyophilised apples. The results showed that the independent variables (temperature, extraction time and ultrasonic power), and the quadratic terms of temperature and extraction time had a statistically significant effect on the efficacy of apple flavonoid extraction. A second-order (quadratic) polynomial model was employed to optimize flavonoid extraction from lyophilised apple samples. The projected optimal extraction conditions following statistical modelling were as follows: temperature 44.61°C, extraction time 26.90 min and ultrasonic power 480 W. The experimental yield of flavonoids was 6.58 mg RE g^{-1}, which was close to the predicted yield value of flavonoids 6.69 mg g^{-1}. By applying RSM selected via statistical modelling, it was determined that the apple flavonoid extraction yield was 37.12% higher compared with sample extraction when extraction conditions were selected empirically. Optimised extraction conditions were applied for the HPLC analysis of the apple samples of six cultivars grown in Lithuania. The highest total amount of identified phenolic compounds (6.430 mg g^{-1}) was determined in the apple sample extracts of the cultivar 'Aldas'.

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PAPER

EFFECTS OF ARCHAIC OLIVE AND OIL STORAGE METHODS STILL USED IN SOUTHERN TUNISIA ON OLIVE OIL QUALITY

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ABSTRACT

The present paper investigated how virgin olive oil quality is influenced by two different storage conditions that residents of Gabes (Southern Tunisia) usually apply to fruits of the Zarazi cultivar: long conservation as oil in glass bottles or traditional storage of olives as sun-dried fruits before processing for oil production. Even if both storage conditions are associated with strong losses in the qualitative characteristics of olive oil, the changes observed were more accentuated for oil stored for two years after its production compared to the oil obtained from olives stored by traditional methods.

Keywords: fatty acid alkyl esters, olives, phenols, traditional storage, virgin olive oil

1. INTRODUCTION

Long known to many generations in the Mediterranean basin populations as essential to their diet, virgin olive oil is now widely appreciated around the world for its healthy and sensory properties. The nutritional benefits of virgin olive oil are firstly related to the fatty acid composition, mainly due to both the high content of oleic acid and the balanced ratio of saturated and polyunsaturated fatty acids (GARGOURI et *al*., 2015; RUIZ-CANELA and MARTÍNEZ-GONZÁLEZ, 2011); in addition, virgin olive oil presents considerable amounts of natural antioxidants. Because of these natural characteristics, virgin olive oil is particularly resistant to storage and more suitable for cooking than other vegetable oils (PELLEGRINI et *al*., 2001). It is also considered important in prevention of many diseases, such as cardiovascular disease, obesity, metabolic syndrome, type 2 diabetes and hypertension (NUNEZ-CORDOBA et *al*., 2009).

It is one of the few vegetable oils that can be consumed in the raw state and contains important nutritional elements (fatty acids, vitamins, sterols, etc.). Extra virgin olive oil (EVOO) is considered the highest quality virgin olive oil due to its organoleptic and chemical characteristics (JABEUR et *al*., 2015).

Nevertheless, oxidation is the main cause of deterioration of EVOOs during the storage period. Auto-oxidation follows a free radical mechanism, wherein a first result of the absorption of oxygen is the formation of hydroperoxydes (FRANKEL, 2005). These labile compounds also decompose to produce a complex mixture of volatile molecules, such as aldehydes, ketones, hydrocarbons, alcohols and esters, among which some are directly responsible for perception of the rancid sensory defect (PRISTOURI et *al*., 2010).

The most important factors affecting the quality of olive oil during storage are environmental, especially temperature, light exposure and contact with oxygen (PRISTOURI et *al*., 2010). Much research has been carried out to study the effects of storage conditions and packaging materials on olive oil quality (DABBOU et *al*., 2011; PRISTOURI et *al*., 2010).

Moreover, one of the primary factors responsible for the low quality and stability of virgin olive oils is linked to the mishandling of the olives during the period between harvesting and processing. Indeed, the storage of olives, which leads to degenerative processes in a short period of time, should be carried out by simple heaping in fruit piles, awaiting their transformation (RABIEI et *al*., 2011). Unfortunately, olive processing is often not well synchronized with crop harvests; in particular, for countries in which the production of olives is very high and concentrated in a short period, if the capacity of the local olive oil mills is not adequate to support such enormous amounts, olives are often piled into large heaps or in plastic sacks. Moreover, olives can be stored in big cumuli outside the olive oil mill for periods that may range from weeks to months prior to oil extraction (KIRITSAKIS et *al*., 1998). During this storage period, mechanical, physical, chemical and physiological alterations occur in the fruit, which can cause the rupture of cellular structures and subsequent negative chemical and sensory changes in the oils obtained (BIASONE et *al*., 2012; INAREJOS-GARCÍA et *al*., 2010; VICHI et *al*., 2009). The olive fruit deteriorates rapidly due to the combined action of pathogenic microorganisms and internal processes of senescence. Both processes are accelerated by temperature increases due to fruit fermentation and mechanical damage due to compression. The degradation of fruit causes the loss of the texture of the flesh and tanning of the skin, and finally complete decomposition (GARCÍA et *al*., 1996). Virgin olive oils obtained from damaged olives usually present high acidity, low oxidation stability and high levels of oxidation, due to the increased peroxide value and specific extinction coefficients at 232 and 270 nm (GARCÍA et *al*., 1996; INAREJOS-GARCÍA et *al*., 2010). Moreover, the content in fatty acid

ethyl esters also increases with storage time of olives due to fruit degradation (BIEDERMANN et *al*., 2008).
In this context, herein we investigated the effects on olive oil quality of *i)* the olive oil immediately processed, and *ii)* of the olive oil and olive fruits stored for two-years following the traditional method of Gabes. Although some data have already been published on the effects of olive storage before oil extraction (BIASONE et *al*., 2012; INAREJOS-GARCÍA et *al*., 2010; VICHI et *al*., 2009), in the present investigation a particular interest was given to a traditional conservation procedure that the residents of Gabes (southern Tunisia) still apply to olive fruits of the *Zarazi* cultivar. In fact, after harvesting, olive fruits are traditionally sun-dried, and when completely dried they are stored in plastic bags at room temperature and then processed to obtain olive oil. Therefore, the objective of this investigation was to compare the deteriorative effects on the oil quality of two different conservation methods: conservation as oil or as intact fruits both carried out for a long period of time (two years). For this aim, different quality parameters were investigated, including composition in minor compounds, such as phenolics and fatty acid alkyl esters.

2. MATERIALS AND METHODS

2.1. Olive fruit samples

The present study was carried out on olives picked up from *O. europaea* L. trees (cv. *Zarazi*) grown in an orchard located in the region of Gabes (southern Tunisia: 33° 32′ N, 10° 06′ E). In particular, olive samples were harvested manually and were divided into two unequal lots:
- About 4 kg of olives were processed (see paragraph 2.3) directly after harvest (about 24 hours between harvest and start of processing) and the virgin olive oil obtained was divided into two samples: one used as a control sample (sample code: "TC") and the other stored in dark glass bottles at room temperature for two years (sample code: "T1").
- About 2 kg of olives were cleaned from leaves, washed and stored following a traditional method described later (see the section 2.2) for two years, oil (sample code: "T2") was then extracted from dried fruit using the same lab-scale mill described in paragraph 2.3, in order to evaluate the effects of both conservation methods (as intact fruits or as oil) on virgin olive oil quality.

2.2. Olive fruit conservation

In this investigation, a traditional storage procedure that the residents of *Gabes* still apply to olive fruits of *Zarazi* cultivar was simulated. After harvesting, olive fruits were sun-dried (thrown singly upon a stone-floor, exposed to the sun in the open air for 10 to 15 days) and, once completely dried, they were stored in plastic bags at room temperature for two years, before processing to obtain the oil.

2.3. Oil extraction

Olive oil was extracted under similar industrial extraction conditions using a bench hammer mill. A sample of 2 kg of olive fruits was firstly crushed with a small hammer crusher, and the paste was mixed at about 25°C for 30 min and centrifuged in a two-phase decanter (3500 rpm for 1 min). After extraction, oils were transferred into 250 mL amber glass bottles and stored in darkness at room temperature (25°C) for further analyses.

2.4. Quality indices determination

Free acidity (FA, expressed as % oleic acid), peroxide value (PV, expressed as milliequivalents active oxygen - meq O_2 kg^{-1} oil) and extinction coefficients (K_{232} and K_{270}) were determined by analytical methods described respectively in ISO 660:2009, ISO 3960: 2010 and ISO 3656: 2011.

2.5. Total phenol and *o*-diphenol content

The total phenol and *o*-diphenol content of olive oil samples were quantified colorimetrically using an UV–Vis 1800 spectrophotometer (Shimadzu Co., Kyoto, Japan), according to the procedures previously described (MATEOS et *al*., 2001; SINGLETON et *al*., 1965). Phenolic compounds were extracted in methanol/water (80:20, v/v), according to IOC/T.20/Doc No 29. Folin-Ciocalteu reagent and sodium carbonate (15% p/v) were added to a suitable aliquot of the extract, and the absorption of the solution at 750 nm was measured to evaluate the total phenolic content, by a specific calibration curve built with different concentrations of gallic acid (Sigma-Aldrich, St. Louise, MO, USA) (r^2 =0.995). For *o*-diphenol content determination, 0.5 mL of the extract was dissolved in 5 mL of a methanol/water solution (50:50, v/v). Four mL of the obtained solution were withdrawn, then 1 mL of sodium molybdate (5% in ethanol/water 50:50) was added. After vigorous mixing, they were centrifuged for 5 min at 3000 rpm. The concentration was determined colorimetrically at 370 nm. A specific calibration curve (r^2 = 0.985) was built using gallic acid for the quantification. Data were expressed as mg gallic acid per kg of oil for both total phenolic compounds and *o*-diphenols.

2.6. Determination of phenolic compounds by HPLC-DAD-MS

HPLC analysis was performed using an Agilent Technologies 1100 series system equipped with an automatic injector, a diode array UV-Vis detector (DAD) and a mass spectrometer detector (MSD) (LERMA-GARCIA et *al*., 2009). A C18 column KINETEX (100 cm x 3.00 mm x 2.6 μm; Phenomenex, Torrence CA, USA) was used, maintained at 40°C during the analyses, with an injection volume of 5 μL and a flow rate of 0.7 mL min^{-1}. The wavelength was set at 280 nm for detection of phenolic acids, phenyl ethyl alcohols and secoiridoids. The mobile phase was a mixture of water/formic acid (99.5:0.5%, v/v) (solvent A) and acetonitrile (solvent B). A linear gradient was run from 95% (A) and 5% (B) to 80% (A) and 20% (B) during 3 min, it changed to 60% (A) and 40% (B) in 1 min, after 1 min it changed to 55% (A) and 45% (B), it changed to 40% (A) and 60% (B) after 4 min, and then in 1 min it becomes 100% (B), finally it changed to 95% (A) and 5% (B) in 3 min (13 min total time). Phenolic compounds were identified by comparison of their relative retention times and maximum absorbance according to IOC/T.20/Doc No 29, and by interpretation of their mass spectra (LERMA-GARCIA et *al*., 2009). Syringic acid (Sigma-Aldrich) was used as an internal standard for the quantification of identified phenols and results are expressed as mg of tyrosol per kg of oil (according to the IOC/T.20/Doc No 29).

2.7. Tocopherol determination

Oils were dissolved in a solution of isopropanol and the isomers of α-tocopherol, β-tocopherol and γ-tocopherol were analysed as described (ANWAR et *al*., 2013). An Agilent Technologies 1100 series HPLC apparatus (Paolo Alto, California, USA), comprising a HP pump series 1050 (Darmstadt, Germany) and a DAD detector set at 292 nm was used.

The eluting solvents were methanol/water (90:10, v/v) acidified with 0.2% H_3PO_4 (solvent A), and acetonitrile (solvent B). Samples were eluted through a Cosmosil column (π NAP 4.6 mm × 150 mm x 5 μm; Nacalai-Tesque, Kyoto, Japan) according to the following gradient: 100% A maintained for 22 min; 100% B for 13 min; 100% A for 15 min (total run = 50 min). The injection volume was 20 μL. The flow rate was 1 mL min^{-1}. The concentrations of different tocopherol isomers were expressed on a calibration curve constructed using solutions of α-tocopherol at different concentrations (r^2 = 0.998). Results are expressed in mg of α tocopherol per kg of oil.

2.8. Oxidative stability measurements

Oxidative stability was measured by a Rancimat 743 apparatus (Metrohm Ω, Schweiz AG, Zofingen, Switzerland) following the method described (TURA et *al*., 2007). Air (20 L h^{-1}) was passed through a sample (3 g) held at constant temperature (120°C). Stability was expressed as the oxidation induction time (h).

2.9. Determination of fatty acid alkyl esters (FAAE) by gas chromatographic analyses

Fatty acid alkyl esters were extracted following the methods described in IOC/T.20/ DOC. NO.28 (2009). Subsequent separation of alkyl esters (methyl and ethyl esters) was performed on a gas chromatograph equipped with an injector port and a FID both set at 325°C. The capillary column was a ZB–5MS (30 m length × 0.25 mm i.d. × 0.25-μm-film thickness; Phenomenex) with a split ratio 1:10. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was programmed from 80°C (kept for 1 min) to 140°C at a rate of 15°C min^{-1}, then raised to 325°C at a rate of 4.5°C min^{-1} and kept for 20 min. The amounts of alkyl esters were expressed as mg of methyl heptadecanoate (C17:0 ME) per kg of oil.

2.10. Statistical analysis

The Statistical Package for Social Sciences (SPSS) program, release 16.0 for Windows (SPSS, Chicago, IL, USA) was used for all statistical analyses. The results were expressed as mean±standard deviation (SD) of three measurements for each analytical determination. Significant differences between the values of all parameters were considered at $p< 0.05$ according to the one-way ANOVA Post Hoc Comparisons (Duncan's test).

3. RESULTS AND DISCUSSIONS

3.1. Effect of storage on basic chemical quality parameters

The basic chemical quality parameters of the olive oil samples were investigated and the results are shown in Table 1. The values of FA, PV, K_{232} and K_{270} for the control sample (TC) were below the legal limits established by IOC for extra virgin olive oils (IOC/T.15/NC No. 3/Rev. 10 November 2015). Regarding the effects of storage, both methods of storage (T1 and T2) negatively affected oil quality (p <0.001) by increasing all four above-mentioned parameters, which all exceeded the limits established by IOC for extra virgin olive oils. Comparing the differences between the two storage methods, the increase of these parameters was more accentuated for the oil extracted from dried fruits (T2) compared to the oil stored for two years (T1). In both cases (T1 and T2), the adopted

storage methods led to the classification of the olive oil samples in the category of "lampante oils".
The results obtained for free acidity agree with a previous study (JABEUR et *al*., 2015), which confirmed the increase of this parameter in olive oil over time. A similar result was reported by (MÉNDEZ and FALQUÉ, 2007) after 3 and 6 months of storage. Moreover, the acidity value was also related with the storage temperature and the percentage of damaged olives (NABIL et *al*., 2012). The increase of oil acidity during storage of oils obtained from stored olives is likely the result of fungal lipase activity (KIRITSAKIS et *al*., 1998).
Regarding the PV, in the present study the olive oil quality was affected by an increase of this parameter after both storage conditions. These results are in accordance with the study performed by MÉNDEZ and FALQUÉ (2007) during 3 and 6 months that showed an increase of PV in olive oil during storage. As reported by SERVILI and GIANFRANCESCO (2002), the PV is basically affected by several factors that damage the fruits (e.g. olive fly attacks or improper systems of harvesting, transport and storage) or the oil (processing technology and oil storage conditions).
Finally, concerning the extinction coefficients, the K_{270} of olive oil increased more slowly than K_{232} over two years of storage. However, the highest increase of K_{232} and K_{270} took place in the oil obtained from stored olives (T2). These results are in accordance with those of (GUTIERREZ et *al*., 1992; VICHI et *al*., 2009) which suggested that K_{232} and K_{270} progressively increased during olive storage. The increase in the extinction coefficient values during storage could be due to the presence of conjugated dienes. As reported by FRANKEL (1993), it is important to consider that the storage temperature can affect the formation of certain volatile compounds resulting in the formation and degradation of different hydroperoxydes formed by oxidation processes. In general, an increase in these parameters can affect the conservation of EVOOs, as well as their nutritive and organoleptic characteristics.

Table 1. Quality indices (free acidity, peroxide value, K_{232} and K_{270}) of *Zarazi* olive oil not stored (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

Quality indices	TC	T1	T2
FA (% oleic acid)	0.77 ± 0.01^{c}	1.68 ± 0.04^{b}	2.40 ± 0.03^{a}
PV (meq O_2 kg oil^{-1})	10.1 ± 0.1^{c}	21.4 ± 0.5^{b}	23.5 ± 1.7^{a}
K_{232}	1.85 ± 0.34^{c}	2.89 ± 0.48^{b}	3.80 ± 0.52^{a}
K_{270}	0.05 ± 0.01^{c}	0.2 ± 0.01^{b}	0.43 ± 0.05^{a}

FA: free acidity; PV: peroxide value; K_{232} and K_{270}: spectrophotometric indices.
Each value represents the mean of three determinations (n = 3) ± standard deviation.
[a-c]Different letters in the same row indicate significantly different values ($p < 0.05$) according to Duncan test.

3.2. Total phenols and *o*-diphenols as influenced by storage methods

Total phenols and *o*-diphenols were quantified in samples and the results are shown in Fig. 1. Statistical analysis showed a significant decrease in both total phenol and *o*-diphenol contents compared to the control sample under the effect of T1 and T2 ($p < 0.001$). Concerning the *o*-diphenol concentration, it also dramatically decreased from 506.8 mg kg^{-1} (sample TC) to 82.0 mg kg^{-1} in T1 and to 80.1 mg kg^{-1} in T2 (Fig.1 B). No significant differences were found between the two storage methods.

The decrease of total phenols with storage time is in accordance with other studies showing that, during storage, phenols undergo qualitative and quantitative variations due to decomposition and oxidation reactions (DABBOU et *al*., 2011; GARGOURI et *al*., 2015). The decrease of the total phenol content in all samples during storage is due to hydrolysis and oxidation processes (SICARI et *al*., 2010). The losses of total phenols could be also be the result of an increase in the oxidative state of fruit. In fact, the oxidation of polyphenols in olive fruits is the result of enhanced activity of oxidative enzymes such as polyphenoloxidase and peroxidase, which contribute to the impairment of health-related qualities and sensory characteristics of olive oil (CLODOVEO et *al*., 2007; SERVILI et *al*., 2003).

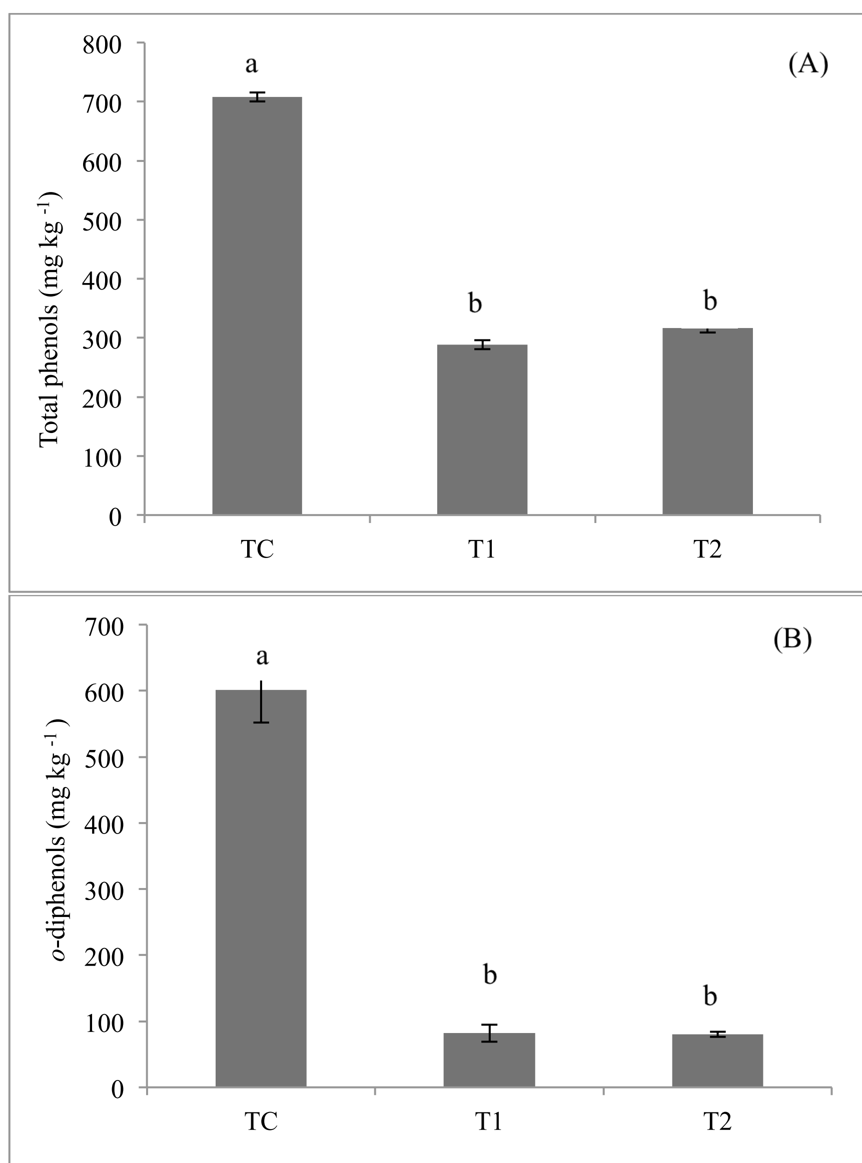

Figure 1. Total phenol (A) and *o*-diphenol (B) content in control olive oil (TC), in olive oil stored for two years (T1) and in olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2). Results are shown as mean ± SD (n = 3).

[a-b]Different letters indicate significantly differences at $p < 0.05$ according to Duncan's test.

3.3. Modification of phenolic profiles during different methods of storage

The analysis of phenolic profiles allowed the separation and identification of several phenolic compounds (Table 2). In all olive oil samples, secoiridoid derivatives were the most abundant. The major secoiridoids detected were oleuropein aglycone (3,4-DHPEA-EA) and its decarboxymethylated derivative (3,4-DHPEA-EDA). The results presented in Table 2 showed significant differences in the concentration of a wide number of phenolic compounds among samples. In general, in both the methods tested, it is possible to observe a significant decrease of all phenolic compounds compared to control samples (TC). In fact, 3,4-DHPEA-EA decreased significantly ($p<0.001$) by 69.87% in T1 and by 58.81% in T2, compared to TC. Another compound (3,4-DHPEA-EDA) also decreased significantly ($p<0.001$) by 84.34% in T1 and 68.40% in T2 compared to TC.
For the other phenolic compounds, T1 had the lowest content of tyrosol (*p*-HPEA), decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA) and luteolin, while T2 oil samples contained the lowest concentration of ligstroside aglycon (*p*-HPEA-EA) and acetoxypinoresinol. On the other hand, hydroxytyrosol (3,4-DHPEA) was not detected in T1 oil, while its concentration was 19.8 mg kg^{-1} and 0.9 mg kg^{-1}, respectively in samples TC and T2.

Table 2. Phenolic compounds (mg of tyrosol kg^{-1} oil) identified and quantified by HPLC-DAD-MS in the control olive oil (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

Phenolic compounds	TC	T1	T2
3,4-DHPEA	19.8±1.0^{a}	ND	0.9±1.5^{b}
p-HPEA	14.9±0.7^{a}	3.4±0.3^{c}	7.8±0.3^{b}
p-HPEA-EA	6.8±0.3^{ab}	8.4±1.4^{a}	5.6±0.7^{b}
3,4-DHPEA-EA	35.3±1.6^{a}	10.6±1.5^{c}	14.5±0.8^{b}
p-HPEA-EDA	15.9±0.9^{b}	9.1±0.5^{c}	19.5±0.4^{a}
3,4-DHPEA-EDA	33.2±1.7^{a}	5.2±1.1^{c}	10.5±0.4^{b}
Acetoxypinoresinol	26.5±1.7^{a}	11.6±0.8^{b}	10.0±0.2^{b}
Luteolin	12.2±0.9 ^{a}	3.2±0.1^{c}	5.3±0.5^{b}

3.4-DHPEA: Hydroxytyrosol; *p*-HPEA: Tyrosol; *p*-HPEA-EA: Ligstroside aglycon; 3.4-DHPEA-EA: Oleuropein aglycon; *p*-HPEA-EDA: Dialdehydic form of elenolic acid linked to tyrosol; 3.4-DHPEA-EDA: Dialdehydic form of elenolic acid linked to hydroxytyrosol; ND: not detected.
Results as expressed as means ± SD (n = 3).
[a-c] Different letters in the same row show statistically significant differences ($p < 0.05$) according to Duncan test.

Given the importance of the phenolic fraction, with regards to antioxidant activities (BENDINI et *al*., 2006), sensory properties and health benefits (BENDINI et *al*., 2007) of olive oil, the content of phenolic compounds could be an important quality control parameter of EVOO. The trends of simple phenols, secoiridoids, lignans and flavones during storage are related to the stability and nature of their molecular structure. Indeed, these compounds may undergo alterations due mainly to hydrolysis, oxidation and increase of decarboxymethylated derivatives during storage of EVOO in the industry or after sales (LERMA-GARCIA et *al*., 2009). The reduced contents of different phenolic compounds observed in this study could be related to hydrolytic processes that may occur in parallel with oxidation, which is in concordance with previous studies (PAGLIARINI et *al*., 2000) that suggested aglycone esters undergo hydrolysis during long term storage.

Several studies have focused on possible hydrolytic and oxidative degradation forms of phenolic compounds present in EVOO during storage (LOZANO-SÁNCHEZ et *al*., 2013). In addition, secoiridoids, 3,4-DHPEA-EDA and 3,4- DHPEA-EA, were the most affected phenolic compounds during the storage period. These results are in accordance with those of other authors (HACHICHA et *al*., 2015).
The results of this study show that under oil storage conditions, almost all phenolic compounds (except *p*-HPEA-EA and acetoxypinoresinol) were lost more in samples of stored oil (T1) than in those produced after fruit storage conditions (T2). It is not easy to provide an explanation for this phenomenon, but it is possible that when conserved as fruits, exposure of the oil to external environmental factors is avoided, which may cause alteration of phenolic profiles, such as availability of oxygen, presence of light and temperature (MÉNDEZ and FALQUÉ, 2007; PRISTOURI et *al*., 2010).

3.4. Modification of the concentrations of tocopherol isomers as an effect of storage methods

As reported in Table 3, α-tocopherol was the most predominant isomer in olive oil samples and its content was 180.4 mg kg^{-1} in the control olive oil (TC), while the β and γ isomers were present at relatively minor concentrations (6.0 mg kg^{-1} and 17.7 mg kg^{-1}, respectively). Statistical analysis revealed a significant decrease of total tocopherol content as well as the individual concentrations of each isomer ($p<0.001$) as an effect of the two storage methods. Comparing the latter two methods, we observed that T1 oil samples had the lowest content of total tocopherols compared to TC and T2, especially because of its significantly lower content of α-tocopherol.
Tocopherols play an important role as antioxidants in the oxidative stability of olive oil by helping to maintain its shelf-life and in preserving oils from rancidity by interrupting the chain reactions involved in the formation of hydroperoxydes (MORELLÓ et *al*., 2004). Our study showed that the loss of α-tocopherol content was about 50% in T1, which confirms the data by KRICHENE et *al*., (2010) who observed a strong reduction in α-tocopherol during storage. As described for most phenolic compounds in paragraph 4.3, α-tocopherol also decreased more in samples of stored oils (T1) than in those obtained from olives stored for two years (T2). However, for both storage methods, it appears that polyphenols and *o*-diphenols were much more sensitive to storage time than tocopherols. This is definitely attributable to the fact that polyphenols, particularly *o*-diphenols, are the preferred substrates for oxidation (BLEKAS et *al*., 1995).

Table 3. Tocopherols isomer contents (mg α-tocopherol kg^{-1}) in the control olive oil (TC), in olive oil stored for two years (T1) and in olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

	Tocopherols (mg kg^{-1})			
	α-tocopherol	**β-tocopherol**	**γ-tocopherol**	**Total tocopherols**
TC	180.4 ± 2.8^{a}	6.0 ± 0.4^{a}	17.7 ± 0.6^{a}	204.2 ± 3.0^{a}
T1	109.0 ± 6.8^{c}	5.3 ± 0.6^{ab}	13.1 ± 0.9^{b}	127.5 ± 8.2^{c}
T2	140.4 ± 2.4^{b}	5.1 ± 0.1^{b}	13.3 ± 0.2^{b}	158.9 ± 2.2^{b}

[a-c] Different letters in the same column indicate significantly different values ($p < 0.05$) according to Duncan test.
Results as expressed as means ± SD (n = 3).

3.5. Effect of the two storage methods on oxidative stability

Oxidative stability was studied by the Rancimat test (Fig. 2). The stability (OS) decreased significantly from 8.47 h (TC) to 1.50 h in T1 and to 2.77 h in T2, reaching a significant lower value in of the latter.
As reported by some studies in the literature (GARCÍA et *al*., 1996; GUTIERREZ et *al*., 1992), oil stability is clearly affected by storage time and conditions. This is in accordance with our results: OSI time decreased more in the stored oil (T1) than in the that obtained from olives stored for two years (T2). This is a quite interesting finding, since it confirms that the traditional storage method of the olives had a lesser negative effect on OSI time than the storage of the oil.

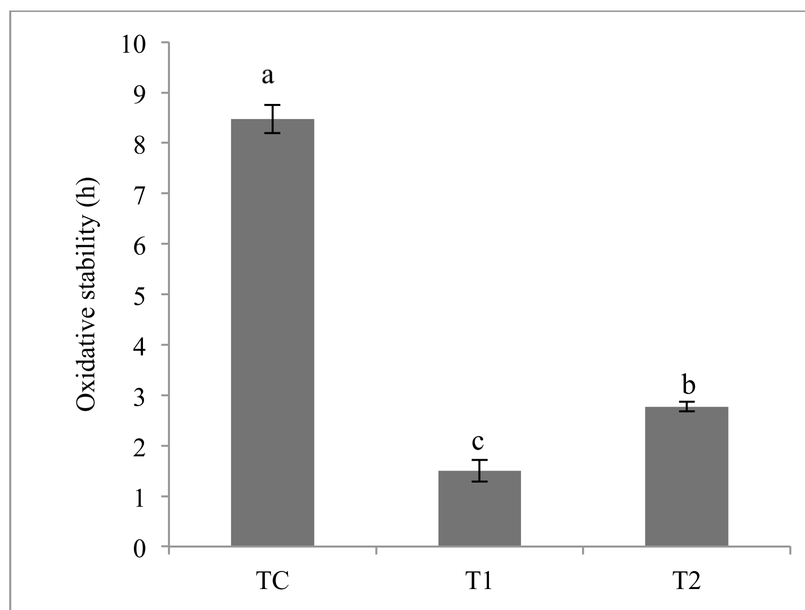

Fig. 2 - Variation of oxidative stability of control olive oil (TC), olive oil stored for two years (T1) and olive oil obtained from intact sun-dried olive fruits stored for two years before being processed (T2). Results are shown as mean ± SD (n = 3).
[a-c] Different letters indicate significantly differences at $p < 0.05$ according to Duncan's test.

3.6. Changes in fatty acid alkyl esters in olive oil samples following the two storage methods

The experimental results of the amounts of FAAEs, FAMEs, FAEEs and FAEEs/FAMEs in oil extracted directly after harvesting (from fresh olives) and in oils stored after two years using the two different methods are summarized in Table 4. For EVOO, the concentration of the FAEEs must be ≤35 mg kg^{-1} (IOC, 2015).
The major fatty acid alkyl esters (FAAEs) present in olive oil are those corresponding to palmitic, oleic and linoleic acids. Indeed, the amount of FAEEs in sample TC did not exceed the quantity of 35 mg kg^{-1}, thus indicating the classification of this olive oil as extra virgin.
Compared with the control oil, the content of FAEEs showed significantly higher values in samples T1 and T2 (p <0.001) with 624.2 mg kg^{-1} and 62.6 mg kg^{-1}, respectively. The two types of storage (T1 and T2) both negatively affected the quality of the oil. This leads to their classification as non-extra virgin olive oils.

Fatty acid alkyl esters (FAAEs), mainly ethyl (FAEEs) and methyl esters (FAMEs), are formed by esterification of free fatty acids (FFAs) with low molecular alcohols, such as methanol and ethanol. In good quality extra virgin olive oils, FAMEs and FAEEs are present in very small amounts (VALLI et *al*., 2013), while they are present in higher amounts in "lampante" olive oils, as confirmed in the current study. The high content of FAAEs in samples T1 and T2 can be due to the presence of specific components, such as free fatty acids (FFAs) and low chain alcohols. These compounds are, respectively, originated through substantial liberation of FFAs from triglycerides by lipolysis and by a parallel consistent formation of ethanol due to the aerobic metabolism of microorganisms and methanol due to the degradation of the pectins linked to the action of endogenous pectinmethyl-esterases (BIEDERMANN et *al*., 2008).
JABEUR et *al*., (2015) found that in lampante oils the amounts of ethyl esters were greater than those of methyl esters: in our study, this was only observed for sample T1. The results also highlighted that the change in the content of FAAEs, and particularly FAEE, that occurred in the stored oil (T1) was significantly higher than that in oils extracted from olives stored for two years (T2). In fact, (CONTE et *al*., 2014; GÓMEZ-COCA et *al*., 2016) reported that the FAEE concentration is a quality parameter that reflects fruit quality at the moment of extraction, and the presence of ethyl esters are markers of fermentation (low quality of olive fruits and extracted oil). It could be assumed that sunlight drying of olives and subsequent long storage could lead to a higher liberation of methanol (from degradation of pectins) respect to a fermentative process, explaining the highest content of FAMEs compared to FAEEs for T2. On the other hand, the high content of FAEEs in sample T1 could be explained by the low quality of olives before processing, as shown by the remarkable content of FAAEs as well as a free acidity value close to the legal limit in sample TC.

Table 4. Fatty acid alkyl esters (mg i.s. kg^{-1}) of olive oil not stored (TC), stored for two years (T1) and obtained from intact sun-dried olive fruits stored for two years before being processed (T2).

	TC	T1	T2
Σ FAMEs	33.7±0.2^{c}	185.2±9.8a	172.4±31.9^{b}
Σ FAEEs	23.6±3.6^{c}	624.2±15.5^{a}	62.6±8.7^{b}
FAEEs/FAMEs	0.7±0.1^{b}	3.4±0.2 ^{a}	0.4±0.1^{c}
Total FAAEs	53.5±8.1^{c}	809.5±20.5 ^{a}	235.0±33.0^{b}

FAMEs: fatty acid methyl esters; FAEEs: fatty acid ethyl esters; FAAEs: fatty acid alkyl esters.
Each value represents the mean of the three determination (n = 3) ± standard deviation.
$^{a-c}$Different letters show statistically significant differences ($p < 0.05$) according to Duncan test.

4. CONCLUSIONS

Overall, from this study it can be concluded that both the storage methods herein investigated had a strong negative influence on the quality of the olive oils. This is a crucial information to be communicated to the local olive oil producers residents in Gabes (Southern Tunisia), in order to make them aware about the strong influence on the quality of the product. In particular, it should be underlined that the basic quality parameters of the stored oils exceeded the limits established by the IOC trade standard for virgin olive oils, thus declassifying the oil as "lampante". A significant degradation (p<0.05) in phenolic compounds also occurred, being more accentuated in the oil conserved for two

years than in that obtained from stored and dried olive fruits. A similar trend was also seen in α-tocopherol content and in oxidative stability time (OSI) of the samples. The high FAAEs level observed in both the two storage conditions suggested that the oils were obtained from olives of suboptimal quality, wherein fermentation and other types of degradations (oxidation) have occurred.
However, the results of the current study highlight that some traditional methods, still applied in Gabes for storage of olives and oil, exerted a notable negative effect on all parameters investigated.

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PAPER

HUMAN HEALTH RISK ASSESSMENT OF ORGANOCHLORINE COMPOUNDS ASSOCIATED WITH RAW MILK CONSUMPTION IN A ROMANIAN INDUSTRIAL AREA

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ABSTRACT

Dietary exposure to organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs), generically named organochlorine compounds (OCCs), now represents a significant health risk for humans due to their endocrine-disrupting and carcinogenic effects. To assess the potential health risk associated with raw milk consumption, 10 milk samples were collected from a local market in the Baia-Mare industrial area, Romania. The concentrations of OCPs and PCB congeners in these samples were determined by capillary gas chromatography with electron-capture detection, after liquid–liquid extraction. In all samples, the OCCs were below the maximum admitted concentrations set by European legislation. The predominant compounds were 4,4′-DDE (11.5 ng/g lipid wt.), β-HCH (10.1 ng/g lipid wt.) and PCB180 (5.08 ng/g lipid wt.). Exposure assessment through milk consumption was performed for male, female and children by calculating the estimated daily intakes (EDIs) and the hazard indices (HIs) for non-carcinogenic effects. The obtained EDIs were lower than the acceptable daily intake values, and HIs were far below 1, indicating no potential health risk for the investigated population.

Keywords: health risk assessment, OCPs, PCBs, raw milk

1. INTRODUCTION

Foods of animal origin play an essential role in human nutrition. Milk and dairy products are a source of micro- and macro-elements and active compounds that play an important role in nutrition and health (CADAR *et al.*, 2015). The microbial and chemical contamination of raw milk during animal feed production, dairy processing or packaging is of great concern for public health, especially for products purchased from local producers.

The primary chemical contaminants in milk and dairy products are antibiotics, anthelmintic drugs, hormones, pesticides, heavy metals, mycotoxins, nitrites, etc. (KHANIKI, 2007; SELIM *et al.*, 2015). Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) are persistent in the environment and have the potential to bioaccumulate along the food chain, and this may cause adverse health effects (TSAKIRIS *et al.*, 2015; YU *et al.*, 2011). Most OCPs have endocrine-disrupting effects and can cause hepatotoxicity, immunotoxicity and developmental abnormalities as well as have neurobehavioral effects (MARTINS *et al.*, 2013). Despite their international ban starting from the 1980s, they are still detected in the environment, food, biota and humans (HENRÍQUEZ-HERNÁNDEZ *et al.*, 2017). The International Agency for Research on Cancer (IARC) has classified most of the OCPs as possibly carcinogenic to humans (group 2B) and "dioxin-like" PCB congeners as carcinogenic to humans (group 1) (KLINCIC *et al.*, 2016).

Bioaccumulation of OCCs through the food chain makes foods of animal origin the main source of human exposure to these contaminants (CASPERSEN *et al.*, 2016). The exposure of human organisms to environmental contaminants, through food, cannot be avoided, and this can lead to acute (short-term exposure) or chronic (long-term exposure) effects. The human risk assessment can be based on deterministic approaches by comparing it to a threshold toxicity value (CERNA *et al.*, 2016). Following the banning of OCCs, the concentrations of OCCs in foodstuff decreased significantly (LI, 1999). There are several papers presenting the level of pesticides in milk and dairy products (SANTOS *et al.*, 2015; SANTOS *et al.*, 2006; LUZARDO *et al.*, 2012; AVANCINI *et al.*, 2013; DETI *et al.*, 2014) all over the world. Also, there are papers on the human health risks associated with various pesticides in foodstuff (ZHANG *et al.*, 2017; CUI *et al.*, 2015; LEI *et al.*, 2015; ZHAO *et al.*, 2014). However, there is only limited information on health risk from pesticides in milk and dairy products (BEDI *et al.*, 2015; WITZAK *et al.*, 2016).

The aim of this study was to establish the level of 19 OCPs and 7 PCBs in raw milk collected from a local market in Baia Mare city, North Western Romania, and to assess the risk associated with raw milk consumption for the local children and adults according to gender (male, female).

2.MATERIALS AND METHODS

2.1. Sampling

A number of 10 raw cow milk samples were purchased from a local market in Baia Mare city, NW Romania, during 2016. The cow milk is widely consumed by the local inhabitants. Samples were collected in chemical-free glass bottles with Teflon seals and frozen at -20°C until chemical analysis according to the procedure described by HECK *et al.* (2007).

2.2. Reagents, standard solution and CRMs

The used solvents (acetonitrile, dichloromethane, ethanol and n-hexane) were of gas chromatography grade (Merck, Germany). Anhydrous sodium sulphate, and Florisil were used after heating overnight at 120°C. Mix standard solution (EN ISO 6468 CERTAN, NE7550) for OCPs and PCBs was purchased from LGC Standards (Germany). The working standard solutions were prepared by diluting accurate volumes of Mix standard solution in dichloromethane. Milk powder certified reference materials (CRMs) purchased from LGC Standards (Germany) - BCR-188 and BCR-450 - were used for the quality control of the results.

2.3. Sample preparation

The extraction and clean-up of milk samples were carried out according to the method described by ENNACEUR *et al.* (2007). The milk samples were thawed at room temperature and homogenized. 20 ml sample was extracted 3 times with a 20/5/1 mixture (v/v/v) of n-hexane/acetonitrile/ethanol. The hexane layers were filtered over anhydrous sodium sulphate, and evaporated to 5 ml. Afterwards, 1 ml was pipetted in a pre-weighed flask and evaporated to dryness. The difference between the final and the initial weight of the empty flask was used to calculate the lipid content of the sample. 4 ml of extract was purified with Florisil and anhydrous sodium sulphate in a chromatographic mini-column. The extract eluted with a 27/3 elution mixture (v/v) of dichloromethane/n-hexane and evaporated to 1 ml, using a EVA-EC1-S sample concentrator (VLM, Germany)

2.4. Sample analysis

In this study the following compounds were determined: α-, β-, γ-, δ-, ε-isomers of hexachlorocyclohexane (expressed as HCHs), 1,1,1-trichloro-2,2-bis-(chlorophenyl)ethane (DDT), 1,1-dichloro-2,2,-bis-(chlorophenyl)ethane (DDD), and dichlorodiphenyl-chloroethylene (DDE), each with their isomers 4,4′- and 2,4′-, expressed as DDTs; aldrin, dieldrin, heptachlor, heptachlor epoxide isomer A, heptachlor epoxide isomer B, *α*-endosulfan, β-endosulfan, hexachlorobenzene (HCB) and PCB congeners: tri (28), tetra (52), penta (101), hexa (138, 153), hepta (180) and octa (194). In order to separate, detect and quantify the OCCs, an Agilent Technologies 6890N gas chromatography equipped with a 63Ni *μ*-electron-capture detector (GC-ECD) and an Agilent J&W, DB-1 capillary column (30 m L × 0.32 mm i.d. with film thickness 3.0 μm) were used. Subsequent to extraction and evaporation, 1 μl of purified extract was injected in splitless mode, at 280°C. The GC oven temperature program consists of 4 stages: from 80°C to 196°C (rate 4°C/min, 2 min), from 196°C to 224°C (rate 4°C/min, 2 min), from 224°C to 240°C (rate 4°C/min, 2 min) and from 240°C to 275°C (rate 4°C/min, 2 min). OCCs were identified by comparison of each relative retention time with the calibration standards. Confirmation of compounds was performed using an Agilent Technologies 6890N coupled with 5975B Agilent Technologies quadrupole GC-MS system. For quantification, multi-level calibration curves were created using standard solutions, and good correlations (r^2=0.995) were achieved.

2.5. Human health risk assessment

For estimating long-term exposure through food intake, the average consumption over a period of time and the ratio between the average consumption and the reference values of

the contaminant amount (μg/kg body weight) that can be consumed daily over a lifetime without appreciable health risks were considered (LEMOS *et al.*, 2016). The exposure to OCCs through food ingestion was evaluated by estimating the dietary exposure, by calculating the estimated daily intake (EDI) from the amount of analyte found in milk and the daily milk consumption by population. EDI is expressed in μg contaminant/person/day. The risk assessment was evaluated by calculation of hazard indices (HIs), expressed as the ratio between exposure and reference dose. If HI<1, the daily exposure does not have potentially adverse effects on the consumers' health over the lifetime (LEMOS *et al.*, 2016; LI *et al.*, 2015).

3. RESULTS AND DISCUSSIONS

3.1. Organochlorine pesticides in milk samples

The obtained results for both CRMs were in agreement with the certified values. OCPs, 4,4'-DDE and 4,4'-DDT metabolites were found in all investigated milk samples. The most frequently found compounds were 4,4'-DDE (11.5 ng/g lipid wt.), β-HCH (10.1 ng/g lipid wt.) and PCB180 (5.08 ng/g lipid wt.). The concentration range, average and standard deviation along the incidence of OCPs in the investigated milk samples are shown in Table 1.

For statistical purposes, the concentrations below the quantification limit (LQ<0.05 ng/g lipid wt.) were considered equal to 0.5LQ (LE FAOUDER *et al.*, 2007). To compare the found OCP concentrations with the legislative threshold values, the maximum admitted concentrations (MACs) expressed in μg/kg (Order 23, 2007) were converted to ng/g lipid wt., considering a mean lipid wt. of 4% in milk (GEBREMICHAEL *et al.*, 2013). Both the individual and the sum of OCP concentrations were below MACs, according to Romanian legislation (Order 23, 2007). The graphical representation of the sum of HCH, sum of cyclodienes, sum of endosulfans and sum of chloro-diphenyl aliphatic compounds concentrations is shown in Fig. 1.

HCB was detected in 80% of samples, with a maximum value of 4.32 ng/g lipid wt., significantly lower than MAC. Endosulfan (α and/or β isomer) was detected in 90% of investigated samples, in relatively low concentrations. The average concentration of α-endosulfan was lower than β-endosulfan β isomer, in accordance with the higher predilection of β isomer compared to α isomer (TSIPLAKOU *et al.*, 2010). HCH compounds were detected in all the analysed samples. The average concentrations of HCH isomers varied in the following order: β-HCH>γ-HCH>α-HCH>δ-HCH>ε-HCH. The total concentrations of HCHs varied between 3.64 and 37.8 ng/g lipid wt., with average and standard deviation values of 6.53 and 3.55 ng/g lipid wt., respectively.

Compounds from cyclodiene group were detected in all milk samples, except one, but the obtained values were low. The average values ranged in the following order: dieldrin>heptachlor>heptachlor epoxide β>aldrin>heptachlor epoxide α. The concentrations of aldrin and dieldrin were much lower than their corresponding MACs.

Chloro-diphenyl aliphatic compounds were determined in all analysed samples, the highest contents of total DDTs were recorded in samples 8 (29.3 ng/g lipid wt.), 9 (18.4 ng/g lipid wt.) and 1 (17.8 ng/g lipid wt.), and the predominant component was 4,4'-DDE. The lowest value was reported in sample 5 (3.26 ng/g lipid wt.).

Table 1. Range, average and standard deviation values of OCPs in milk samples.

Compound	Range (ng/g lipid wt.)	Average (ng/g lipid wt.)	Standard deviation (ng/g lipid wt.)	MAC (µg/kg / ng/g lipid wt.)	Samples > MAC (%)	Incidence (%)
HCB	<0.05-4.32	2.18	1.61	10*/250	0	80
Hexachlorcyclohexanes (HCH)						
α-HCH	<0.05-6.49	1.65	2.01	4*/100	0	90
β-HCH	<0.05-17.4	10.14	5.21	3*/75	0	90
γ-HCH (Lindane)	<0.05-7.16	2.92	2.81	8*/200	0	90
δ-HCH	0.25-4.34	1.70	1.37	-	-	90
ε-HCH	<0.05-2.41	0.68	0.78	-	-	80
ΣHCHs	3.64-37.8	17.1	10.3	-	-	
Cyclodienes						
Aldrin	<0.05-1.71	0.63	0.62	6*/150	0	70
Dieldrin	<0.05-6.49	2.45	2.04	6*/150	0	80
Heptachlor	<0.05-6.25	2.30	1.93	-	-	90
Heptachlor epoxide β	<0.05-2.03	0.94	0.66	-	-	90
Heptachlor epoxide α	<0.05-0.69	0.22	0.23	-	-	80
Endosulfans						
β-endosulfan	<0.05-1.82	0.57	0.44	-	-	80
α-endosulfan	<0.05-1.27	0.42	0.47	-	-	70
ΣEndosulfan	<0.10-2.63	0.98	0.69	4*/100**	0	
Chloro-diphenyl aliphatic compounds						
2,4'-DDE	<0.05-0.13	0.07	0.04	-	-	70
4,4'-DDE	2.93-28.4	11.52	8.15	-	-	100
2,4'-DDD	<0.05-0.48	0.12	0.15	-	-	70
4,4'-DDD	<0.05-0.44	0.13	0.14	-	-	70
2,4'-DDT	<0.05-0.24	0.12	0.08	-	-	80
4,4'-DDT	0.05-3.67	0.58	1.17	-	-	100
ΣDDTs***	3.21-28.9	12.3	7.96	40*/1000	0	
ΣOCPs	12.7-68.1	37.1	16.6	-	-	

*according to Romanian legislation (Order 23, 2007).
**according to Codex Alimentarius (FAO/WHO, 2006).
***Σ(4,4'-DDE+4,4'-DDD+2,4'-DDT+4,4'-DDT).

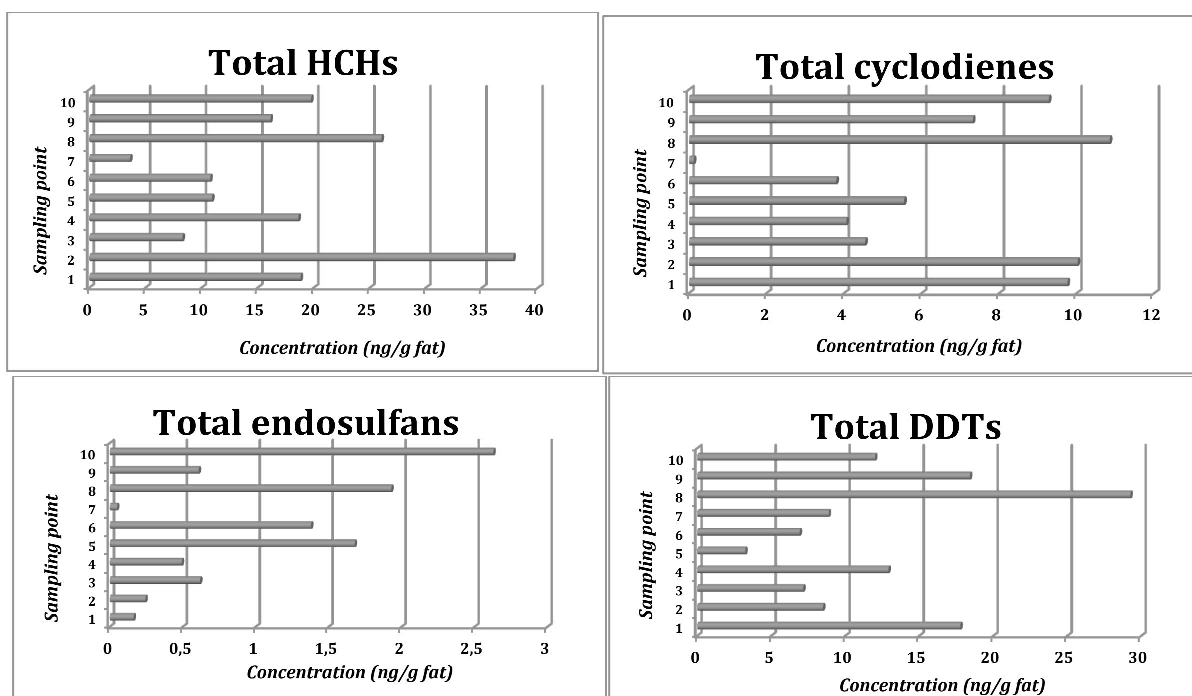

Figure 1. Concentrations of total HCHs, cyclodienes, endosulfans and total DDT (ng/g lipid wt.) in milk samples.

The obtained values for OCPs in milk samples were comparable with those reported by HECK *et al.* (2007) in milk samples consumed in Rio Grande do Sul, Brazil, for HCB, α-HCH, 4,4'-DDE and lower for lindan, aldrin, 2,4'-DDD and 2,4'-DDT. Also, the average values obtained for ΣDDT were comparable with the average values reported in Yugoslavia and Canada and lower than in Egypt, Mexico, Ethiopia, Ghana, India, Tunisia and Iran (GEBREMICHAEL *et al.*, 2013). The obtained average values were comparable with those reported by MICLEAN *et al.* (2011) in milk samples collected in Cluj-Napoca area (Romania), except for β-HCH and β-endosulfan, which were lower, while dieldrin, heptachlor, 4,4'-DDD and 4,4'-DDT were higher.

3.2. Polychlorinated biphenyls in milk samples

PCBs were detected in all samples. The range, average and standard deviation values of PCB concentrations in milk samples are shown in Table 2. The highest contribution to the total PCB content is represented by congener PCB180.

The total concentrations of PCBs (expressed as the sum of the seven congeners: PCB 28, 52, 101, 138, 153, 180 and 194) varied between <0.60 ng/g lipid wt. (sample 7) and 15.3 ng/g lipid wt. (sample 4), with average and standard deviation of 9.12 ng/g lipid wt. and 4.99 ng/g lipid wt., respectively.

In the case of non-dioxin like PCBs, the European Commission set the MAC to 100 ng/g lipid wt. from milk for the sum of the seven PCB congeners (PCB 28, 52, 101, 138, 153, 180, 194) (EFSA, 2005; EC, 2006a,b) and recently set the MAC as 40 ng/g lipid wt. from milk for the sum of six congeners (PCB 28, 52, 101, 138, 153 and 180) (EC, 2011) (PÉREZ *et al.*, 2012). In the analysed milk samples, the MAC was not exceeded.

The contribution of the PCB congeners to the total PCB content in analysed milk samples is shown in Fig. 2.

Table 2. Range, average and standard deviation values of PCBs in milk samples

Compound	Range (ng/g lipid wt.)	Average (ng/g lipid wt.)	Standard deviation (ng/g lipid wt.)	Incidence (%)
PCB28	<0.05-0.44	0.18	0.17	70
PCB52	<0.05-0.23	0.09	0.07	80
PCB101	<0.05-3.71	0.89	1.42	50
PCB138	<0.05-2.26	0.54	0.75	80
PCB153	<0.05-5.37	1.09	1.88	40
PCB180	<0.05-10.4	5.08	3.30	80
PCB194	<0.05-5.32	1.25	2.11	30
ΣPCB*	<0.60-15.3	9.12	4.99	

*ΣPCB – sum of PCB congeners (PCB 28, 52, 101, 138, 153, 180, 194).

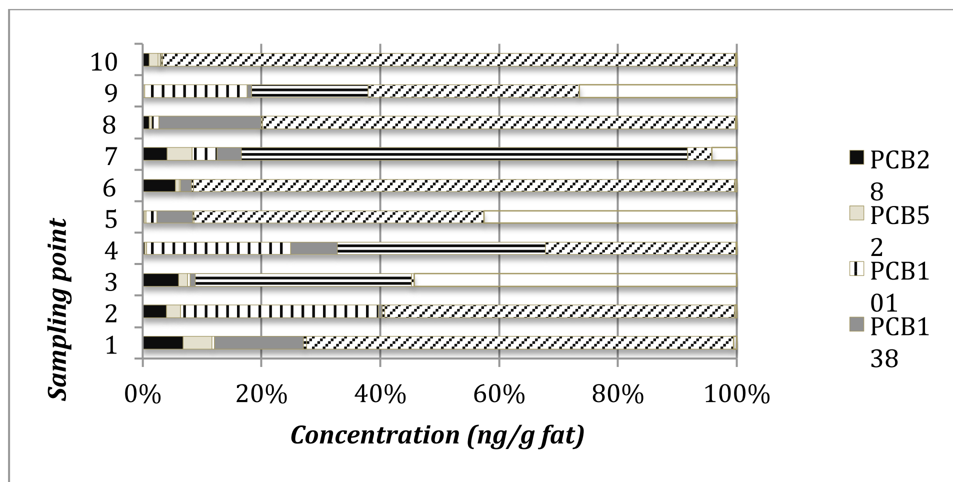

Figure 2. PCB congeners contribution of total PCB content in milk samples.

3.3. Estimated daily intake

In this study, the long-term exposure was assessed by determination of the average intake of OCPs and PCBs through milk consumption for the three population groups - male, female and children - and their comparison with the reference values, namely acceptable daily intake (ADI), set by the Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 2006).

The daily intake of milk for the three investigated population groups was determined based on milk consumption frequency questionnaires. These questionnaires were filled out by 75 individuals - 55 adults (18-75 years old), among them 28 male, 27 female and 20 children (6-10 years old) - and indicated an average milk daily intake of 300 g milk/person/day for male, 200 g milk/person/day for female and 500 g milk/person/day for children and the average body weight - 75 kg for male, 65 kg for female and 30 kg for children.

The values obtained for the estimated daily intake (EDI) associated with milk consumption are shown in Table 3. In calculations, the pesticides concentrations lower than LQ were considered zero (LUZARDO *et al*., 2012).

Table 3. Average estimated daily intake (EDI) and acceptable daily intake (ADI) of organochlorine compounds (μg/kg bw*/day).

Compound	EDI, μg/kg bw/day			ADI, μg/kg bw/day***
	Female	Male	Children	
Hexachlorbenzene	0.00033	0.00043	0.00181	-
Hexachlorcyclohexanes (HCH)				
α-HCH	0.00034	0.00044	0.00185	-
β-HCH	0.00138	0.00180	0.00748	-
γ-HCH (Lindane)	0.00039	0.00051	0.00213	5
δ-HCH	0.00023	0.00030	0.00124	-
ε-HCH	0.00010	0.00013	0.00056	-
ΣHCH	0.00223	0.00255	0.01207	0.3
Cyclodienes				
Aldrin	0.00011	0.00014	0.00060	0.1
Dieldrin	0.00037	0.00048	0.00199	0.1
Heptachlor	0.00032	0.00041	0.00171	-
Heptachlor epoxide β	0.00013	0.00017	0.00071	-
Heptachlor epoxide α	0.00004	0.00005	0.00020	-
ΣHeptachlor	0.00043	0.00056	0.00232	0.1
Endosulfans				
α-endosulfan	0.00009	0.00011	0.00046	-
β-endosulfan	0.00007	0.00009	0.00038	-
ΣEndosulfans	0.00016	0.00020	0.00084	6
Chloro-diphenyl aliphatic compounds				
2,4'-DDE	0.00001	0.00001	0.00006	-
4,4'-DDE	0.00142	0.00184	0.00768	-
2,4'-DDD	0.00002	0.00003	0.00011	-
4,4'-DDD	0.00002	0.00003	0.00011	-
2,4'-DDT	0.00002	0.00002	0.00009	-
4,4'-DDT	0.00008	0.00009	0.00039	-
ΣDDT**	**0.00154**	**0.00200**	**0.00834**	10
ΣOCPs	**0.00495**	**0.00644**	**0.02682**	-
PCB2013				
PCB28	0.00003	0.00004	0.00017	-
PCB52	0.00001	0.00002	0.00008	-
PCB101	0.00022	0.00028	0.00118	-
PCB138	0.00008	0.00010	0.00043	-
PCB153	0.00035	0.00045	0.00187	-
PCB180	0.00077	0.00100	0.00417	-
PCB194	0.00053	0.00069	0.00286	-
ΣOCCs	**0.00607**	**0.00789**	**0.03287**	-

*bw=body weight.
** 2,4'-DDE + 4,4'-DDE + 2,4'-DDD + 4,4'-DDD + 2,4'-DDT + 4,4'-DDT.
*** EFSA, 2013.

The obtained values for the estimated daily intake of investigated organochlorine compounds, through milk consumption, for adults (male and female) and children, in Baia Mare area were far below ADI values set by the European Food Safety Authority (EFSA), according to Table 3 (EFSA, 2013).
For each investigated analyte, the average estimated daily intake varied in the following order: $EDI_{children}$>EDI_{male}>EDI_{female}, according to the daily milk consumption and body weight. For the investigated children, the EDI value calculated for ΣHeptachlor provides 2.32% from ADI value, set for the sum of these cyclodienes.
The values obtained for EDI of total DDT were below the EFSA recommended value (10 μg/kg bw) and were much lower (4 orders of magnitude). The obtained EDI values for total DDT were comparable (the same order of magnitude) with the values reported in Spain, Poland and China and were lower (with an order of magnitude) than those obtained in Ethiopia, Mexico, Egypt and Iran, through cow milk consumption (GEBREMICHAEL *et al.*, 2013).
The EDIs obtained in this study were comparable with those determined by LUZARDO *et al.* (2012) for adult and child consumers of milk in Spain for hexachlorobenzene, dieldrin, heptachlor and higher (but the same order of magnitude in both studies) for α-, β, γ-, δ-HCH, aldrin, endosulfan, 4,4'-DDT. The average EDI values for total OCPs were comparable in both studies for adults and children.

3.4. Risk assessment

In order to assess the non-carcinogenic risk from exposure to milk through consumption of the three investigated population groups, the HIs were calculated by dividing the EDI values to the US EPA Reference Doses (RfDs) (TSAKIRIS *et al.*, 2015). US EPA provides RfD values only for γ-HCH (0.3 μg/kg bw/day) and 4,4'-DDT (0.5 μg/kg bw/day) (US-EPA, 2006). The obtained values for HIs are shown in Table 4.

Table 4. Hazard indices for lindane and 4,4'-DDT.

Compound	Hazard Index		
	Female	Male	Children
γ-HCH (Lindane)	0.0013	0.0017	0.0071
4,4'-DDT	0.00016	0.00018	0.00078

The values of HIs for lindane and 4,4'-DDT were far below 1, indicating no health risk for the investigated population groups through raw milk consumption, in the studied area.

4. CONCLUSIONS

The tendency of organochlorine compounds to accumulate in fatty tissues, their long persistence and high acute health risk raise concerns about their impact on health due to chronic dietary exposure to low concentrations. This study revealed low dietary intake of organochlorine compounds from milk consumption, in females, males and children. The health risk assessment was calculated based on specific values for the investigated area and the intake of organochlorine pesticides. The specific exposure factors were obtained using questionnaires regarding the structure of the diet for three consecutive days. For each investigated analyte, the average estimated dietary exposure varied in order - $EDI_{children}$>EDI_{men}>EDI_{female} - according to the daily consumption of milk. The average daily

intake of organochlorine compounds through milk for the investigated residents exposed to chronic pollution was lower than the reference values, indicating no health risks.

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PAPER

PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF WILD GRAPE (*VITIS TILIIFOLIA*)

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ABSTRACT

Vitis tiliifolia is a tropical grape with a deep purple colour and a high content of pigments. Total polyphenols content in the skin and pulp was 400.35 and 171.26 mg GAE/g dry sample of *Vitis tiliifolia*, respectively, which coincides with DPPH radical scavenging for skin (91.39%) and in the pulp (19.57%). The predominant individual phenolic compounds found in the skin were quercetin-3-glucoside (39.86 μg/g), rutin (37.01 μg/g) and *trans*-resveratrol (32.88 μg/g). The DPPH radical scavenging and reducing power revealed a high antioxidant activity. This study demonstrates that wild grape can thus be utilised as a novel functional resource.

Keywords: *Vitis*, wild grapes, anthocyanins, food composition, polyphenols

1. INTRODUCTION

Consumers have focused increased attention on functional foods, especially those containing antioxidants, which decrease reactive oxygen species (ROS) (MANACH *et al.*, 2005). It is reported that some fruits such as grapes can dramatically increase the balance between the production and manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage is interrupted (STAGOS *et al.*, 2006). At the same time, grape extracts and wine have been recognised to contain polyphenol compounds that have beneficial effects on human health. It is known that grapes are anti-mutagenic, antineoplastic, reduce human low-density lipoprotein (LDL) oxidation and allergic inflammation, decrease cardiovascular diseases (LEKAKIS *et al.*, 2005), exhibit antimicrobial (JAYAPRAKASHA *et al.*, 2001), antihypertensives (SOARES DE MOURA *et al.*, 2002), and antiulcer activities (CUEVAS *et al.*, 2011). On the one hand, anthocyanins are a type of polyphenol, and it is reported that they present strong antioxidant activity, inhibit the growth of cancerous cells and inflammation, and act as vasoprotectors and anti-obesity agents, in addition to having effects on diabetes and cardiovascular disease prevention, as well as the improvement of visual and brain functions (TSUDA, 2012). On the other hand, resveratrol (3,5,4-trihydroxy-*trans*-stilbene) is a natural polyphenolic that acts as a defense mechanism against deleterious microorganisms. These compounds are present in several fruits as grapes, and their manufactured products, especially red wine. Anthocyanins are primarily located in the skin and have pharmacological benefits. It has been known to exert its protective effect against cardiovascular disease, ischemia-reperfusion injury and diabetes mellitus through the modulation of adipocyte/fibroblast biology, platelet activation, blood vessel function, oxidative stress, inflammation, serum glucose maintenance, cardiomyocyte biology, the maintenance of cell structure, and serum lipid activity, cause body fat loss, and confer protection against disease or injury (TSUDA, 2012).
There are many types of grapes that have been widely studied. However, *Vitis tiliifolia* is a wild grape resource that has not yet been adequately recognised by researchers and winemakers. It is a small to very large climbing shrub with thick, woody stems that can be 10-35 m long and up to 20 cm in diameter, which commonly grows in wet to dry forest or thickets, often in pine-oak forest; It grows regularly around 1700 meters above sea level. *Vitis tiliifolia* grows in the southern states of Mexico and the Antilles to Colombia (FERNANDEZ, 2009). In Mexico, it is located in the states of Chiapas, Colima, Guerrero, Hidalgo, Nuevo Leon, Oaxaca, Querétaro, San Luis Potosí, Tabasco and Veracruz, where it is known by different names such as: wild grape, Gunhi, loobabi-chuli, uvilla, xocomecatl, tecamate and others, according to the region and growing area (ARELLANO *et al.*, 2003). Flowers are seen from May to June and fruits are harvested from August to November (IBARRA and SINACA, 1996). The fruits have been used as raw materials for juice and wine (ARELLANO *et al*., 2003). Fresh fruit is commonly used to make vinegar and soft drinks (FERNANDEZ, 2009), while the root and leaves are used empirically against haemorrhoids. Therefore, these products may be useful as a source of potentially functional ingredients providing the opportunity to develop innovative added value products. However, the further application of this wild grape requires the evaluation of their composition and there is little information on the physicochemical and antioxidant properties of *Vitis tiliifolia*. Therefore, the aim of this work was to investigate the physicochemical properties and antioxidant activity in the pulp and skin of *Vitis tiliifolia* fruit to provide sufficient experimental evidence for the antioxidant activity and potential for further development and utilisation of this species.

2. MATERIALS AND METHODS

2.1. Chemicals

2,2′-Diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, *trans*-resveratrol, Folin-Ciocalteu reagent and 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-hydroxybenzoic acid, (+) catechin, vanillin acid, scopolin, chlorogenic acid, caffeic acid, (-) epicatechin, vanillin, 4-coumaric acid, quercetin 3-glucoside, ferulic acid and *trans*-cinnamic acid were purchased from Extrasynthese (Lyon, France). The rest of the standards were bought at Sigma-Aldrich (USA). The solvents used for the extraction was analytical grade and MS grade for the ultra-high performance liquid chromatography (UPLC) procedures and other standards were also purchased from Sigma-Aldrich (USA). All the stock solutions, samples, solvents and reagents were filtered through 0.20 μm PTFE membrane filters (Phenomenex, USA) before separation or injection in the instrument.

2.2. Samples

The samples of *Vitis tiliifolia* were collected at "Cafetal" ranch, located in the Veracruz state, situated at 19° 37' 0.4 " north latitude and 96° 50' 2.7" west longitude, at an elevation of 734 meters above mean sea level. Ten kg of grapes were harvested during the month of August of the years 2015 and 2016 with the optimum stage of maturity and with a concentration of soluble solids between 12 and 14 °brix. Samples were washed, drained and subsequently, skins, seeds and pulp were directly obtained by manual separation. One part of the samples was frozen at -40°C for the analysis of the composition and physicochemical properties of the pulp and skin and the other part was subjected to lyophilization for the preparation of the extracts.

2.3. Determination of some basic physicochemical parameters

Total nitrogen was determined by the micro-Kjeldahl method and protein was calculated as nitrogen·6.25. Oil was extracted for 24 h with diethyl ether in a Soxhlet system. Ash was determined by incineration in a furnace at 550°C and weight, moisture, titratable acids, reducing sugars and total dietary fibre and pH were determined following the AOAC (2000) methods. The water activity was measured at 25ºC using Aqualab 4 TE (Decagon 142 Devices, Pullman, WA, USA) and °Brix were measured with a hand refractometer. The colour was measured with a colorimeter (ColorFlex V1-72 SNHCX 1115 s/n: Cx1115 Hunter Lab, USA) using parameters a° (yellow-red), b° (blue-green) and L° (intensity and brilliance) on the scale of the system CIE Lab (International Commission on Illumination, Vienna). Browning index was determined according to the method reported by BUERA *et al.* (1986). Equations 1 and 2 were used to calculate hue angle (H°) and Chroma, respectively.

$$Hue\ angle\ =\ \tan^{-1}\frac{b}{a} \qquad \text{Eq. (1)}$$

$$Chroma = \sqrt{a^2 + b^2} \qquad \text{Eq. (2)}$$

2.4. Chemical compounds and antioxidant analysis

2.4.1. Extraction

The dry sample pulp and skin (approximately 10 g each one) were mechanically homogenised with 10 mL of acidified methanol/0.1% HCl in a manual blender and sonicated in an Ultrasonic bath (Branson model 2510) for 30 min and agitated in a horizontal shaker at room temperature (24°C) for 1.5 h. Then, the sample was centrifuged (Hettich, Mod. Universal 32R) at 2200 g for 15 min. The supernatant was removed and the residue was re-extracted twice with 10 mL of a mixture of methanol: HCl 0.1 v/v according to CHIOU *et al.,* (2014). The three supernatants were pooled and brought to a final volume of 100 mL with the same solvent used in the last two extractions. This concentration was considered by quantification of the components present in the sample. This extract was prepared in triplicate and used for the analysis of individual phenolic compounds, total phenolics, monomeric anthocyanins, and antioxidant activity.
The identification and quantitation of individual phenolic compounds, it was established by Ultra High Performance Liquid Chromatography (Agilent 1290 series) and dynamic multiple reaction monitoring (dMRM) following the protocol conditions of DURAND-HULAK *et al.* (2015). The chromatographic analysis were carried out on a ZORBAX SB-C18 column (1.8 µm, 2.1 × 50 mm) (Agilent Technologies) with the column temperature at 40°C. The mobile phase consisted of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The gradient conditions of the mobile phase were: 0 min 1% B, 0.1-40 min linear gradient 1-40% B, 40.1-42 min linear gradient 40-90% B, 42.1-44 min isocratic 90% B isocratic, 44.1-46 min linear gradient 90-1 %B, 46.1-47 min 1% B isocratic (total run time 47 min). The flow rate was 0.1 mL/min, and 5 µL of sample injection volume. dMRM were obtained on an Agilent 6460 Triplequadropole (QqQ) mass spectrometer. The ESI source was operated in positive and negative ionization modes, desolvation temperature of 300°C, Cone gas (N_2) flow of 5 L/min, nebulizer 45 psi, sheath gas temperature 250°C, sheath gas flow of 11 L/min, capillary voltage (positive and negative) 3,500 V, nozzle voltage (positive and negative) 500 V. For quantitation of each phenolic compound a calibration curve in a concentration range of 0.3 to 30 μM was prepared (R^2 values ≥ 0.97 were considered for the linearity range) and quantities were established by using MassHunter Workstation Software version B.06.00 (Agilent Technologies) (Table 1). The results were expressed as μg/g of sample (dry weight).

2.4.2. Anthocyanins Profile

Anthocyanins were identifying according to LIANG *et al.* (2008). Twenty grams of dry methanol pulp and skin extracts were dissolved in 1 mL of MeOH with 0.1% of formic acid (LCMS grade, SIGMA). The samples were filtered in PTFE filters and 1 μL injected in a UPLC-MS system (Acquiti Class-I coupled to mass spectrometer Synapt G2 Si, Waters™) for high resolution mass analysis. The mobile phases were water (A) and acetonitrile (B), both with 0.1% of formic acid. The elution gradient was: at T= 0 minutes, 1% of B, then in 13 minutes changes from 1 to 80% of B. Isocratic in 80% of B for 1 minute and finally change in 1 minute from 80 to 1% of B and remains for 5 minutes. The flow rate of the mobile phase was 0.3 mL/min and the column oven temperature was 40°C. The mass spectrometer was operated in positive mode, with capillary, sampling cone and source offset voltages of 3, 40 and 80 kV, respectively. The source and desolvation temperatures were 100 and 450°C, respectively. The gas flows of desolvation was 600 L/h and the nebulizer pressure was 6.5 Bar. The data were analyzed with the Waters Masslynx

software v4.1 and the mass spectra compared with the public databases Metlin and Massbank and analyzed with the Masslynx tool named Massfragment (v4.1).

Table 1. Protocol used in the analysis of the compounds was a dynamic MRM (Multiple Reaction Monitoring).

Reference compounds	Precursor ion	Product ion	Retention time	Collision energy	Polarity	R^2	Linearity range (μM)
Gallic acid	168.9	125	1.5	10	Negative	0.996	0.3 - 24
4-hydroxybenzoic acid	137.02	93.03	9.4	10	Negative	0.997	0.3 - 24
(+)-Catechin	291.1	139.03	11.3	10	Positive	0.971	0.3 - 12
Vanillic acid	169.04	151.04	12	10	Positive	0.998	0.3 - 12
Scopolin	355.1	193	12.2	20	Positive	0.998	0.3 - 12
Chlorogenic acid	353.08	191.05	12.3	10	Negative	0.998	0.3 - 12
Caffeic acid	179	135	12.5	10	Negative	0.999	0.3 - 12
(-)-Epicatechin	291.1	139.1	14.6	10	Positive	0.998	0.3 - 12
Vanillin	153	93	15.3	10	Positive	0.998	0.3 - 12
4-Coumaric acid	163.05	119	16.4	10	Negative	0.996	0.3 - 12
Quercetin 3,4-di-O-glucoside	627.15	303.04	17.7	10	Positive	0.997	0.3 - 12
Scopoletin	193.04	133.02	18.6	10	Positive	0.995	0.3 - 12
Ferulic acid	193.1	133.9	18.8	5	Negative	0.998	0.3 - 12
Rutin	611.16	465.1	20.4	10	Positive	0.994	0.03 - 12
Quercetin 3-D-galactoside	465.1	303.04	20.6	10	Positive	0.999	0.3 - 12
Quercetin 3-glucoside	465.2	303.04	20.9	10	Positive	0.987	0.03 - 12
Luteolin 7-O-glucoside	449.1	287.05	21.3	10	Positive	0.993	0.3 - 12
Kaemperol 3-O-glucoside	449.1	287.05	23.3	10	Positive	0.986	0.03 - 12
2,4-Dimethoxy-6-methylbenzoic acid	197.08	79.05	23.4	10	Positive	0.992	0.3 - 12
Trans-resveratrol	229.08	135.04	25.9	10	Positive	0.999	0.3 - 24
Trans-Cinnamic acid	147.01	103.05	28.7	10	Negative	0.999	1.5 - 24
Quercetin	303.05	153.1	29.4	35	Positive	0.990	0.03 - 12
Piperine	286.14	201.05	43.8	10	Positive	0.981	0.03 - 12

The retention time variation allowed for the search of the compounds was 2 min in each case. The fragmentor voltage was 100 V and the cell accelerator voltage was 7 V for each compound. It was made a calibration curve for each compound in a concentration range of 0.03 to 30 μM.

2.4.3. Total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu method (SINGLETON AND ROSSI, 1965). Briefly, the grape extracts were mixed with Folin-Ciocalteu reagent, and sodium carbonate solution (10%) was added. The mixture was allowed to react at room temperature in the dark for 120 min, and then the absorbance was measured at 765 nm in a UV/VIS spectrophotometer (JENWAY, model 6305, Japan). The result was then referred to a calibration curve obtained with a similarly prepared set of different Gallic acid concentrations, and was expressed as mg of Gallic acid equivalent (GAE) per g of dry sample (R^2=0.980).

2.4.4. Total flavonoid content

Each grape extract was analysed for total flavonoid content according to a previously reported colorimetric method with modifications (VELURI *et al.*, 2006). Specifically, 10 mg of lyophilised grape extract or 1 mL of quercetin standard (Sigma, St. Louis, MO) was mixed with 0.3 mL of 0.7 mol/L sodium nitrite ($NaNO_2$), 0.3 mL of 0.8 mol/L aluminium chloride ($AlCl_3$), and 2 mL of 1 mol/L sodium hydroxide (NaOH). All samples were analysed in duplicate and compared against a blank at an absorbance of 510 nm in a UV/VIS spectrophotometer (JENWAY, model 6305, Japan). Results were expressed as milligram quercetin equivalent per gram of dry sample (mg/g).

2.4.5. Total monomeric anthocyanin content

The total monomeric anthocyanin (TMA) content was estimated using the pH differential method (WROLSTAD, 2001). Here, 10 mg of grape extract was diluted with buffers at pH 1.0 and 4.5 to obtain the same dilution. Absorbance was measured in a UV/VIS spectrophotometer (JENWAY, model 6305, Japan) at 510 and 700 nm in both pH 1.0 and 4.5 buffers. The TMA content (expressed in terms of cyanidin-3-glucoside) was calculated using the following formula:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5} \qquad (3)$$

$$TMA\ content = (Ax\ MWx\ DFxVx1000)/(\varepsilon\ x\ 1\ x\ M) \qquad (4)$$

Where *MW* is the molecular weight of cianindin-3-glucoside (449 g mol^{-1}), *DF* is the dilution factor, *V* is the extract volume, ε is the molar extinction coefficient of cyanindin-3-glucoside (29,600), and *M* is the mass of *Vitis tiliifolia* extracted.

2.4.6. Condensed tannins determination

The determination of condensed tannins was performed according to the method described by PORTER *et al.* (1986). The dry extract of the pulp or skin (200 mg) and ten mL of aqueous acetone (70%) were added and suspended in an ultrasonic water bath, then the content was centrifuged for 10 min at approximately 3000g at 4°C, then 0.50mL of the supernatant was diluted with 70% acetone, 3.0 mL of butanol-HCl reagent and 0.1 mL of the ferric reagent. This sample was boiled for 60 min and measure the absorbance at 550 nm was obtained in the cool sample.

2.4.7. Determination of ascorbic acid content.

Ascorbic acid contents were carried out by using a colorimetric method. The extract (10 mg) was mixed with metaphosphoric acid (10 mL, 1.0%) for 45 min at R.T. and filtered through Whatman No. 1 filter paper. The filtrate (1.0 mL) was mixed with 2,6-dichlorophenolindophenol (9.0 mL) and the absorbance was measured within 30 min against a blank at 515 nm in a UV/VIS spectrophotometer (JENWAY, model 6305, Japan). Ascorbic acid contents were calculated on the basis of the calibration curve of authentic ascorbic acid (R^2= 0.927). The results were expressed as l g of ascorbic acid of extract.

2.4.8. DPPH free radical scavenging capacity

DPPH free radical-scavenging capacity was estimated using the method of GÜDER AND KORKMAZ (2012). Briefly, grape extracts and DPPH methanol solution were mixed and kept in the dark for 30 min. The absorbance of the reaction mixture was measured at 517 nm in a UV/VIS spectrophotometer (JENWAY, model 6305, Japan). The calibration curve was made with standard solutions of Gallic acid in the range 1-100 mg mL (R^2=0.935).

2.4.9. Reducing power

The determination of the reducing power was made according to the method of JAYAPRAKASHA *et al.* (2001). 0.125 mL of the sample in methanol (1 mg/mL), 1.25 mL of phosphate buffer (200 mM, pH 6.6) and 1.25 mL of potassium ferricyanide (1%) were added. The mixture was incubated at 50°C for 20 minutes. Then, 1.25 mL of 10% trichloracetic acid was added to the mixture, which was centrifuged at 650 *g* for 10 minutes. An aliquot of 2.5 mL was taken, 2.5 mL of distilled water and 0.5 mL of ferric chloride were added, and the absorbance measured at 700 nm in UV/VIS spectrophotometer (JENWAY, model 6305, Japan). Solutions of Trolox (6-hydroxy-2.5.7.8-tetramethyl-chroman-2- carboxylic acid) in a range of concentrations were used for calibration of the FRAP assay. The values were expressed as mg of Trolox/L of seed extracts. All determinations were performed in triplicate.

2.5. Statistical analysis

Data were subjected to ANOVA and Tukey tests (Statistica 7.0 software) at a 0.05 level of significance. Five samples ($n=3$) of pulp and skin were analysed.

3. RESULTS AND DISCUSSIONS

3.1. General composition and physicochemical properties

The grapes of *Vitis tiliifolia* are round and dark violet; with an average weight around 0.15 g, their dimensions were: length 3.74 mm and width 3.14 mm. Physicochemical parameters of the pulp and skin in *Vitis tiliifolia* fruits are described in Table 2. Pulp and skin showed moisture content of 84.88 and 82.00%, respectively. Reducing sugars, ash and total dietary fibre were the major components in the pulp and skin of the grape. Proteins content varied from 0.45 to 0.95 % in the pulp and skin, respectively; both values were lower to those reported for red and white grape (BRAVO and SAURA-CALIXTO, 1998). The grape had a weight, brix, titratable acidity percentage and dimensions lower than those reported for other species, but a similar pH to those reported by JIANG-FEI *et al.* (2012) for four varieties of grapes, three red varieties (Junzi #1, Junzi #2 and Liantang) and one white variety (Baiyu). These differences are due to these properties being influenced by cultivar, ripening stage and environmental factors (CORDENUNSI *et al.*, 2002).
Fruit colour is a tool that is commonly used by winemakers as a selection parameter to define the optimal moment for harvesting during the wine production process. However, this parameter generally is estimated visually and there is not enough information about relations among fruit colour, different harvest dates and some chemical parameters of the *Vitis tiliifolia* fruit (OBREQUE-SLIER *et al.*, 2012). In this work, colour parameters showed significant differences ($p<0.05$) for the pulp (L*=6.23, a*=17.13 and b*=2.28) and skin (L*=38.42, a*=10.28 and b*=4.74). Hue value of the skin (H=24.71°) was located in the first

quadrant of the colour plane which corresponds to a red-violet colour. Parameter a*and b* assumed a positive values, indicating a characteristic violet colour. These colour parameters has been associated with the colour of anthocyanins present in grapes. All colour parameters of this fruit exhibited significant differences with respect to the other grape varieties reported (PÉREZ-MAGARIÑO and GONZÁLEZ-SAN JOSÉ, 2003), but similar values to those reported for fruits such as chagalapoli (JOAQUÍN-CRUZ *et al.*, 2015).

Table 2. Composition of pulp and skin of *Vitis tiliifolia* fresh fruit.

	Pulp	**skin**
Moisture (%)	84.88±0.14^{b}	82.00±0.96^{a}
Brix (°)	12.7±0.17^{b}	8.16±0.28^{a}
pH	3.20±0.10^{a}	3.30±0.50^{a}
Titratable acid (%)	3.00±0.20^{a}	3.50±0.20^{b}
Reducing sugars (%)	15.82±0.16^{b}	4.41±0.31^{a}
Protein (%)	0.45±0.10^{a}	0.95 ±0.05^{b}
Oil	0.37±0.10^{a}	0.50 ±0.10^{b}
Ash	0.28 ±0.01^{a}	0.85± 0.07^{b}
Total Dietary Fiber	0.73±0.25^{a}	1.53±0.10^{b}
a_w	0.98±0.06^{b}	0.64±0.05^{a}
Color parameters		
L	6.23±1.69^{a}	38.42±0.04^{b}
a	17.13±0. 57^{b}	10.28±0.16^{a}
b	2.28±0.26^{a}	4.74±0.09^{b}
Hue angle (°)	82.37±1.12^{b}	24.71± 0.74$_{a}$
Chroma	17.28±0.52^{b}	11.31±0.12^{a}
Browning index	81.63±7.94^{b}	26.3±1.91^{a}

Results are expressed as the mean (n=3)±SD. Note: Diameter of single grape was calculated on the basis of the mean of random 100 grapes. Values of other parameters are mean±SD values of three replicates. Means followed by different letters in column are significantly different by Tukey's test 5%.

3.2. Chemical compounds and antioxidant properties

The antioxidant and functional properties of the different types of grapes depend to a great extent on the bioactive compounds it possesses. So that, in order to determine the compounds that may be responsible for the high antioxidant activity, we investigated the chemical constituents in pulp and skin of the grape (Table 3). Twelve compounds were identified and quantified in the skin, and only two were found in the pulp (vanillin and quercetin-3-D-galactoside). The most abundant compounds identified in skin were: quercetin-3-glucoside (39.86 μg/g dry sample), rutin (37.01 μg/g dry sample) and *trans*-resveratrol (32.88 μg/g dry sample). The majority of these compounds contain double bonds in their aromatic ring structure, reported to be responsible for electron delocalisation, which is attributed to their radical scavenging activity (RICE-EVANS *et al.*, 1996). These compounds may contribute to the antioxidant activity of this grape. Moreover, it has been reported that the bioactivity of the grape is strongly correlated with the composition and the presence of polyphenol compounds (BURIN *et al.*, 2014), which form an important group of secondary metabolites that is abundant and play an important

role in the quality and nutritional value of grapes. Some of these polyphenol are synthesised in the skin of the fruit (JEANDET *et al.*, 1991) and their concentration depend of several factors such as climate, geographical area of cultivation, growing conditions and storage conditions (GEROGIANNAKI-CHRISTOPOULOU *et al.*, 2006).

Table 3. Phenolic compounds (μg/g dry sample) presents in pulp and skin from *Vitis tiliifolia* grape.

Compounds	Pulp	Skin
4-hydroxybenzoic acid	-	0.16±0.06
(+)-Catechin	-	8.29±0.35
Vanillic acid	-	12.60±0.18
Caffeic acid	-	3.68±0.15
(-)-Epicatechin	-	5.17±0.06
Vanillin	0.004±0.00	0.33±0.01
4-Coumaric acid	-	3.37±0.21
Rutin	-	37.01±0.13
Quercetin-3-D-galactoside	1.86±0.82	13.91±0.29
Quercetin-3-glucoside	-	39.86 ±1.36
Trans-resveratrol	-	32.88±0.72
Quercetin	-	22.08±0.67

Data are expressed as means±SD (*n=3*).

Anthocyanins were tentatively identified based on their mass spectra fingerprint (exact mass values and fragmentation pattern) in high resolution compared with public metabolomics databases (Table 4). Overall, the *V. tiliifolia* skin presented higher level of anthocyanins compared to the pulp (Fig. 1). In total, five and seven anthocyanins were identified in pulp and skin, respectively. The most abundant anthocyanin tentatively detected in the skin was malvidin 3-glucoside, while that in pulp was malvidin 3,5-diglucoside. The result that malvidin derivatives were the major anthocyanins agreed with the data reported by LIANG *et al*. (2008).

Total polyphenols concentration found in the skin (400.35 mg GAE/g dry sample) was higher than in the pulp (171.26 mg GAE/g dry sample) (Table 5). This difference in the total polyphenol concentration between pulp and skin might be attributed to the different inherent components present in each part of the grape. The total soluble polyphenolic content of our grape was higher than those of other fruits, such as apple, melon, peach, pear, prune and strawberry (ISHIWATA *et al.*, 2004) and similar to those reported in previous works with other varieties of grapes grown in various parts of the world (BURIN *et al.*, 2014), but lower than reported by the red grape variety (APOSTOULO *et al.*, 2013). These differences probably depend on the variety of grape and are influenced by climatic and geographical factors, cultural practices, and the stage of ripeness (BURIN *et al.*, 2014). By other hand, flavonoids are secondary metabolites presents in plants and fruit such as grapes, which possess biological activities and have an impact on human health. The flavonoid content of the pulp (17.22 mg QE/g dry sample) was lower than quantified in the skin (282.57 mg QE/g dry sample) of the grape.

Table 4. Anthocyanins Profile by Ultra high resolution Liquid chromatography and high-resolution mass spectrometry (UPLC-HRMS-ESI-QTOF).

RT (min)	Mass detected (m/z)	Formula	Fragments (m/z)	Tentative identification	Formula	Ion type	Mass calculated	Error (ppm)
Pulp								
2.36	655.187	$C_{29}H_{35}O_{17}$	493.1341, 331.0816, 287.0543	Malvidin 3,5-diglucoside	$C_{29}H_{35}O_{17}$	$[M]^+$	655.1874	-0.6
2.74	479.1181	$C_{22}H_{23}O_{12}$	317.0662	Petunidin-3-O-β-glucoside	$C_{22}H_{23}O_{12}$	$[M]^+$	479.119	-1.9
3.09	493.1341	$C_{23}H_{25}O_{12}$	331.0809, 287.0541	Malvidin 3-O-glucoside	$C_{23}H_{25}O_{12}$	$[M]^+$	493.1346	-1
3.77	757.1971	$C_{36}H_{37}O_{18}$	449.1087, 287.0550	Cyanidin 3-O-(6-O-p-coumaroyl)glucoside-5-O-glucoside	$C_{36}H_{37}O_{18}$	$[M+H]^+$	757.198	-1.2
4.02	801.2236	$C_{38}H_{41}O_{19}$	639.1685, 493.1356, 331.0816, 287.0551	Malvidin 3-O-(6-O-(4-O-caffeoyl-alpha-rhamnopyranosyl)-beta-glucopyranoside)	$C_{38}H_{41}O_{19}$	$[M+H]^+$	801.2242	-0.7
Skin								
2.36	655.1872	$C_{29}H_{35}O_{17}$	493.1346, 331.0818, 287.0546	Malvidin 3,5-diglucoside	$C_{29}H_{35}O_{17}$	$[M]^+$	655.1874	-0.3
2.69	479.1183	$C_{22}H_{23}O_{12}$	317.0652	Petunidin-3-O-glucoside	$C_{22}H_{23}O_{12}$	$[M]^+$	479.119	-1.5
3.02	493.1347	$C_{23}H_{25}O_{12}$	331.0818, 287.0551	Malvidin 3-O-glucoside	$C_{23}H_{25}O_{12}$	$[M]^+$	493.1346	0.2
3.73	757.1961	$C_{36}H_{37}O_{18}$	287.0551	Cyanidin 3-O-(6-O-p-coumaroyl)glucoside-5-O-glucoside	$C_{36}H_{37}O_{18}$	$[M+H]^+$	757.198	-2.5
4.01	801.2236	$C_{38}H_{41}O_{19}$	639.1699, 493.1339, 331.0815, 287.0556	Malvidin 3-O-(6-O-(4-O-caffeoyl-alpha-rhamnopyranosyl)-beta-glucopyranoside)	$C_{38}H_{41}O_{19}$	$[M+H]^+$	801.2242	-0.5
4.18	463.1234	$C_{22}H_{23}O_{11}$	301.0706	Peonidin 3-O-glucoside	$C_{22}H_{23}O_{11}$	$[M+H]^+$	463.124	-0.6
4.73	639.1705	$C_{32}H_{31}O_{14}$	331.0813	Malvidin 3-(6''-p-coumarylglucoside)	$C_{32}H_{31}O_{14}$	$[M+H]^+$	639.1714	-1.4

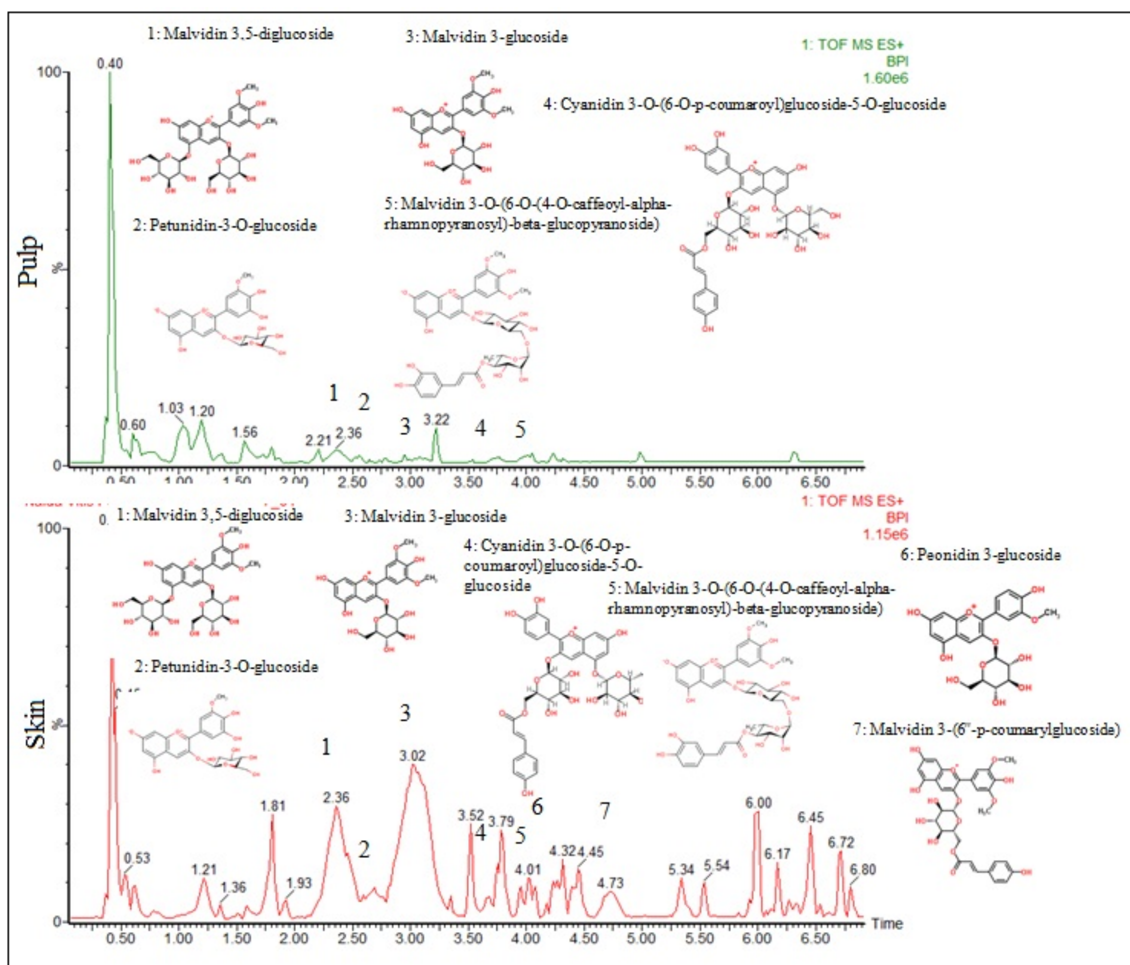

Figure 1. Chromatograms and structure of anthocyanins tentatively identified based on their mass spectra fingerprint (exact mass values and fragmentation pattern).

These data were lower than those reported in other varieties of grapes (GÜDER *et al.*, 2014). Similarly, the total monomeric anthocyanins content was significantly higher ($p < 0.05$) in skin (188.11 mg Cy3/g dry sample) than in pulp (150.93 mg Cy3/g dry sample), and these values were similar to the values of anthocyanins reported for other varieties of grapes (DE PASCUAL-TERESA *et al.*, 2010).

Vitis tiliifolia skin had a high ratio of anthocyanins/total polyphenols close at 0.5, whereas pulp had a ratio close 1.0 indicating than more than half of the polyphenols present in pulp and skin are anthocyanins. A high proportion of the total polyphenols content presents in the grape correspond to anthocyanins, which are considered important groups of plant pigments that contribute to the coloration and sensorial attributes and diverse biological properties; therefore, these are considered secondary metabolites with potential nutritional value, as chronic diseases can be reduced by the regular consumption of anthocyanins in the diet. Anthocyanins are regarded as important nutraceuticals due to their antioxidant activity (KALLITHRAKA *et al.*, 2005). At the same time, total tannins were analysed in the pulp and skin, with a higher concentration found in the skin (188.37 mg Leucocyanidin/g dry sample) than in the pulp (60.26 mg Leucocyanidin/g dry sample). The result in total tannins concentration was consistent with other research that has reported a higher concentration of tannins in the skin and seeds of grapes, playing a

relevant role to define the sensory characteristics of red wines, contributing to bitterness and astringency, in addition to providing antioxidant and antibacterial activity (FIGUEROA-ESPINOZA *et al.*, 2015). By last, in the present work, the pulp and skin from *Vitis tiliifolia* presented a content of ascorbic acid of 130.88 and 5.75 mg AA/g dry sample, respectively, which could contribute to the recommended dairy dietary intakes (0.04-0.09 g/day) suggested by the United Kingdom Food Standards Agency or the United States National Academy of Science (DEL BUBBA *et al.*, 2009). Ascorbic acid is a good reducing agent present in grape juices that is associate with the biosynthesis of tartaric acid (DEBOLT *et al.*, 2006). In plants, ascorbic acid as vitamin C provides protection against free radicals generated during photosynthesis and respiration processes, and is also involved in cell growth; in addition, it is a co-factor of several enzymes participating in the synthesis of anthocyanidins and several secondary metabolites (BRAVO and SAURA-CALIXTO, 1998).

Table 5. Antioxidant activity of dry pulp and skin of *Vitis tiliifolia*.

	Pulp	Skin
Total polyphenols (mg GAE/g dry sample)	171.26±7.90^{a}	400.35±5.90^{b}
Total monomeric anthocyanins (mg Cy3/g dry sample)	150.93±5.55^{a}	188.11±3.15^{b}
Total Flavonoids (mg QE/g dry sample)	17.22±2.40^{a}	282.57 ±2.20^{b}
Condensed Tannins (Leucocyanidin/g dry sample)	60.26±0.34^{a}	188.37±0.20^{b}
Ascorbic acid (mg AA/g dry sample)	130.88±9.60^{a}	5.75±1.20^{b}
DPPH radical scavenging activity (%)	19.57±2.13^{a}	91.39±3.04^{b}
FRAP (mg TE/g dry sample)	40.67±1.17^{a}	7.24±1.80^{b}

GAE: Gallic Acid Equivalents, Cy3: Cyanidin-3-glucoside, QE: Quercetin Equivalents, AA: Ascorbic Acid, TE: TROLOX Equivalents. Results are expressed as the mean ($n=3$)±SD. Means followed by different letters in column are significantly different by Tukey's test 0.05.

The presence of these compounds has been demonstrated confer antioxidant activity. One of the techniques used to evaluate this capacity is through the percentage inhibition of the DPPH radical and reducing power. DPPH is a stable free radical and the effect of antioxidants on DPPH scavenging is thought to be due to their hydrogen- or electron-donating abilities. In its radical form, DPPH radical absorbs at 517 nm, but this absorbance value decreases in the presence of an antioxidant or a radical species due to the reaction between antioxidant molecules and the DPPH radical (GÜDER and KORKMAZ, 2012). The radical scavenging activity of pulp and skin showed values from 19.57% and 91.39%, respectively, at a concentration of 10 mg/mL, showing that the skin was highly antioxidant than pulp, which is consistent with the concentration of some polyphenols compounds, such as flavonoids, tannins and anthocyanins. Therefore, the data obtained reveal that these compounds present in this grape act as free radical inhibitors that confer antioxidant activity. Similarly, the reducing power measured by the FRAP value was 40.67 and 7.24 mg TE/g of dry pulp and skin, respectively. The value of reducing power were lower than those reported by red globe grapes (TAGLIAZUCCHI *et al.*, 2010), but FRAP values were consistent with other antioxidant techniques evaluated. FRAP assay does not react fast with some antioxidants, such as glutathione, but some authors consider the FRAP assay to still be suitable for assessment of the antioxidant activity of fruit samples because only limited amounts of plant glutathione are absorbed by humans (SCHAFER and BUETTNER, 2001). On the other hand, it is reported that the antioxidant activity determined by this technique corresponds to approximately 55% of the bioavailability at

the end of digestion (TAGLIAZUCCHI *et al.*, 2010). Therefore, these reports are based on an estimate that approximately 13% of the total antioxidant is used by the human body. The reducing properties are generally associated with the presence of reductones, which also react with certain precursors of peroxide, thus preventing peroxide formation; a higher absorbance of the reaction mixture indicates greater reducing power (PIN-DER, 1998).
Table 6 shows the correlation analysis in the pulp and skin of *Vitis tiliifolia* grape. A linearly relation of DPPH radical scavenging activity with anthocyanins (R^2=0.728), polyphenols (R^2=0.878) and condensed tannins (R^2=0.680), suggesting a strong antioxidant effect of these mixtures of components from *Vitis tiliifolia* pulp. Instead, ascorbic acid had a positive correlation (R^2=0.850) with reducing power, and revealed a moderately strong relationship between ferric ion reducing power and ascorbic acid content. It is reported that in several fruits including grapes, over 80% of the FRAP value was from vitamin C contribution (GUO *et al.*, 2003).
The results on the antioxidant activity of *Vitis tiliifolia* pulp seems to be due to the presence of polyphenols and anthocyanins which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable products and terminate the free radical chain reaction (JAYAPRASKASHA *et al.*, 2001), which may serve as significant evidence of their potential antioxidant activity. Instead, in the skin, the antioxidant activity is mainly due to the presence of polyphenols, anthocyanins, resveratrol, tannins and ascorbic acid.

Table 6. Correlation coefficient (R^2) between antioxidant activity and chemical components presents in *Vitis tiliifolia* pulp.

	DPPH	Reducing power (FRAP)
Total polyphenols	0.878	0.650
Total Flavonoids	0.650	0.500
Total Monomeric anthocyanins	0.728	0.567
Resveratrol	0.320	0.450
Condensed tannins	0.680	0.720
Ascorbic acid	0.576	0.850

Correlation was statistically significant at $p < 0.05$.

4. CONCLUSIONS

The results of the present study showed that skin from this wild grape has a higher concentration of polyphenols than pulp. The more abundant individual polyphenols in skin were quercetin-3-glucoside, rutin and *trans*-resveratrol. Instead, the pulp has a large amount of ascorbic acid. Malvidin 3-glucoside and malvidin 3,5-diglucoside were the most abundant anthocyanins identify in skin and pulp, respectively. All this compounds confer a strong antioxidant activity comparable to other grape varieties, and may explain in part the benefits for human health. In addition, the skin has an intense violet blue color that could be exploited to obtain pigments that can be used as food colorant, as food additives or as food supplements.

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PAPER

UBIQUINONE IN ITALIAN HIGH-QUALITY RAW COW MILK

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ABSTRACT

The content of ubiquinone (UBN) was evaluated in Italian high-quality (HQ) raw cow milk. Samples were collected from four cowsheds in two different days during summer and winter. The fat content in HQ raw cow milk ranged between 2.86% and 3.46%, while UBN content varied between 0.15 and 0.45 μg/g milk. The fat content was significantly influenced by the cowshed only, whereas the UBN content was significantly more affected by both season and sampling days. Although UBN is a lipophilic antioxidant, no statistically significant correlation was found between UBN and fat content in HQ raw cow milk.

Keywords: ubiquinone, coenzyme Q_{10}, lipophilic antioxidant, Holstein-Friesian cows, high-quality raw cow milk, season, cowshed

1. INTRODUCTION

Cow milk is undoubtedly the most frequently consumed dairy product (MERDJI *et al.*, 2015), due to its nutritional composition and properties (ASSOLATTE, 2006). The dairy industry offers many product categories with diverse characteristics, based on consumers' requests and nutritional requirements. The quality of raw milk is greatly affected not only by the technology used for the preservation and diversification of milk products, but also by the characteristics of raw milk itself. The EC Regulation 853/2004 defines raw milk as the product of the mammary gland secretion of farmed animals that has not been heated to more than 40 °C and has not been subjected to any treatment; the same regulation also states the health requirements for raw milk production and its standards of quality. Raw drinking milk can contain pathogenic microflora, so consuming it can pose a significant public health risk (EFSA, 2015). In some EU countries (EC Regulation 853/2004; Intesa Stato-Regioni 25/01/2007), the sale of raw milk is allowed through vending machines, but current law clearly states that it must be heat-treated before being consumed (EC Regulations 852/2004 and 853/2004). The overall quality of raw milk will thus be highly dependent on the breeding conditions and hygienic controls, which will give rise to diverse quality-labelling categories among which that labelled 'high-quality' (HQ) milk is considered the best commercialised one; in fact, the compositional profile of HQ milk is most similar to that of raw milk. The Italian Ministerial Decree 185/1991 imposes rigorous breeder management and hygienic controls aimed to obtain HQ milk, which should fulfil the following stringent sanitary and quality requirements: fat content and protein content $\geq$ 3.50% and 32.0 g/L, respectively; bacterial load $\leq$ 100.000/mL at 30 °C; somatic cells $\leq$ 300.000/mL; lactic acid content $\leq$ 30 ppm; level of non-denatured soluble serum proteins $\geq$ 15.50% of the total protein (when ready for consumption). The Italian Legislation n.169/1989 actually defines HQ fresh pasteurised milk, stating that to further preserve the quality of HQ milk, pasteurization is always necessary and must be performed within 48 h after milking.

Cow milk contains many health promoting compounds, such as vitamins (CLAEYS *et al.*, 2014) and ubiquinone (UBN), also known as coenzyme Q_{10} (MATTILA and KUMPULAINEN, 2001). UBN is present in all cells and membranes, and it has been reported to increase the energy level, to augment the immune system, to act as an antioxidant, to exert a protective effect on the cardiovascular system, and to guard against skin aging and neurodegenerative diseases (HEMAT, 2004; PRAHL *et al.*, 2008; KEWAL, 2011; SAINI, 2011, HECHTMAN, 2011; AMAR-YULI *et al.*, 2009; QUINZII and HIRANO, 2010). As a lipophilic substance, UBN is absorbed following the same process as that of lipids in the gastrointestinal tract, being first incorporated into chylomicrons, followed by absorption and transportation via the lymphatics to the circulatory system (BHAGAVAN and CHOPRA, 2006). Due to its high molecular weight and low water solubility, UBN is poorly and slowly absorbed (Tmax 2-10 h) from the gastrointestinal tract (SEO *et al.*, 2009). PAKAMULA *et al.* (2005) in fact observed different regional permeability of UBN in isolated rat gastrointestinal tracts, suggesting that UBN formulations should target the duodenum to get maximum dosage effect; to compensate for its low absorption rate, diverse strategies can be adopted to enhance UBN bioavailability, such as particle size reduction, solubility improvement (i.e. solid dispersion, complexation, ionization), and use of carriers (i.e. liposomes, microspheres, nanoemulsions, nanoparticles, self-emulsifying systems) (BEG *et al.*, 2010). Once UBN is slowly absorbed from the small intestine, it passes into the lymphatics, and finally to the blood and tissues (GARRIDO-MARAVER *et al.*, 2014). Once UBN reaches the tissues, it is quickly broken down (short half-life of 49-125 h) and degraded by ω-oxidation and β-oxidation of its side-chain (THELIN *et al.*, 1992). The main breakdown product found in tissues, urine and faeces has an intact, fully substituted

ring, a short side-chain (5-7 carbon atoms) and a carboxylated ω-terminus (NAKAMURA *et al*., 1999). By using labelled UBN, it has been demonstrated that UBN is metabolised in all tissues. The metabolites are converted into more hydrophilic compounds (mostly in their phosphorylated form) in the cells, transported in the blood to the kidney, and excreted into the urine (BENTINGER *et al*., 2003); however, other minor metabolites were also detected in the faeces, which contained non-metabolised labelled UBN, excreted through the bile. Under certain physical conditions (such as aging, cardiomyophathies, degenerative muscle diseases, and carcinogenesis), UBN concentration can diminish greatly (BENTINGER *et al*., 2003). Ubiquinone deficiency may be due to insufficient dietary intake, impairment in UBN biosynthesis, excessive utilization by the body or a combination of any of these three (FEDACKO *et al*., 2011). UBN deficiencies are clinically and genetically heterogeneous. This syndrome has been associated with five major clinical phenotypes: (1) encephalomyopathy, (2) severe infantile multisystemic disease, (3) cerebellar ataxia, (4) isolated myopathy, and (5) nephrotic syndrome (QUINZII and HIRANO, 2011).
Several studies show that milk is a natural source of UBN, whose concentration level varies from species to species and is influenced by the lactation stage and the heat treatment during processing (MATTILA and KUMPULAINEN, 2001; STRAZISAR *et al*., 2005; NIKLOWITZ *et al*., 2005; TANG *et al*., 2006; QUILES *et al*., 2006). MATTILA and KUMPULAINEN (2001) assessed the levels of coenzyme Q_9 (CoQ_9) and UBN in milk purchased from major dairies, finding only UBN at a concentration level equal to 0.1 μg/g in milk (1.5% fat). STRAZISAR *et al*. (2005) also evaluated the UBN content of fresh cow milk produced in a Slovenian farm (3.6% fat), cow milk from the alpine region (3.5% fat), and ultra-heat-treated homogenised milk (3.5% fat), finding a UBN content equal to 1.90 μg/g, 1.57 μg/g, and 1.70 μg/g, respectively. As reported earlier, most studies have been carried out on pasteurised milk, but to the best of our knowledge, there is no report available in the literature about the UBN content in HQ raw cow milk.
The aim of this survey was to evaluate the level of UBN in Italian HQ ("Alta Qualità") raw cow milk. To this purpose, milk samples were collected from four different Italian cowsheds producing HQ milk, in two different days during both summer and winter. Raw cow milk samples were analysed for both fat and UBN content.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents and solvents used were analytical grade chemicals. Potassium hydroxide pellets (≥ 85%) and pyrogallol (≥ 98%), were purchased from Carlo Erba (Milan, Italy). Commercial standards of UBN (≥ 98%, HPLC) and CoQ_9 (≥ 96%, HPLC) were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA), whereas bidistilled water (100%) was purchased from Panreac (Barcelona, Spain). Petroleum ether with a boiling range 40-60°C (≥ 95.0%), diethyl ether (≥ 99.5%), ethanol (≥ 99.9%), acetonitrile (≥ 99.9%, HPLC), *n*-pentane (≥ 99.0%), anhydrous sodium sulphate (≥ 99.9%), ammonia solution 14 M, and 2-propanol (≥ 99.8%, HPLC), were supplied by Merck KGaA (Darmstadt, Germany). Acetonitrile and 2-propanol were degassed before use by filtering under vacuum through a 0.20 μm nylon membrane filter (Phenomenex, Westboro, MA, USA).

2.2. Sampling and experimental design

Milk samples were obtained from Holstein-Friesian cows that were bred in four different cowsheds. The latter belonged to medium/large-sized Italian farms focusing on the production of HQ milk and located in the valley area between the provinces of Bologna, Mantova, and Modena (Italy). The management of these farms is characterised by a particular attention to hygienic conditions, nutrition and animal welfare, which are the basic requirements for obtaining HQ milk as regulated by the Italian D.M. 185/1991. In these farms, *unifeed* or "single pot" is used for feeding, with the objective of providing a food ration that is homogeneously mixed, properly formulated, and nutritionally balanced. The daily dose administered consisted of 20-25 Kg of unifeed per milk cow. UBN in the food ration is provided mainly by the forage, which is almost exclusively hay from *Medicago sativa L.* UBN was evaluated in hay specimens at different mowing times and was found to be present in an average content of 11 mg/Kg; therefore, the amount of presumed UBN taken in the daily ration could be estimated around 46-58 mg.
Two milk samples were collected from each cowshed (CS) in two different sampling days (SD, i and ii) and two diverse seasons (S, summer and winter), thus giving a total of 16 (4x2x2) samples that were analysed in duplicate (32 analysis in total). Raw cow milk samples were collected from bulk tanks containing the morning and evening milk of the entire herd. The collected milk was placed in 1-L PET bottles and kept at 2-6°C during sample delivery. The milk samples were then divided into 100-mL PET bottles, frozen and stored at -20°C until subsequent analysis. All milk samples were analysed for fat content (by cold extraction), as well as for UBN content (by direct cold saponification followed by extraction of the unsaponifiable matter and high-performance liquid chromatography-ultraviolet diode-array (HPLC-UV/DAD) analysis.

2.3. Lipid extraction

Lipids were extracted according to the ISO 14156:2001 method (IDF 172:2001). An aliquot of 100 mL (at 20 °C) milk was introduced into a 500-mL separatory funnel. Eighty mL of ethanol, 20 mL of 14 M ammonia aqueous solution and 100 mL diethyl ether were added, and the funnel was shaken vigorously for 1 min. Thereafter, 100 mL of *n*-pentane were added and the funnel was gently shaken. After phase separation, the aqueous phase was discarded. The organic phase (lipid-containing one) was washed twice with 100 mL of 10% (w/v) sodium sulphate aqueous solution. The organic phase was transferred into a 250-mL Erlenmeyer flask fitted with a ground glass stopper and approximately 10 g of anhydrous sodium sulphate were added. The flask was stoppered, well shaken, and allowed to stand for 10 min. The organic phase was then filtered into a 100-mL round-bottom flask through a Whatman No. 1 filter paper, dried at 40°C using a vacuum rotary evaporator and at the end dried under a gentle stream of nitrogen. The fat content was gravimetrically determined. Two replicates for each sample were performed.

2.4. Saponification and extraction of the unsaponifiable matter

The sample preparation method for the quantification of UBN in milk consisted of a direct cold saponification of milk followed by the extraction of the unsaponifiable matter. The direct cold saponification was performed according to the modified method of RENKEN and WARTHESEN (1993). About 35 g of milk were weighed into a 100-mL glass bottle with screw cap. Fifteen micrograms of CoQ_9 (internal standard), 35 mL of 2 N potassium hydroxide in 85% ethanol, and 20 mL of 1% (w/v) pyrogallol in ethanol were added. The headspace of the bottle was flushed with a nitrogen stream to remove the oxygen,

stoppered, and kept at room temperature for 18-20 h, in the dark and under continuous agitation (180 oscillations/min). After the saponification had taken place, the alcoholic soap solution was transferred into a 250-mL separatory funnel to extract the unsaponifiable matter containing UBN. The unsaponifiable portion was extracted by adding consecutively 15 mL of bidistilled water, 5 mL of ethanol, and 35 mL of a petroleum ether:diethyl ether mixture (9:1, v/v), under shaking. After phase separation, the aqueous phase was transferred into a 150-mL separatory funnel. Five mL of ethanol and 35 mL of a petroleum ether:diethyl ether mixture (9:1, v/v) were added, shaken, and allowed to stand until phase separation. The two ethereal fractions were combined in the 250-mL separatory funnel and washed until neutrality was reached by using cold bidistilled water (approximately 3 x 30 mL). The ethereal extract was transferred into a 100-mL Erlenmeyer flask fitted with a ground glass stopper; next, approximately 5 g of anhydrous sodium sulphate were added. The flask was stoppered, shaken well, and allowed to stand for 60 min. The ethereal extract was then filtered into a 100-mL round-bottom flask through a Whatman No. 1 filter paper and dried at 40 °C using a vacuum rotary evaporator. The unsaponifiable matter was dissolved in 1 mL of 2-propanol and filtered through a 0.45 μm nylon syringe-type filter (Econofilter, 25-mm diameter, Agilent Technologies, Wilmington, DE, USA), before injection into a HPLC system. Two replicates for each sample were performed.

2.5. HPLC-UV/DAD determination of UBN

The separation of the compounds of interest (UBN and CoQ_9) was performed as suggested by RAO *et al*. (2008), with minor modifications. A HPLC system (HP 1050 series; Hewlett-Packard, Palo Alto, CA, USA) consisting of an autosampler (Series 1100), a quaternary pump, a UV/DAD detector and a Poroshell 120 EC-C18 (3.0 x 50 mm x 2.7 μm particle size) analytical column (Agilent Technologies, Santa Clara, CA, USA), was used. Ten microliters of the sample solution were injected in isocratic mode, using a mixture of acetonitrile:2-propanol (70:30, v/v) at a flow rate of 1.2 mL/min. The column temperature was maintained at room temperature (25°C). The detection wavelength of the UV/DAD detector was set at 275 nm as suggested by KOMMURU *et al*. (1998). Data were acquired using Chemstation for LC3D software (Agilent Technologies, Palo Alto, CA, USA). Standard solutions (CoQ_9 and UBN) were prepared in 2-propanol and stored at -20°C in amber vials until further analysis. Their concentrations were periodically checked by measuring the absorbance at 275 nm using a UV-Visible spectrophotometer (V-550; Jasco, Tokyo, Japan) and using the known molar extinction coefficients for CoQ_9 ($E^{1\%}_{1cm}$ 185) and UBN ($E^{1\%}_{1cm}$ 165) as reference (HATEFI, 1963; SOUCHET and LAPLANTE, 2007). Two replicates were performed for each sample. The limits of detection (LOD) and quantification (LOQ) were determined according to VIAL and JARDY (1999), with a signal-to-noise ratio equal to 3 and 10, respectively. LOD of UBN was 0.35 μg/mL, while its LOQ was equal to 1.18 μg/mL.
The content of UBN was calculated using the following equation [1]:

$$\text{UBN } (\mu\text{g/g milk}) = \frac{A_a \times C_{IS}}{A_{IS}} \times \frac{1}{w_m} \quad (1)$$

Where:
A_a is the peak area of the analyte;
A_{IS} is the peak area of the internal standard;
C_{IS} is the concentration of the internal standard, in μg;
W_m is the weight of the milk sample, in g.

To identify CoQ_9 and UBN in milk samples, commercial standards of both compounds were individually injected into the HPLC system and their corresponding chromatographic retention times were compared with those of the unknown peaks in milk samples. The identification of UBN in milk samples was further confirmed by LC-MS analysis. An analytical column Kinetex 5 μm (C18 100A) (Phenomenex, Torrance, CA, USA) was used. Fifty microliters of the sample solution were injected in isocratic mode. The eluent mixture was made up of methanol:2-propanol (70:30, v/v) at a flow rate of 1.0 mL/min. The HPLC flow was split in two detectors in parallel, DAD and electrospray interface (ESI), through a three-way valve. ESI was used in positive mode at a voltage of 4.4 kV (LCQ Duo Mass Spectrometer, Thermo Finnigan, San José, CA, USA). The flow was 0.1 mL/min and the temperature was 200°C. The presence of UBN was monitored at *m/z* 880, which corresponds to the molecular weight of UBN (863.3) + NH_4–H. The DAD detector (Varian mod. 330) was set at 275 nm.

2.6. Statistical analysis

To perform the statistical tests, Minitab software (version 16.1.0; LEAD Technologies, Inc., Charlotte, NC, USA) was used. The analysis of variance (ANOVA) on the whole set of sample data was assessed, as well as the effects of season (S), cowshed (CS), sampling day (SD), their first-degree interactions (S × CS, S × SD, and CS× SD), and second-degree interaction (S × CS × SD) on fat and UBN contents. Tukey's honest significance test was carried out at a 95% confidence level ($p < 0.05$). The percentage contribution of each factor and interaction was calculated using eta-squared values from the ANOVA summary table. The Pearson's correlation (α= 0.05) with two-tailed probability value was used to estimate the strength of association between fat content and UBN content.

3. RESULTS

The fat and UBN contents of sixteen samples of HQ raw cow milk were analysed in duplicate.
Table 1 reports the fat content (%) and the UBN content (expressed as μg/g milk) of HQ raw cow milk samples. Data correspond to the mean of two analytical determinations. A three-factor experimental design was used. Each factor was set at different levels (season (S)-2 levels, cowshed (CS)-4 levels, and sampling day (SD)-2 levels).
The fat content ranged from 2.9% to 3.5%, whereas the UBN content varied from 0.15 to 0.45 μg/g milk.

Table 1. Contents of fat (%) and UBN (μg/g milk) in raw cow milk.

Season (S)	Cowshed (CS)	Sampling Day (SD)	Fat	UBN
Summer	1	i	2.89 ± 0.08^{d}	0.177 ± 0.004^{hi}
		ii	3.22 ± 0.00^{bc}	0.149 ± 0.001^{i}
	2	i	3.34 ± 0.02^{ab}	0.198 ± 0.013^{gh}
		ii	2.95 ± 0.02^{d}	0.451 ± 0.000^{a}
	3	i	2.97 ± 0.03^{d}	0.236 ± 0.019^{fg}
		ii	3.43 ± 0.02^{a}	0.274 ± 0.015^{ef}
	4	i	3.01 ± 0.05^{d}	0.192 ± 0.009^{ghi}
		ii	2.94 ± 0.02^{d}	0.312 ± 0.002^{de}

Winter	1	i	3.00±0.10^{d}	0.351±0.007^{cd}
		ii	2.98±0.06^{d}	0.378±0.010^{bc}
	2	i	3.46±0.03^{a}	0.355±0.020^{cd}
		ii	3.31±0.00^{abc}	0.413±0.003^{ab}
	3	i	2.93±0.00^{d}	0.294±0.015^{e}
		ii	2.92±0.01^{d}	0.236±0.006^{fg}
	4	i	3.18±0.03^{c}	0.421±0.019^{ab}
		ii	2.86±0.01^{d}	0.420±0.003^{ab}

Values are expressed as mean±standard deviation of two replicates. i and ii correspond to 2 independent sampling days. Different letters within the same column denote statistically significant differences at $p < 0.05$ (Tukey's test) between the milk samples; the letter "a" indicates the highest value and "e" the lowest one.

Figure 1 shows the HPLC-UV/DAD chromatograms (at 275 nm) of CoQ_9 standard solution, the unspiked and spiked (with UBN) unsaponifiable fraction of HQ raw cow milk. Since CoQ_9 was absent in HQ milk, it was therefore used as internal standard (IS) in the quantitative determination of UBN.

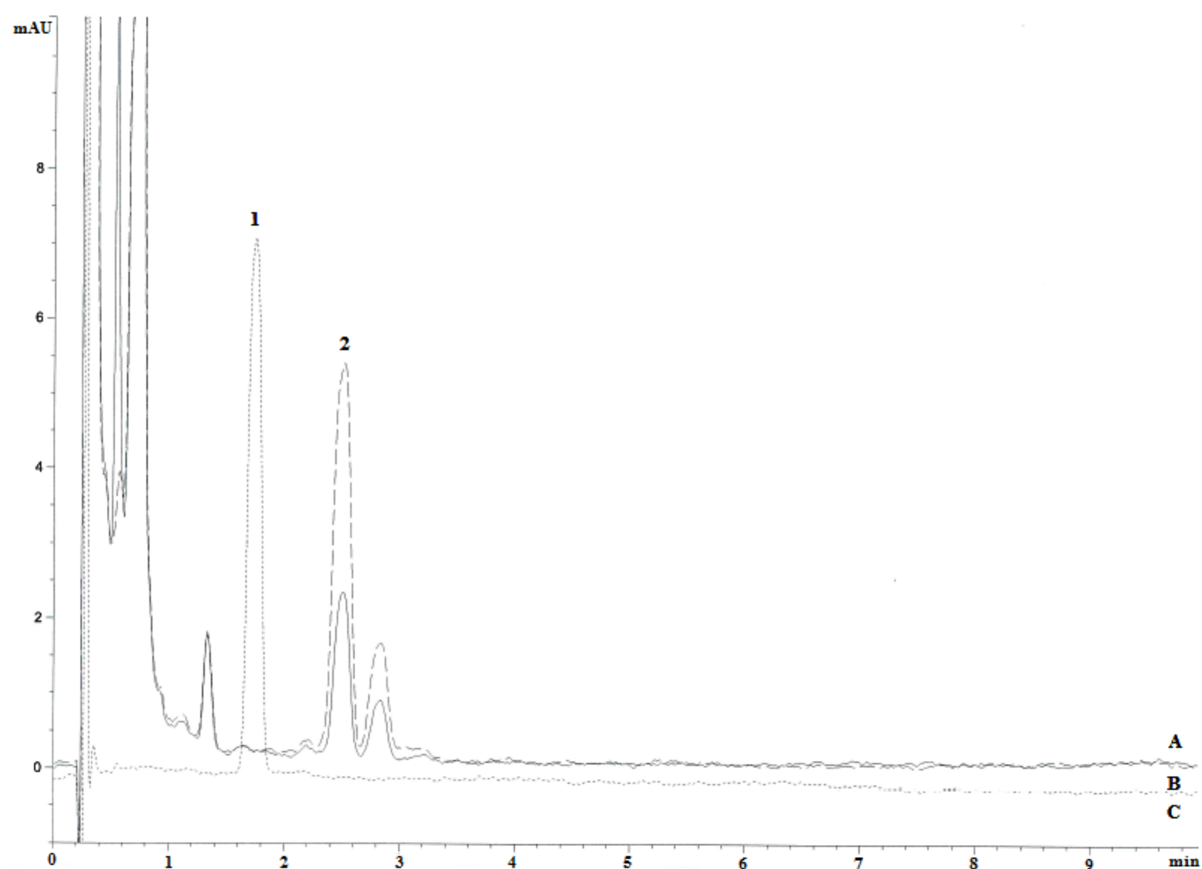

Figure 1. Overlaid HPLC-UV/DAD chromatograms (at 275 nm) of the unspiked (A) and the spiked (with UBN) unsaponifiable fraction of raw cow milk (B), as well as the CoQ_9 standard solution (C). Peak identification: 1, CoQ_9 internal standard; 2, UBN.

4. DISCUSSION

The fat content was determined to find out whether there was a correlation with the level of UBN in the HQ milk samples. Data analysis (Table 2) was carried out to emphasise the effect of each individual factor and their first-and second-degree interactions on fat and UBN contents of HQ raw cow milk.

Regarding milk fat content, data processing showed that it was significantly influenced by cowshed (CS) (28.1%) and by all interactions. However, season (S) and sampling day (SD) factors had no significant effects on the HQ raw cow milk fat content. The fat content of HQ milk significantly varied among cowsheds; in general, the highest milk fat content was found at CS no. 2, followed by 3, 1, and 4. This variation may be due to feeding with different fodders and concentrates, the lactation stage of individual animals from herds, the variability among animals and/or the CS location. The average fat content of milk varies considerably through lactation, from approximately 3% in early lactation to more than 4.5% in late lactation, and among individuals (FOX and KELLY, 2012).

Concerning the UBN content of HQ milk, it was influenced by all factors and their interactions (Table 2); however, the factors that exerted the greatest influence were S (32.9%) and CS (19.5%). The highest content of UBN was found in milk from cowshed no. 2, in which the CS x SD interaction indicated that the UBN milk content varied according to the sampling day.

Table 2. Effects of season, cowshed, sampling day, and their first- and second-degree interactions on fat (%) and UBN contents (μg/g milk).

Factor	Fat	UBN
Season (S)		
Summer	3.09	0.249^{b}
Winter	3.08	0.358^{a}
p/contribution (%)	*0.353 n.s./0.1*	*< 0.001***/32.9*
Cowshed (CS)		
Cowshed 1	3.02^{bc}	0.264^{c}
Cowshed 2	3.27^{a}	0.354^{a}
Cowshed 3	3.07^{b}	0.260^{c}
Cowshed 4	3.00^{c}	0.336^{b}
p/contribution (%)	*< 0.001***/28.1*	*< 0.001***/19.5*
Sampling Day (SD)		
i	3.10	0.278^{b}
ii	3.08	0.329^{a}
p/contribution (%)	*0.148 n.s./0.3*	*< 0.001***/7.1*
S x CS		
Summer x cowshed 1	3.06^{cd}	0.163^{e}
Summer x cowshed 2	3.15^{bc}	0.324^{c}
Summer x cowshed 3	3.20^{b}	0.255^{d}
Summer x cowshed 4	2.98^{de}	0.252^{d}
Winter x cowshed 1	2.99^{de}	0.364^{b}
Winter x cowshed 2	3.38^{a}	0.384^{b}
Winter x cowshed 3	2.93^{e}	0.265^{d}
Winter x cowshed 4	3.02^{de}	0.420^{a}
p/contribution (%)	*< 0.001***/21.6*	*< 0.001***/16.7*

S x SD		
Summer x i	3.06[b]	0.201[c]
Summer x ii	3.13[a]	0.297[b]
Winter x i	3.14[a]	0.355[a]
Winter x ii	3.02[b]	0.361[a]
p/contribution (%)	*< 0.001***/6.5*	*< 0.001***/5.5*
CS x SD		
Cowshed 1 x i	2.94[c]	0.264[d]
Cowshed 1 x ii	3.10[b]	0.263[d]
Cowshed 2 x i	3.40[a]	0.276[d]
Cowshed 2 x ii	3.13[b]	0.432[a]
Cowshed 3 x i	2.95[c]	0.265[d]
Cowshed 3 x ii	3.18[b]	0.255[d]
Cowshed 4 x i	3.10[b]	0.306[c]
Cowshed 4 x ii	2.90[c]	0.366[b]
p/contribution (%)	*< 0.001***/29.6*	*< 0.001***/11.9*
S x CS x SD		
p/contribution (%)	*< 0.001***/11.7*	*< 0.001***/5.6*

Values are expressed as mean of two replicates. Abbreviations: CS, cowshead; S, season; SD; sampling day; i and ii correspond to 2 independent sampling days. Different letters within the same column denote statistically significant differences (Tukey's test $p < 0.05$) between the milk samples; the letter "a" indicates the highest value and "e" the lowest one. Significant differences are denoted by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $p \geq 0.05$, non-significant.

The level of UBN in milk may be influenced by genetic characteristics, diet, duration of lactation, length of gestation (TANG *et al*., 2006), and metabolic changes (SOUCHET and LAPLANTE, 2007). Previous studies on the quantification of UBN were carried out using various dairy product categories that contained different fat percentages and that underwent diverse heat treatments (MATTILA and KUMPULAINEN, 2001; STRAZISAR *et al*., 2005). Thus, the comparison with our results is difficult. STRAZISAR *et al*. (2005) reported a higher UBN content (1.90 μg/g milk) in fresh cow milk (3.6% fat) than that detected in the present study (0.15-0.45 μg/g milk). The same authors noticed that UBN levels were lower in UHT milk having diverse fat content (0.46 μg/g milk in skimmed milk (0.5% fat), 1.16 μg/g milk in semi-skimmed milk (1.6% fat), and 1.70 μg/g milk in whole milk (3.5% fat)). However, MATTILA and KUMPULAINEN (2001) found a significantly lower UBN content (0.1 μg/g milk) in commercially available semi-skimmed milk (1.5% fat), probably due to the heat treatment. As already mentioned, UBN is known to be a temperature sensitive molecule; MILIVOJEVIC FIR *et al*. (2009) in fact showed that pure UBN is degraded by 72.3% after being exposed at 80°C for 120 min in the presence of UV light.

Regarding the correlation of the fat and UBN contents in HQ raw cow milk samples, the results of the current study did not show any significant relationship (r= - 0.034, p = 0.855). This lack of correlation is further confirmed by the scatter plot (**Figure 2**) developed with the fat and UBN contents (linear regression and no fit intercept). This is in contrast to the data of STRAZISAR *et al*. (2005), who noticed a positive correlation trend between the UBN and the fat contents of milk, even though it was not statistically confirmed. On the other hand, NIKLOWITZ *et al*. (2005) found a high level of UBN in human colostrum, even though its fat content was low.

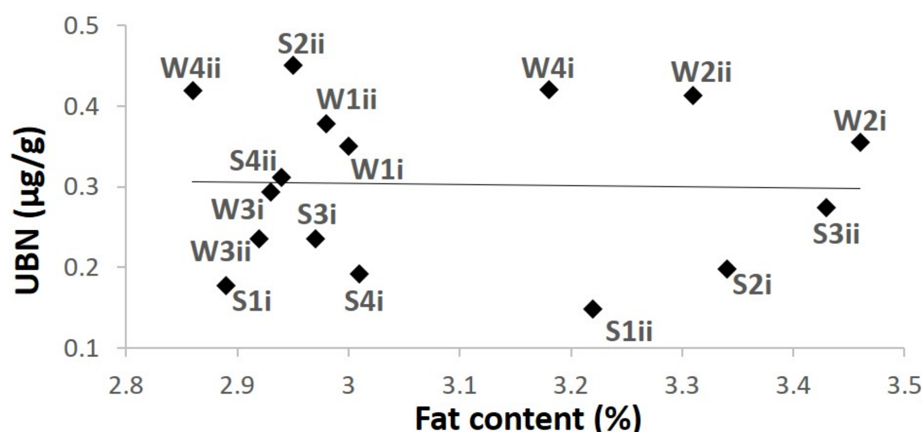

Figure 2. Scatter plot of fat content vs. UBN content. Abbreviations: S, summer; W, winter; 1-4, number of single cowsheds; i, sampling day 1; ii, sampling day 2.

The scatter plot of Fig. 2 also displays the distribution of the samples according to both variables (UBN and fat content). Only two groups can be distinguished in terms of fat content: one below 3.02% (10 samples) and the other above 3.17% (6 samples). No more clustering was evident as related to the rest of variables (cowshed, season, sampling day).

5. CONCLUSIONS

The content of UBN in Italian HQ ("Alta Qualità") raw cow milk was for the first time determined in the present study, ranging from 0.15 to 0.45 μg/g milk. Although health authorities have not yet established specific dietary intake recommendations for UBN, some researchers suggest a daily dose of 30-200 mg UBN for 19-year adult and older (EFSA, 2010). Considering that the guidelines for healthful Italian food habits published by the Italian National Institute for Research on Food and Nutrition (INRAN, 2003) recommend an average consumption of three 125-mL portions of milk and/or yogurt a day, a consumption of 250 mL of HQ raw milk would potentially provide an intake of 0.04-0.11 mg UBN. However, UBN content in pasteurised HQ milk may suffer a decline due to the heat treatment (especially UHT) and storage, even though modern pasteurization technologies applied to HQ milk with relatively low temperatures and short times (72°C for 15 sec) should limit significant thermal degradation of UBN. The results of this study indicate that the UBN content in HQ raw cow milk was significantly affected by both seasons and sampling days within the same cowshed. However, no significant correlation was found between UBN and fat content in HQ raw cow milk, even though UBN is a lipophilic antioxidant. Although an investigation involving a greater number of different cowshed types from diverse locations and product categories would be necessary to better understand the contribution level of the various factors on the increase of UBN content in milk, the adoption of the Italian HQ regulation by the EU could be a way to further improve the nutritional quality of European milk, including its UBN content.

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PAPER

THE EFFECT OF BLANCHING PRE-TREATMENT ON THE DRYING KINETICS, THERMAL DEGRADATION OF PHENOLIC COMPOUNDS AND HYDROXYMETHYL FURFURAL FORMATION IN POMEGRANATE ARILS

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ABSTRACT

This study examined the effect of blanching pre-treatment on the drying kinetics, thermal degradation of phytonutrients and hydroxymethylfurfural (HMF) formation in pomegranate arils. Pre-treated and untreated arils were dried in a cabinet dryer operated at temperatures of 55, 65 and 75°C. The efficiency of the drying process was assessed according to the effective moisture diffusivity and activation energy values. Effective moisture diffusivities ranged from 0.59×10^{-9} to 5.62×10^{-9} m^2s^{-1} and activation energies for drying were 31.82 and 76.11 kJ/mol for pre-treated and untreated arils, respectively. Six thin-layer drying models were tested, and Page and Modified Page models were found to be the most suitable. The final quality of pomegranate arils was evaluated according to their total phenolic and total anthocyanin contents, their antioxidant capacities and the rate of HMF formation. The blanching pre-treatment prior to drying produced higher retention of antioxidant compounds with less HMF content and superior sensory properties. Sensory analysis results revealed that pre-treated arils were preferred to untreated arils.

Keywords: pomegranate, drying kinetics, antioxidants, HMF, thermal degradation, blanching

1. INTRODUCTION

The pomegranate is a native plant to an area covered by Persia, Anatolia, Mesopotamia and India, and has been cultivated in the USA and Mediterranean countries (MENA *et al.*, 2013). Significant attention has been given to pomegranate fruits in recent years because of the desirable aroma, flavor and characteristic bright red color (SHAHBAZ *et al.*, 2014). Pomegranate fruits are used for the manufacture of various food products and fruit juice concentrates. Fresh pomegranates are harvested during late September, October and November. Even though there is significant demand for fresh pomegranates during the whole year, geographical and seasonal restrictions limit the fresh pomegranate supply to the market. Therefore, many food preservation techniques have been investigated to extend the shelf life of the pomegranate fruits such as canning, freezing, modified-atmosphere packaging and controlled storage of the products in addition to drying (VIUDA-MARTOS *et al.*, 2012). Traditionally dried pomegranate arils are called 'Anardana', which is widely consumed in Southern Asia and Persia. Anardana is an alternative pomegranate product enabling people to serve dried arils the whole year round. Anardana production keeps increasing together with increasing acres of pomegranate plantations in other parts of the world. Drying is one of the oldest food preservation methods that have been used from ancient times up to the modern day. The drying process extends the shelf life of the products by lowering water activity, and therefore inhibiting microbial and biochemical decay of the food (KAMİLOGLU *et al.*, 2014). However, the drying process may negatively affect food quality parameters such as the nutritional quality, the bioactive compound content, the color, and the texture (FAZAELİ *et al.*, 2013). Therefore, it is necessary to investigate the optimum drying conditions for the production of high quality, desirable and shelf life –extended foods [AKDAŞ and BAŞLAR, 2015; STURM *et al.*, 2012). Pre-treatment applications, and different drying methods and conditions, are currently being investigated for the production of high quality products (CHAETHONG and PONGSAWATMANİT, 2015; ADEDEJİ *et al.*, 2008]. The balance between low cost, fast production techniques and high quality of the final product should be established to ensure consumer acceptability. The consumer acceptability and the market value of the products are influenced by both qualitative and quantitative factors. In general, consumers tend to prefer foods at a reasonable price with a high functional compound content and improved color characteristics. Especially in recent years, together with the improvements in welfare and education, most people from all over the world have become more and more demanding with regard to functional foods (SIRO *et al.*, 2008). The major functional compounds in fruit and vegetable products are vitamins, pigments, flavonoids and phenolic acids (ZULUETA *et al.*, 2007). Phenolic compounds have been shown to be associated with inhibiting cardiovascular diseases, tumor formation and cancer development (ACOSTA-ESTRADA *et al.*, 2014; BONDIA-PONS *et al.*, 2009). However, food-processing steps can also exert negative effects on vulnerable food compounds such as vitamins, pigments and phenolic acids (CHONG *et al.*, 2009; MOUSA *et al.*, 2002). Even though most of the drying process is completed in cabinet-type dryers in the food industry, there are few studies in published literature evaluating the bioactive-compound degradation kinetics and the formation of harmful compounds. Thus information on the final quality parameters is essential for enabling better process control and the manufacture of dried goods with a higher antioxidant capacity. The objective of this study is to evaluate the drying kinetics of pomegranate arils and determine the proper drying conditions to produce dried arils with higher nutritional value. Blanched and unblanched pomegranate arils were dried using cabinet dryers at different temperatures.

The association between the thermal-processing steps and the phytonutrient content of pomegranates were determined at different temperatures. The heat and mass transfer characteristics of the dried foods determine their drying mechanisms. Hence, mathematical methods are useful in predicting the drying behavior of food commodities. This study also looked at mathematical models to determine which model best described the kinetics of the drying process in the context of degradation of phenolics and anthocyanins, as well as the formation of HMF during the process.

2. MATERIALS AND METHODS

2.1. Materials

Fresh pomegranates (*Punica granatum* L., cv. *Hicaz*) were purchased from a wholesale market hall in Şanlıurfa, Turkey, and immediately transported to the laboratory. Fruits were kept at 4°C before commencement of the tests. Folin-Ciocalteu, gallic acid and DPPH (1,1-diphenyl-2-picrylhydrazyl) were obtained from the Merck Co. (Darmstadt, Germany), and 5-(hydroxymethyl)furfural (HMF) and acetone from the Sigma Chemical Co. (St. Louis, Mo, USA). All other chemicals used were of analytical grade.

2.2. Preparation of pomegranate arils

The pomegranates were peeled manually, and the arils were separated from the fruits. The arils were separated into two groups. Half of the group was pre-treated by dipping into water with 0.1% citric acid at 80 ± 2°C for 2 minutes and afterwards immediately transferred to the cabinet dryer (i.e. pre-treated). The ratio of blanching water and pomegranate arils was 20 mL/g. After blanching, the arils were drained using soft filter papers. The other half of the arils was dried directly without any pre-treatment.

2.3. Drying procedure

The pre-treated and untreated arils were immediately transferred for cabinet drying. Cabinet drying was carried out in the dryer (Kendro Laboratory Products, Germany) at three different temperatures (55, 65 and 75°C) at an air velocity of 1.2 m/s. During drying, moisture loss was recorded and almost 10 g of samples were removed from the dryer at thirty-minute intervals. The samples were stored at −20°C until the extraction procedure.

2.4. Mathematical modeling

Page, Modified Page, Henderson and Pabis, Wang and Singh, Two Term and Logarithmic models (Table 1) were used for modeling the drying kinetics of the pomegranate arils. In these models, the moisture ratio (*MR*) was simplified to Equation 1, below, instead of $(M-M_e)/(M_0-M_e)$, as the value of M_e is relatively small compared to M or M_0.

$$MR=M/M_0 \tag{1}$$

where M is the moisture content at time t, M_0 is the initial moisture content and M_e is the equilibrium moisture content. Goodness of fits were determined according to the evaluation of R^2 and root mean square error (RMSE). Higher values of R^2 and smaller RMSE values (Equation 2) indicated a better fit of the experimental data to the model. The

correlation coefficient (R^2) and root mean square error (RMSE) were taken into consideration to select the best model.

$$RMSE = \left[\frac{1}{N}\sum_{i=1}^{N}(MR_{exp.i} - MR_{pre.i})^2\right]^{1/2} \quad (2)$$

Table 1. Models used for determining thin-layer drying curves.

Model	Equation	Reference
Page	*MR* = exp(-kt^n)	(PAGE, 1949)
Modified Page	*MR* = exp[-(kt)^n]	(OVERHULTS *et al.,* 1973)
Henderson and Pabis	*MR* = *a* exp(-kt)	(HENDERSON and PABIS, 1961)
Wang and Singh	*MR* = 1+*at*+*bt*^{28}	(WANG and SINGH, 1978)
Two Term	*MR* = *a* exp(k_0t)+ *b* exp(k_1t)	(HENDERSON, 1974)
Logarithmic	*MR* = *a* exp(-kt) + c	(YAGCİOGLU *et al.,* 1999)

2.5. Computation of effective moisture diffusivity and activation energy

Fick's second law was adapted to spherical shapes for unstable diffusion conditions as follows (CRANK, 1975):

$$MR = \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}exp(-n^2\pi^2\frac{D_{eff}t}{r^2}) \quad (3)$$

For extended drying periods (Equation 4) can be further simplified as follows (DOYMAZ, 2012):

$$ln(MR) = ln(\frac{6}{\pi^2}) - (\frac{\pi^2 D_{eff}t}{r^2}) \quad (4)$$

Plotting $\ln(MR)$ versus time enables calculation of the effective moisture diffusivity and a straight line can be obtained with a slope of (K) as expressed in (Equation 5) below:

$$K = \frac{\pi^2 D_{eff}}{r^2} \quad (5)$$

The activation energy for diffusion was determined from the slope of the Arrhenius-type equation (Equation 6):

$$D_{eff} = D_0 exp\left(-\frac{E_a}{RT}\right) \quad (6)$$

2.6. Modeling the degradation of total phenolic and total anthocyanin contents

Thermal degradation of most bioactive compounds follows first order kinetics (Equation 7):

$$C_t = C_0 \, x \, exp\,(\pm k_0 \, x \, t) \quad (7)$$

Where C_t and C_0 are the total phenolic (TP) and total anthocyanin (TA) contents after heating time t and t=0 minutes, respectively, and k is the kinetic constant. The natural logarithms of the ratios of C_t and C_0 against time were plotted, and by using the slope of this graph, half-life ($t_{1/2}$) was deduced according to Equation 8:

$$t_{1/2} = \frac{ln2}{k} \qquad (8)$$

2.7. Extraction of bioactive compounds

The arils were firstly homogenised with 80% acetone (0.01% HCl) using a pestle and mortar. Extraction was carried out in a shaking incubator (Labline, USA) operated at 50°C and 180 rpm for 60 minutes. Then, the slurry was centrifuged at 6000 rpm for 8 minutes (Hitachi CT6E, Taiwan), and the supernatant was collected in falcon tubes and stored at −40°C until analysis.

2.8. Determination of total phenolic (TP) content

The TP contents of the samples were determined by the Folin-Ciocalteu method (SINGLETON and ROSSI, 1965) using the gallic acid standard curve. The absorbance of each sample was read at 750 nm in a spectrophotometer (Libra, Biochrom, UK) against the blank, and the results were expressed as mg of gallic acid equivalent per kilogram of pomegranate aril (mg GAE kg^{-1}).

2.9. Determination of total anthocyanin (TA) content

The TA contents of the samples were determined using a pH-differential method (GIUSTI and WROLSTAD, 2001) by a UV-vis spectrophotometer (Biochrom Libra, UK). The pomegranate extracts were mixed with pH 1.0 (0.025 M potassium chloride) and pH 4.5 (0.4 M sodium acetate) buffers, and the absorbance values were recorded at 520 and 700 nm. The results were expressed as mg of cyanidin 3-glucoside per kilogram dry weight of the pomegranate aril.

2.10. Determination of antioxidant capacity

The antioxidant capacity of the samples was estimated by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay as described by KARAASLAN *et al.*, 2004a. An 0.1 mL amount of various concentrations of the extracts diluted in ethanol was added to 2.9 mL of 0.1 mM of the DPPH solution. The decrease in absorbance at 517 nm was measured after 30 minutes of incubation at room temperature. The inhibition concentration (EC_{50}) – the amount of sample concentration (g/mL db) necessary to decrease the initial DPPH concentration by 50% – was calculated after plotting the percentage inhibition versus the extract concentration curve.

2.11. Determination of hydroxymethylfurfural (HMF)

The HMF contents of the arils were determined using a spectrophotometric method (ACAR *et al.*, 1999). Briefly, 5 g of dried sample was crushed and diluted with distilled water up to 50 mL. Then, 5 mL of this solution was mixed with p-toluidine solution (10 g/100 mL) and 1 mL of barbituric acid solution was added to the mixture after 2 minutes.

The absorbance of the mixture was noted on the spectrophotometer at 55 nm and calculations were done using the HMF standard calibration curve.

2.12. Sensory analysis

Dried samples (pre-treated and untreated at three different temperatures for a total of 6 samples) were randomly coded and served to panelists. Samples were analyzed organoleptically by 20 trained panelists in terms of color, shape, texture, flavor-aroma and overall acceptability. Evaluation was scored on a ten-point scale (0–4, very bad – bad; 5–9, acceptable – excellent), according to GOULD (1977).

2.13. Statistical analyses

Experimental data were subjected to analysis of variance (ANOVA) using SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA) and *P* values less than 0.05 were taken into consideration. The Duncan test was used as a post hoc test after applying the homogeneity test. The parameters of kinetic models were determined by using Sigma Plot software (Sigma Plot 10.0 Windows version, SPSS Inc.). All the experiments were repeated three times.

3. RESULTS AND DISCUSSIONS

3.1. Drying kinetics and modelling

The initial moisture content of the fresh and pre-treated pomegranate arils were 79.82 ± 0.44% and 80.13 ± 0.67% (w.b.), respectively. The samples were dried until they had 16% (w.b.) moisture content by considering sensory properties and current literature data. A graphical representation of MR values versus time belonging to pre-treated samples is shown in Fig. 1.

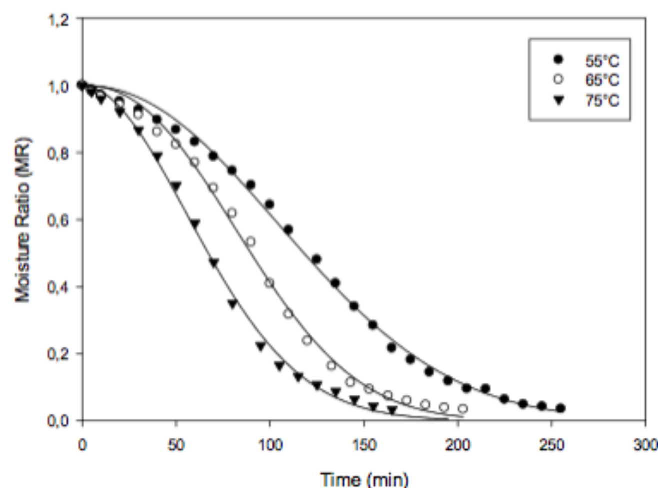

Figure 1. Drying curve of pre-treated pomegranate arils (plotted by the page model) in cabinet dryer.
(*MR* Moisture Ratio: The ratio of dry basis moisture content at any time to initial dry basis moisture content).

As expected, the drying temperature had a significant effect on the drying kinetics of the samples. The time required to dry pre-treated pomegranate arils in a cabinet dryer were 168, 204 and 237 minutes at 75, 65 and 55 °C, respectively, while for untreated samples the values were 244, 448 and 945 minutes at the same temperatures. It is clear that pre-treatment application had a significant effect on the drying time of the arils. In a pomegranate drying study using an air circulating oven (v = 1.3 m/s), drying times were reported to be 330, 520 and 1020 minutes at 55, 65 and 75°C, respectively (BAŞLAR *et al.*, 2014). Six thin layer drying models were evaluated by fitting the experimental data and considering the highest R^2 and lowest RMSE values. The results showed that Page and Modified Page models were the most relevant models in all drying conditions. Model constants with statistical evaluations are demonstrated in Table 2. Mathematical models used in drying applications are useful for designing new or improved existing drying systems. Such models are directly related to the temperature and velocity of the drying medium inside the mechanical dryer as well as the energy cost (BABALIS and BELESSIOTIS, 2004).

3.2. Effective moisture diffusivity and activation energy of the drying process

Knowing the effective moisture diffusivity is necessary for designing and modeling mass transfer processes (SHARMA and PRASAD, 2004). The *Deff* values of the pre-treated samples in the cabinet dryer ranged from 2.87×10^{-9} to 5.62×10^{-9} m^2/s and the untreated samples from 0.59×10^{-9} to 2.92×10^{-9} m^2/s (Table 3). Higher *Deff* values were observed in pre-treated samples that are associated with faster removal of moisture and thus the faster drying of samples. Similar effective moisture diffusivity results were demonstrated by studies investigating pomegranate aril drying (DOYMAZ, 2012; BAŞLAR *et al.*, 2014; KARAASLAN *et al.*, 2014b; MINAEI *et al.*, 2011;HII *et al.*, 2009). The *ln(Deff)* value was plotted against the reciprocal of absolute temperature to determine the activation energy and Equation (6) was used to determine the activation energy. The activation energy may be defined as the energy barrier that must be overcome in order to activate moisture diffusion. Therefore, determination and comparison of the *Ea* values are important in drying applications (KARAASLAN *et al.*, 2014b). The activation energy was 31.82 kJ/mol for the pre-treated arils and 76.11 kJ/mol for untreated arils. Pre-treatment of the samples brought about a significant decrease in the activation energies of the samples. BAŞLAR *et al.*,(2014) reported the *Ea* value as 44.798 kJ/mol for cabinet drying of pomegranate arils. In a pomegranate aril drying study, pre-treated (dipping in alkali emulsion of ethyl oleate) samples had lower activation energy than the control group (DOYMAZ, 2012).

3.3. The changes in total phenolic compounds and anthocyanin content

Anthocyanins are responsible for giving the characteristics red color to pomegranate arils, and the arils are rich in anthocyanins and other colorless phenolic compounds. Numerous studies have showed the health benefits of phenolics present in pomegranate arils (KARAASLAN *et al.*, 2014a). However, the stabilities of these compounds can easily be affected by temperature increases and the presence of oxygen (VERBEYST *et al.*, 2010). Therefore, the determination of such substances is important for process optimization. The anthocyanin content of the samples declined from 824.65 ± 87.27 (mg kg^{-1}, d.b.) to 813.83 ± 79.91 (mg kg^{-1}, d.b.), and total phenolic compounds declined from 7433.04 ± 685.51 (mg kg^{-1}, d.b.) to 6863.86 ± 630.27 (mg kg^{-1}, d.b.) as pre-treatment was applied.

Table 2. Statistical data of the six thin-layer mathematical models applied to the drying data.

Condition	T (°C)	Model	R^2	RMSE	k	n	a	b	k_0	k_1	c
UNTREATED	55	**1**	**0.9914**	0.0203	3.1471	1.9017					
		2	**0.9914**	0.0203	0.0010	1.8922					
		3	0.9124	0.0637	0.0014		1.0919				
		4	0.9897	0.0458			0.0004	5.3849			
		5	0.9114	0.0651			0.5394	0.5448	0.0009	0.0009	
		6	0.9108	0.0039	0.0029		6.2423				-5.348
	65	**1**	**0.9955**	0.0309	4.6778	2.076					
		2	**0.9955**	0.0310	0.0080	2.076					
		3	0.9068	0.0779	0.0074		1.1079				
		4	0.9845	0.0614			0.0027	1.8017			
		5	0.9073	0.0774			0.5481	0.5561	0.0078	0.0078	
		6	0.8345	0.0547	0.0058		4.2418				-4.154
	75	**1**	**0.9873**	0.0323	8.8847	1.8517					
		2	**0.9873**	0.0323	0.0058	1.8488					
		3	0.9271	0.0738	0.5724		1.1417				
		4	0.9577	0.0617			0.0029	2.1854			
		5	0.9303	0.0716			0.5673	0.5673	0.0060	0.0060	
		6	0.9668	0.0515	0.0073		5.2346				-4.132
PRE-TREATED	55	**1**	**0.9964**	0.0183	6.0977	1.9132					
		2	**0.9962**	0.1830	0.0063	1.9132					
		3	0.9249	0.0770	0.0061		1.1105				
		4	0.9840	0.0369			0.0023	0.5421			
		5	0.9248	0.0762			0.5537	0.5487	0.0061	0.0061	
		6	0.8978	0.0184	0.6913		1.2710				-2.026
	65	**1**	**0.9960**	0.0187	4.6787	2.0874					
		2	**0.9960**	0.0188	0.0081	2.0874					
		3	0.9528	0.0854	0.0799		1.1104				
		4	0.9852	0.0451			0.0034	1.8446			
		5	0.9061	0.0839			0.5572	0.5572	0.0071	0.0071	
		6	0.8632	0.1239			3.0045				-0.353
	75	**1**	**0.9816**	0.0425	0.0014	1.4628					
		2	**0.9816**	0.0425	1.4631	1.4628					
		3	0.9611	0.0849	0.0110		1.0921				
		4	0.9633	0.0624			0.0082	1.4365			
		5	0.9616	0.0850			0.5473	0.5473	0.0103	0.0103	
		6	0.9684	0.0058	0.0068		1.3641				-0.285

As demonstrated in Table 4, more anthocyanin and phenolic contents were preserved in the samples dried at 55°C compared to other samples, while the increase in temperature caused more phenolic and anthocyanin degradation. The TA concentration of the pre-treated samples decreased to 19% in a cabinet dryer at 75°C, to 25% at 65°C, and to 29% at 55°C. The TA in untreated samples in a cabinet dryer declined to 13%, 19% and 22% under the same temperature regimes. A decrease of TP content of the arils was also observed.
The TP content of the pre-treated samples in the cabinet dryer dropped to 36% in the cabinet dryer at 75°C, to 41% at 65°C and to 54% at 55°C. The TP content of the untreated

samples in the cabinet dryer declined to 22%, 28%, and 33% under the same conditions at 75, 65, 55°C, respectively (Table 4). It is clear that both TP and TA are preserved better in pre-treated samples. BAŞLAR *et al*. (2014) reported increasing TP retention with increasing temperature. The TP retentions of pomegranate arils in their study were 78.61, 81.82 and 84.11% for 55, 65 and 75°C, respectively. BCHIR *et al.* (2012) reported that the phenolic content of the arils decreased to 40% (w.b.), and the anthocyanin content decreased to 25% (w.b.) for cabinet drying of pomegranates, which are much lower than our results.

Table 3. Effective Moisture Diffusivities and Activation Energies of Drying.

Experimental Samples	Temp (°C)	D_{eff} (m^2/s)	*Ea* (kJ/mol)	R^2
Untreated Cabinet	55	0.59×10^{-9}	76.11	0.9708
	65	1.71×10^{-9}		
	75	2.92×10^{-9}		
Pre-treated Cabinet	55	2.87×10^{-9}	31.82	0.9844
	65	3.77×10^{-9}		
	75	5.62×10^{-9}		

Table 4. Effect of Drying Temperatures and Pre-treatment Application on Half-Life ($t_{1/2}$), Drying Time and Total Phenolic (TP) – Total Anthocyanin (TA) Contents of Pomegranate Arils Dried in a Cabinet Dryer.

Pre-treated					**Untreated**				
Temperature (°C)	$t_{1/2}$ (min)	R^2	Drying Time (min)	C_t/C_0 at drying time	Temperature (°C)	$t_{1/2}$ (min)	R^2	Drying Time (min)	C_t/C_0 at drying time
Kinetic Results of TP degradation									
55	211	0.982	237	0.54	55	629	0.958	945	0.33
65	173	0.964	204	0.41	65	190	0.984	448	0.28
75	140	0.938	168	0.36	75	147	0.965	244	0.22
Kinetic results of TA degradation									
55	124	0.974	237	0.29	55	793	0.988	945	0.22
65	75	0.988	204	0.25	65	154	0.970	448	0.19
75	53	0.990	168	0.19	75	83	0.975	244	0.13

3.4. Thermal degradation kinetics of total phenolic compounds and anthocyanins

The time-dependent TP and TA degradation data were used to develop an Arrhenius model to predict the bioactive compounds' degradation during the drying. Table 5 shows that the model adequately fits the degradation kinetics ($0.9131 < R2 < 0.9754$). A higher *Ea* value implies the increasing temperature dependence of TP–TA degradation. Table 5 shows the *Ea*, half-life ($t_{1/2}$) and final TP–TA contents at the drying point. TA degradation was less sensitive to heat treatment under both these conditions compared to TP degradation. In addition, pre-treated samples have a higher degradation rate compared to untreated samples. However, the final TP and TA contents were higher in pre-treated samples despite the higher degradation rate they experienced (Table 4). The final concentrations of TP and TA in untreated samples were lower than pre-treated samples, even if *Ea* values were lower. So, it is important to evaluate drying kinetics and phytonutrient degradation kinetics in combination. Also, the necessity to control the time

to terminate the drying at a desired point is crucial. BAŞLAR *et al*. (2014) reported higher retention of TP and TA with increasing temperature, while on the other hand they demonstrated higher degradation rates of TP and TA at higher temperatures. They also concluded that the combination of time and temperature in the drying process is important.

Table 5. Activation Energy (E_a) Values Obtained from Arrhenius Model for Total Phenolic (TP) and Total Anthocyanin (TA) Degradation.

Condition	Arrhenius model E_a ($kJ\ mol^{-1}$)	R^2
TP degradation kinetic		
Pre-treated	32.53	0.9428
Untreated	72.65	0.9641
TA degradation kinetic		
Pre-treated	41.60	0.9131
Untreated	102.27	0.9754

3.5. Antioxidant capacities of dried pomegranate arils

The antioxidant capacities of the final products were significantly affected by pre-treatment application. Pre-treated arils had a higher antioxidant capacity than untreated arils (Fig. 2). The resulting higher antioxidant capacity of the pre-treated arils is linked to the shorter drying operation, which may protect the bioactive compounds. The temperature also had a significant effect on the antioxidant capacities of dried arils (Fig. 2).

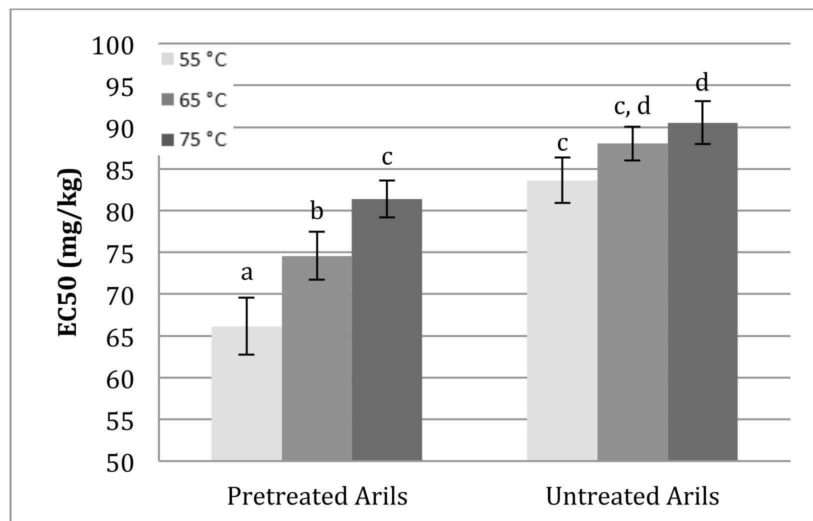

Figure 2. Antioxidant capacities of dried pomegranate arils depending on the drying conditions.

The highest antioxidant capacity value belonged to the pre-treated arils dried at 55°C. The antioxidant capacities were decreased as temperature increases, and this may result from degradation of heat sensitive bioactive compounds. The correlation between the antioxidant capacity and antioxidant compounds of the dried pomegranates were evaluated individually. The correlations among the data were determined using Pearson's correlation coefficient. The correlations between the TPC and antioxidant capacity (r = - 0.833) and TA and antioxidant capacity (r = - 0.774) were found to be statistically significant ($P < 0.01$).

3.6. Formation of HMF (hydroxymethylfurfural)

Several factors, such as dry matter content (or °Bx), aw, processing or storage temperature may affect the HMF formation in the dried fruits and concentrated juices (LAVELLI and VANTAGGI, 2009). The content of HMF in the dried pomegranate arils is depicted in Fig. 3. A higher HMF content was found in untreated samples in this study.. In addition, increasing temperature led to higher HMF content in the final product. HMF contents of the dried samples were between 5.22 and 16.34 mg/kg. The lowest HMF content was found in pre-treated samples dried at 55°C. The harsh effect of temperature increase on the HMF content of dried samples has been illustrated in other studies (ZANONİ *et al.,* 1999; PEKKE *et al.,* 2013; WOJDYŁO *et al.,* 2014).

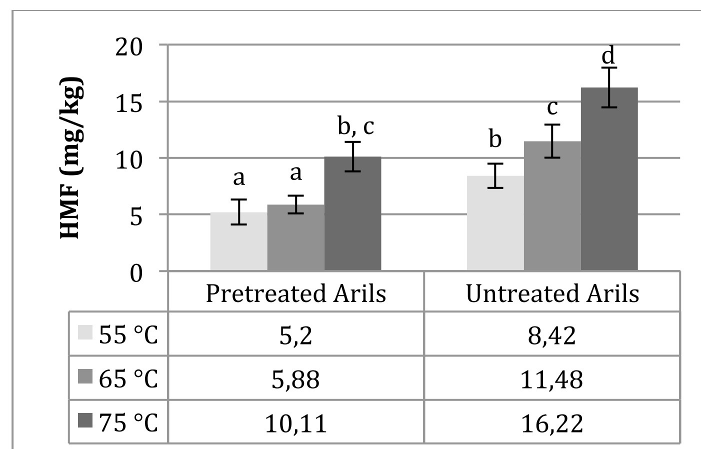

Figure 3. The effect of pre-treatment conditions and temperature on HMF formation.

3.7. Sensory analysis

The untreated and pre-treated samples had statistically significant differences in color, shape, texture and overall acceptability, while there were no statistically significant differences in flavor and aroma ($P < 0.05$). The pre-treated samples were more *acceptable–excellent* to the panelists across all sensory properties. Apart from color, the temperature had no statistically significant effect on the sensory properties of the dried samples. The average score for the overall acceptability of pre-treated samples was 8.76, and 5.34 for untreated samples.

4. CONCLUSIONS

The use of controlled drying conditions and pre-treatment applications may assist the production of better quality dried arils. Drying rates were considerably higher for pre-treated arils under all drying conditions. The drying process was completed in a shorter time for pre-treated samples, and thus, pre-treated arils had a higher phenolic and anthocyanin content in comparison to untreated samples, even if the thermal degradation rate of the bioactive compounds were higher in the pre-treated samples. Pre-treated samples had a higher antioxidant capacity and lower HMF content at all temperatures. This study is a good example that reflects the necessity of evaluating the kinetics of drying and the bioactive compounds' degradation together.

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NOMENCLATURE

Symbol	Description	Unit
M	Moisture content	g/g dry solid
M_0	Initial moisture content	g/g dry solid
M_e	Equilibrium moisture content	g/g dry solid
MR	Moisture ratio	dimensionless
t	Time	min
D_{eff}	Effective moisture diffusivity	m^2/s
RMSE	Root mean square error	
N	Number of observations	
n	constant, positive integer	
r	radius	m
$MR_{exp,}$ i^{th}	moisture ratio value experimentally determined	
$MR_{pre,}$ i^{th}	predicted moisture ratio value	
R	Gas constant	8.314 $J.mol^{-1}.K^{-1}$
R^2	Determination of coefficient	
E_a	Activation energy	$kJ.mol^{-1}$
C	Concentration of total phenolic compound or anthocyanin	mg/kg d.b.
C_t	Concentration of total phenolic compound or anthocyanin at time t	mg/kg d.b.
C_0	Initial concentration of total phenolic compound or anthocyanin	mg/kg d.b.
d.b.	Dry basis	
w.b.	Wet basis	
rpm	revolution per minute	

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PAPER

AN ANCIENT CROP REVISITED: CHEMICAL COMPOSITION OF MEDITERRANEAN PINE NUTS GROWN IN SIX COUNTRIES

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ABSTRACT

The aim of the study was to analyze the proximate composition of pine nuts harvested from 15 growing areas in Chile, Argentina, Italy, Spain, Turkey, and Israel. The main component was fat, followed by protein. Pine nuts from Chile and Argentina were similar, zones with the highest thermal oscillation and rainfall. Italian pine nuts had the highest fiber content, while Spanish nuts had the highest fat content. Israel presented the highest number of dry months, where pine nuts contained more protein and minerals, while nuts from Turkey showed an intermediate position. Minimum and maximum average temperatures, amount of dry months, and thermal oscillation affected the chemical composition.

Keywords: agro-climatic conditions, chemical composition, growing zone, pine nuts, *Pinus pinea*

1. INTRODUCTION

Pinus pinea L., also known as stone pine, is one of the nine most important tree nut species in the world. Pine nut is the most expensive nut worldwide, while stone pine is one of the oldest fruit trees, as demonstrated by archaeological remains that evidence its cultivation in the pre-Christian era (ROTTOLI and CASTIGLIONI, 2011). A risk for depletion and degradation of European stone pine forests has appeared, in relation to emerging threats such as climate change (MILANO *et al*., 2015) and demographic dynamics (UNITED NATIONS, 2015). Climate change has affected the production of pine nuts worldwide (MUTKE *et al*., 2005), and efforts are being made to improve the growth of valuable Mediterranean forests in non-traditional areas. The species is endemic to the Mediterranean basin, cultivated mainly in Spain, Portugal, Italy, Turkey, and Tunisia. Pine nuts are part of the traditional Mediterranean diet, which is well recognized for reducing cardiovascular risk factors (REES *et al*., 2014; ROS, 2015).

As part of the Mediterranean diet, pine nuts contribute to reducing risk factors of cardiovascular disease (CVD), type-2 diabetes, and some types of cancer (ALASALVAR and BOLLING, 2006; SABATÉ and ANG, 2009; BAO *et al*., 2013; ESTRUCH *et al*., 2013; SORLÍ *et al*., 2013). In general, nuts are energy-dense foods, since they contain 4.4 to 7.4 g kg^{-1} fat (ROS and MATAIX, 2006; RYAN *et al*., 2006; KORNSTEINER-KRENN *et al*., 2013; USDA, 2016). However, nut consumption improves blood lipid levels (SABATÉ *et al*., 2010) and reduces risk factors of CVD (KRIS-ETHERTON *et al*., 2008). The cardio-protective constituents of pine nut oil include unsaturated fatty acids, phytosterols, tocopherols, and squalene, among other bioactives (WOLFF and BAYARD, 1995; MAGUIRE *et al*., 2004; ALASALVAR and BOLLING, 2006; BOLLING *et al*., 2011). Moreover, although nuts are high fat and energy dense foods, their consumption has been associated with reduced body mass index (BMI) (BES-RASTROLLO *et al*., 2009; IBARROLA-JURADO *et al*., 2013; LUTZ and LUNA, 2016). Pine nuts are not only a good source of fat, but they also contain high levels of proteins, they supply various vitamins (E, B_6, niacin, folic acid), minerals (potassium, phosphorus, magnesium, zinc, iron, copper), and a variety of phytochemicals, including phenolic compounds (NERGIZ and DÖNMEZ, 2004; EVARISTO *et al*., 2010; BOLLING *et al*., 2011; LUTZ *et al*., 2016). EVARISTO *et al.* (2010) reported significant differences in the mineral profile and other chemical components of pine nuts grown in different regions, suggesting that environment and soil types have an important influence. VANHANEN and SAVAGE (2013) also reported differences in minerals probably due to soil conditions, climate and growing practices.

Pine nuts supply has been extremely affected by *Leptoglossus occidentalis* Heideman, an insect spread in all the major producing countries, which represents an elevated economic impact in global markets (BLOOMBERG BUSINESS, 2013). On the other hand, given the high nutritional and outstanding organoleptic quality of pine nuts, its demand is increasing worldwide, reaching high prices (INTERNATIONAL NUT AND DRIED FRUITS, 2016), which represents an opportunity to produce the seeds in non-conventional growing areas, including the southern hemisphere.

Models at different scales have been developed to establish relationships between stone pine productivity and several variables, including climatic ones (CALAMA *et al*., 2011), but no reference has been found on their impact on pine nut quality.

The chemical characterization of pine nuts grown in different areas is important due to health, commercial, and genetic concerns. Taking into account that most available composition data have been obtained from the main productive countries, the aim of the study was to assess the current pine nut proximate composition across six countries: three located in the traditional growing areas (Italy, Spain, and Turkey), and three in areas where there is no current commercialization of pine nuts (Israel, Argentina, and Chile),

accessible to authors for seed collection. The study focuses on the chemical composition, and the impact of climate variables on the composition of pine nuts harvested in these countries.

2. MATERIALS AND METHODS

2.1. Study area

Fifteen areas were selected for collecting pine nuts in Italy, Turkey, Spain, Israel, Argentina, and Chile. Average climatic variables of locations from where pine nuts were grown are summarized in Table 1, and their distribution is shown in Fig. 1.

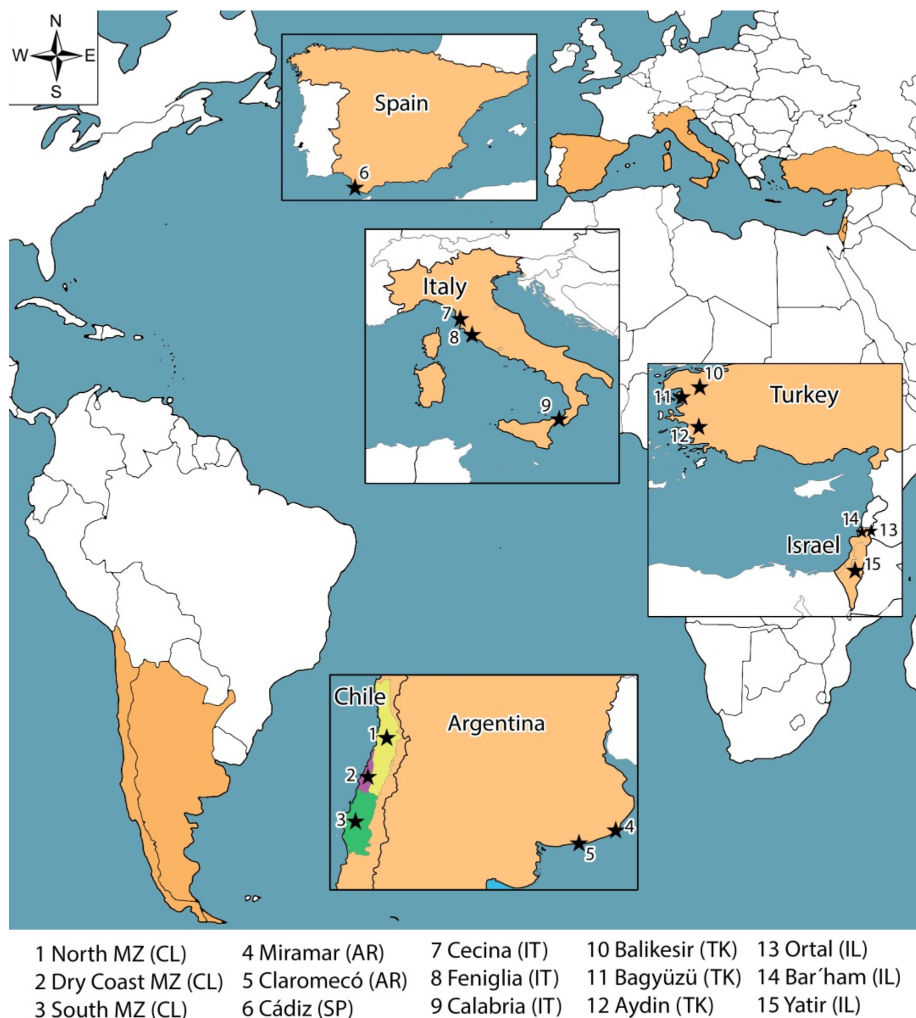


Figure 1. Distribution of locations in six countries where pine nuts were collected.

Table 1. Location and mean climatic variables of harvest sites of *Pinus pinea* L. nuts.

Location	Country	Annual rainfall (mm)	Annual average temperature (°C)	Dry months† (N°)	Maximum average temperature (°C)	Minimum average temperature (°C)	Thermal oscillation (°C)	Latitude	Longitude	Soil type
Miramar		660.7	15.1	5	22.1	8.0	14.1	38°16'S	57°50'W	Dunes afforested with conifers, poor in organic matter. Entisol (FREDES *et al.*, 2009)
Claromecó		758.4	14.5	4	21.2	7.9	13.3	38°51'S	60°04'W	Clayey granular soils with 3-5% of organic matter, moderately acids. Molisols. Imperfect drainage (CARBONE and PICCOLO, 2002).
Mean	**Argentina**	**709.6**	**14.8**	**5**	**21.7**	**8.0**	**13.7**			
North MZ		383.7	14.1	9	21.9	7.5	14.3	33°26'S	71°04'W	Alluvial origin (alfisols, mollisol, entisols) (ALBERS, 2012).
Dry Coast MZ		648.3	13.6	8	21.0	7.0	14.0	35°14'S	72°12'W	Granitic origin (alfisols, inceptisols (ALBERS, 2012).
South MZ		1,047.0	13.2	7	19.8	7.5	12.3	37°54'S	72°40'W	Volcanic origin (altisols, red clay) (ALBERS, 2012).
Mean	**Chile**	**693.0**	**13.6**	**8**	**20.9**	**7.3**	**13.5**			
Ortal		830.0	14.7	6	20.6	8.9	11.7	33°05'N	35°50'E	Volcanic origin (ORENSTEIN *et al.*, 2001). Basaltic brown Mediterranean soils and basaltic lithosols (DAN *et al.*, 1975).
Bar'ham		682.1	16.2	7	20.4	12.0	8.4	33°04'N	35°26'E	Volcanic origin (ORENSTEIN et al., 2001). Pale rendzinas (DAN *et al.*, 1975),
Yatir		275.0	17.6	8	22.4	12.8	9.6	31°18'N	35°01'E	Aeolian origin loess with a clay-loam texture, overlying chalk and limestone bedrock (NAAMA *et al.*, 2012). Brown lithosols and loessial arid brown soil (DAN *et al.*, 1975)
Mean	**Israel**	**595.7**	**16.2**	**7**	**21.1**	**11.2**	**9.9**			

Table 1. Continues.

Cecina (LI)		833.6	14.5	4	19.8	9.1	10.7	43°19'N	10°30'E	Alluvial soils, sometimes with shallow water table (calcaric cambisols, fluvisols and gleysols), clay accumulation along the profile (COSTANTINI *et al.*, 2004).
Feniglia (GR)		455.1	13.2	4	16.0	10.3	5.7	42°25'N	11°12'E	Sandy soils (PIRAINO *et al.*, 2012).
Calabria (RC)		546.8	18.3	6	22.5	14.1	8.3	39°19'N	16°21'E	Eroded soils (eutric and calcaric regosols) with accumulation of carbonates and soluble salts, rich in iron oxides and clay. Volcanic soils (umbric andosols) (COSTANTINI *et al.*, 2004).
Mean	**Italy**	**611.8**	**15.3**	**5**	**19.4**	**11.2**	**8.2**			
Cádiz	**Spain**	**524.0**	**18.7**	**5**	**21.7**	**15.6**	**6.1**	36°32'N	06°17'W	Litoral dunes (entisols). Sandy, poor soils (MUÑOZ and GRACÍA, 2009).
Balikesir		576.8	14.6	6	20.5	8.9	11.5	39°39'N	27°53'E	Sandy-loamy soil texture, neutral pH, non-calcareous or slightly calcareous, salt-free and organic matter weak (YILMAZ and SATIL, 2017).
Bagyuzu		743.2	13.1	5	18.9	8.8	10.1	39°18'N	26°58'E	Rough broken land (brown forest soil material) (OAEKS and ARIKOK, 1954).
Aydin		651.7	17.5	7	24.5	11.9	12.6	37°50'N	27°51'E	Alluvial and youthful soils (OAEKS and ARIKOK, 1954).
Mean	**Turkey**	**657.2**	**15.1**	**6**	**21.3**	**9.9**	**11.4**			

Climatic data sources: Argentine Meteorological Service (www.smn.gov.ar); Chilean Environmental Information System (www.inia.cl); The Israel Meteorological Service (www.ims.gov.il); Italian Army Aeronautic Meteorological Service (www.meteoam.it); State Meteorological Agency (www.aemet.es); Turkish Meteorological Service (www.mgm.gov.tr).

†Dry months were calculated as those with monthly rainfall/monthly potential evapotranspiration <0.5.

2.2. Materials

Stone pine seeds were collected from planted trees (none corresponds to cultivars) in 2013/14 in Turkey (n=3 zones), Israel (n=3 zones), Spain (n=1 zone), Italy (n=3 zones), Argentina (n=2 zones) and Chile (n=3 zones) in 2013; a minimum of 500 pine nuts from 10 trees were harvested in each collection area. Chilean stone pine seeds were collected from *Pinus pinea* L. planted trees distributed in three macrozones located between 30.82°N and 38.99°S (LOEWE *et al*., 2015). 167 trees were sampled taking into account the macrozone size and variability of the most variable chemical contents. From each tree, 500 pine nuts were harvested.

Samples were harvested during winter since it corresponds to the maturation season according to ABELLANAS and PARDOS (1989). Once obtained, in-shell pine nuts were kept in plastic nets individually tagged at room temperature until manually shelled. For sample preparation, shelled nuts were dried at 40°C until moisture reached 4 g kg^{-1}. All the seeds were ground with a kitchen processor (Moulinex®) and sieved to 0.5 mm, and then frozen in sealed plastic bags at -20°C until analyses.

2.3. Methods

All reagents and solvents were analytical grade chemicals from Merck (Darmstadt, Germany). Proximate analyses were performed using AOAC methodologies (AOAC, 2012). Protein content was determined by Kjeldahl assay (AOAC 920.54) using a nitrogen digestor DK6 (VELP®) and a nitrogen distiller UDK 129 (VELP®), applying factor of 5.3 to convert nitrogen to proteins (GREENFIELD and SOUTHGATE, 1972). Crude fat was assessed using the AOAC method 920.39, moisture was determined using the AOAC method 945.15, and ash was determined using the AOAC method 942.05. Total dietary fiber (TDF) was measured using the AOAC enzymatic-gravimetric method 991.43 using the MEGAZYME K-TDRF 05/12 kit supplied by Megazyme®.

2.4. Statistical analysis

Chemical analyses were done in triplicate; each replicate was quantified in duplicate, unless stated otherwise. All data given represent mean values ± standard error (SE). Data were compared using heteroscedastic ANOVA, and statistical significance was determined with an LSD test ($P<0.05$). The relative contribution of climatic variables to chemical components was estimated using CART (Classification and Regression Trees) algorithms (BREIMAN, 1999). As confirmatory analysis, the groups suggested by the identified climatic variables thresholds were also compared by ANOVA. Finally, a principal component analysis (PCA) was applied, generating a biplot for chemical composition of pine nuts and climate variables of different countries. Analyses were performed using the software Infostat® and its interface with the software R® (DI RIENZO *et al*., 2014).

3. RESULTS AND DISCUSSIONS

In this comparative study, we took into consideration geographic zones and agro-climatic conditions that may affect the chemical composition of pine nut seeds, considering that LOEWE *et al.* (2016a) reported marked differences on stone pine cone productivity along the climatic gradient in Chile, which could also be translated to chemical composition, as in fact has been determined by using a discriminant analysis by Near Infrared

Spectroscopy (NIRS) in stone pine nuts collected in different macrozones of Chile (LOEWE *et al.*, 2016b).
The chemical composition of pine nuts collected in six countries is shown in Table 2.

Table 2. Chemical composition of pine nuts by location and country (g/100 g)[1].

Location/Country	Moisture	Protein	Lipids	Ashes	Total Dietary Fiber
Miramar	4.6	33.1	42.0	4.2	10.5
Claromecó	3.0	31.2	41.6	4.6	9.1
Argentina	3.8±0.8ab	32.1±0.9b	41.8±0.2a	4.4±0.2ab	9.8±0.7c
North MZ	4.1	34.9	42.3	4.7	11.6
Dry Coast MZ	4.5	35.3	46.9	4.7	11.6
South MZ	4.3	32.1	43.6	4.6	11.8
Chile*	4.3±0.07a	34.1±0.5b	44.3±0.7a	4.7±0.03a	11.7±0.1b
Ortal	3.5	35.3	31.0	4.8	13.0
Bar'ham	3.7	37.2	30.1	4.7	12.3
Yatir	4.0	37.0	42.9	4.6	11.9
Israel	3.7±0.12b	36.6±0.5a	34.7±4.1a	4.7±0.06a	12.4±0.3b
Cecina	5.2	33.2	37.0	4.2	14.7
Feniglia	4.9	32.8	37.9	3.9	13.9
Calabria	5.2	30.3	36.4	4.7	15.2
Italy	5.1±1.0a	32.1±0.9b	37.1±0.4a	4.3±0.2ab	14.6±0.4a
Spain (Cádiz)	4.8	33.8	45.3	4.1	12.4
Balikesir	3.7	33.3	45.0	4.3	12.2
Bgyuzu	4.0	34.0	43.5	4.0	12.6
Aydin	4.0	37.0	40.1	4.1	14.4
Turkey	3.9±0.1ab	34.8±1.1ab	42.9±1.4a	4.1±0.1b	13.1±0.7ab

[1]Data are expressed as means±SE (n=3). Different letters in a column indicate statistically significant differences ($P<0.05$).
*LUTZ *et al.* (2016).

Significant differences were found among countries for protein ($p=0.0053$), TDF ($p=0.0003$), ash ($p=0.0008$), and moisture ($p=0.0001$). The main chemical component in pine nut seeds is lipids. In the analyzed seeds, fats ranged from 34.7% (Israel) to 45.3% (Spain) ($p<0.05$). These values are in agreement with NERGIZ and DÖNMEZ (2004) and RYAN *et al*. (2006), but lower than the fat content reported for this nut by KORNSTEINER-KRENN *et al*. (2013) and ESCHE *et al*. (2013), including the USDA Database (USDA, 2016), which reports a mean value of 68.4%. According to the principal components analysis, the lipid content of the pine nuts was enhanced in Chile by low minimum temperature, and across countries by high maximum temperatures. The lipid quality is relevant to the energetic and nutritive values of pine nuts, while the fatty acid profile, as well as phytosterols, phytostanols, tocopherols and other lipid bioactives contents play major roles in their healthy properties (KORNSTEINER *et al*., 2006; BOLLING *et al*., 2010; ESCHE *et al*., 2013).

Pine nuts are recognized as a good dietary source of proteins, and the average protein content ranges from 13% to 30% dry matter, depending on the *Pinus* species (EVARISTO *et al*., 2010; USDA, 2016). The protein content observed in the seeds collected from six countries ranged from 32.1% (Italy and Argentina) to 36.6% (Israel), which are above the reported averages. In three Chilean macrozones across 1,300 km with varying climatic conditions, proteins ranged from 32.1% to 35.3% (LUTZ *et al*., 2016). These results demonstrate that protein content can be significantly affected by the agro-climatic conditions in which the species grow. Moisture was highest in Italian pine nuts (5.1%), while it was lowest in the Israeli samples (3.7%); ashes varied from 4.1% (Spain and Turkey) to 4.7% (Israel and Chile), and TDF varied between 9.8% (Argentina) and 14.6% (Italy).
Fig. 2 represents the biplot of the two principal components by country, which explained 66% of the variability. Differences were observed between pine nuts grown in Chile and Argentina, and between Turkey and Israel, presenting a chemical composition that differs from Italy and from Spain. South American pine nuts, which grew in areas with the highest thermal oscillation and rainfall, showed similar chemical composition. Italian pine nuts exhibited the highest TDF and moisture contents. Spanish pine nuts showed a different composition, which would be related to the minimum average temperature and average temperature. Israeli and Turkish pine nuts showed a similar composition, exhibiting high protein content. Mineral content related to the number of dry months, and lipids to the maximum average temperature. Lipids content was superior when maximum average temperature was high.

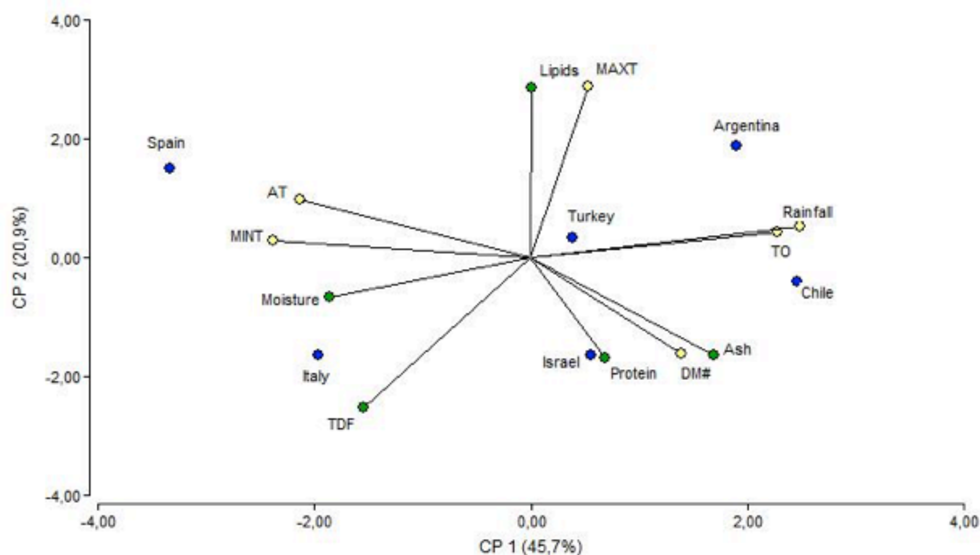


Figure 2. Biplot for chemical composition of pine nuts and climate variables according to country.
MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: dry month number, TO: thermal oscillation, TDF: total dietary fiber.

Fig. 3 shows the biplot of the two principal components, explaining 55.6% of the variability. It also shows that the Italian samples hold a high content of TDF and moisture, with Calabria (IT) and Cádiz (SP) being characterized by a high minimum average temperature. South American pine nut samples grown in areas with high thermal oscillation –especially those from northern and central zones in Chile – and rainfall –

especially those from the southern zone of Chile - showed a similar composition. Turkish and Israeli pine nuts showed some differences in the second component, being Yatir (IL) and Aydin (TK) characterized by a high maximum average temperature and dry month number, and high protein content. Bagyuzu (TK), an area with a high rainfall, holds a similar lipid content to the ones from southern Chile.

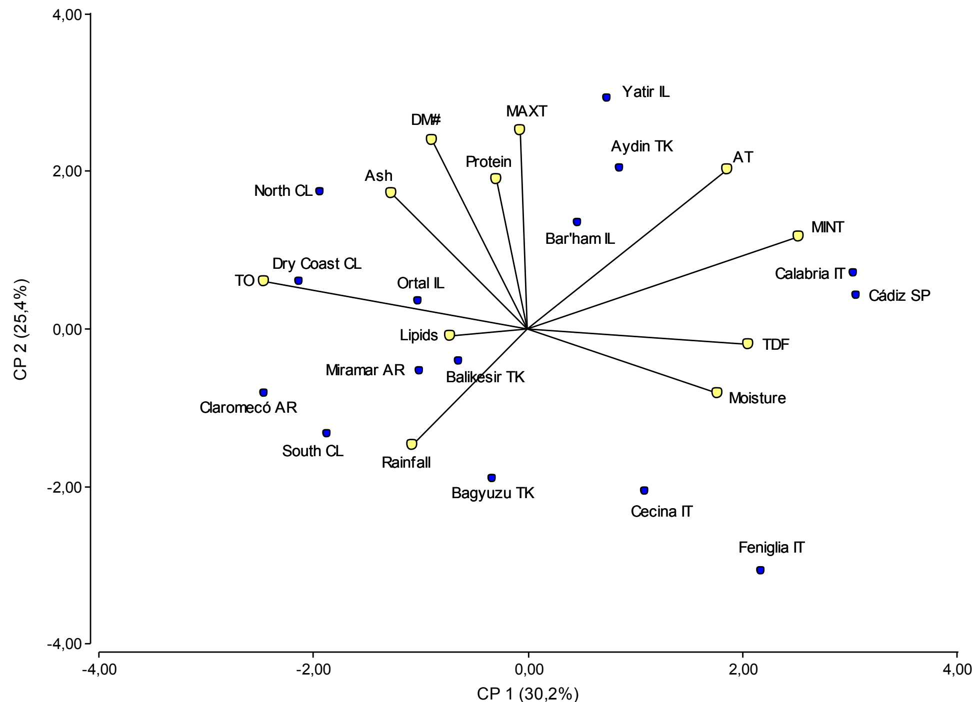


Figure 3. Biplot for chemical composition of pine nuts and climate variables by location.
MAXT: maximum average temperature, AT: average temperature, MINT: minimum average temperature, DM#: dry month number, TO: thermal oscillation, TDF: total dietary fiber.

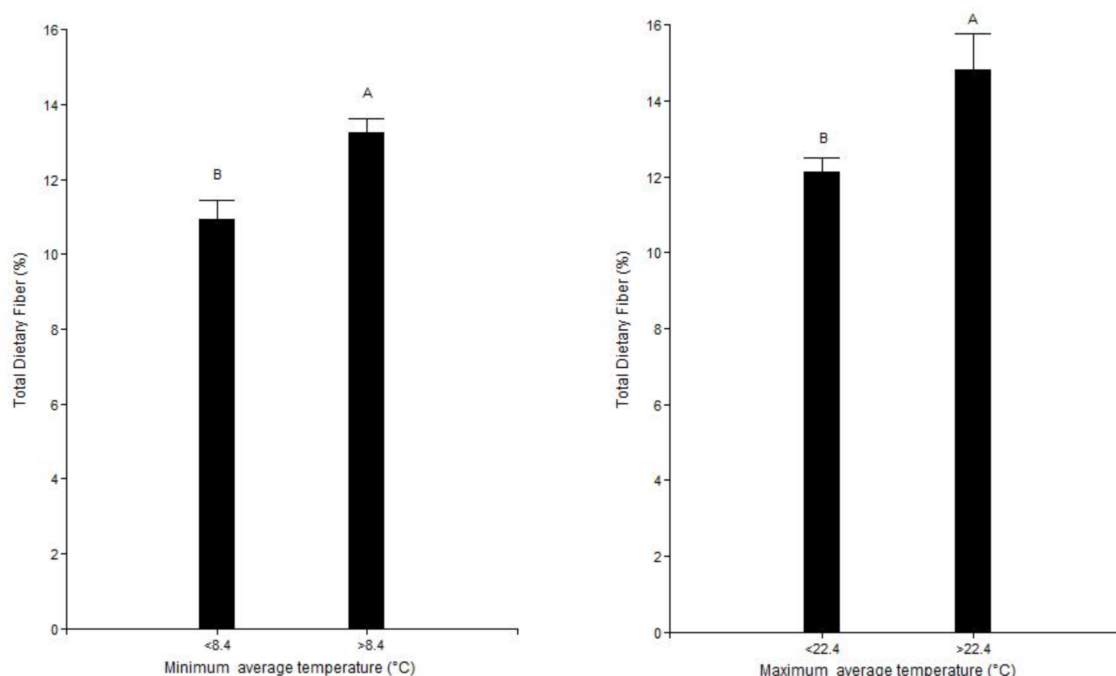
In the CART analyses for each component (Table 3), data were first split into two subsets based on the predictor variable (MINT for TDF; TO for Moisture and DM# for Ash) and its thresholds (8.4°C, 8.4°C, and 5.5 months, respectively). Each subset, or node, for TDF was then analyzed independently using the same procedure (MAXT, 22.4°C). Top nodes are the most important to explain the chemical composition.
Interestingly, the influence of climate on some chemical components was observed. A significant negative influence of thermal oscillation on moisture was detected, as well as significant positive effects of dry months on minerals, and of minimum and maximum temperatures on TDF. In particular, the TDF content in the seeds of the six countries varied from 9.8% (Argentina) to 14.6% (Italy) ($p<0.05$). It was affected by climatic variables such as the minimum average temperature and maximum average temperature ($p<0.05$), increasing with minimal temperatures above 8.4°C and maximum temperatures above 22.4°C. A different situation was observed in the ash content, which increased in presence of longer dry periods (over 5.5 months) by 9.5%.
Fig. 4 depicts the effect of climatic variables on pine nut composition. Across locations, TDF was significantly influenced by climatic variables, with an increase of 21.1% at a minimum average temperature above 8.4°C (13.2% vs 10.9%, $p=0.0032$), and an increase of 22.3% at a maximum average temperature above 22.4°C (14.8% vs 12.1%, $p=0.0203$). Minerals were influenced by the number of dry months ($p=0.0063$), with an increase of

9.5% when dry months exceeded 5.5 (4.6% vs 4.2%). Moisture was influenced by thermal oscillation, being 22.5% higher when thermal oscillation was below 8.4°C (4.9% vs 4.0%, $p=0.0101$).

Table 3. Climatic variables that best explain Total Dietary Fiber (TDF), Moisture and Ash determined by CART analyses.

Node	Predictor variable	Average content (g/100 g)	n	Standard Error
		Total Dietary Fiber		
1	MINT≤8.4°C	10.9	5	1.33
2	MINT>8.4°C	13.2	10	1.42
2.1	MAXT≤22.4°C	12.1	8	0.90
2.2	MAXT>22.4°C	14.8	2	0.33
		Moisture		
1	TO≤8.4°C	4.9	3	0.05
2	TO>8.4°C	4.0	12	0.31
		Ash		
1	DM#≤5.5	4.2	6	0.05
2	DM#>5.5	4.6	9	0.05

MINT: annual average minimum temperature; MAXT: annual average maximum temperature; TO: thermal oscillation (annual average maximum absolute temperature minus annual average minimum absolute temperature); DM#: dry month number.



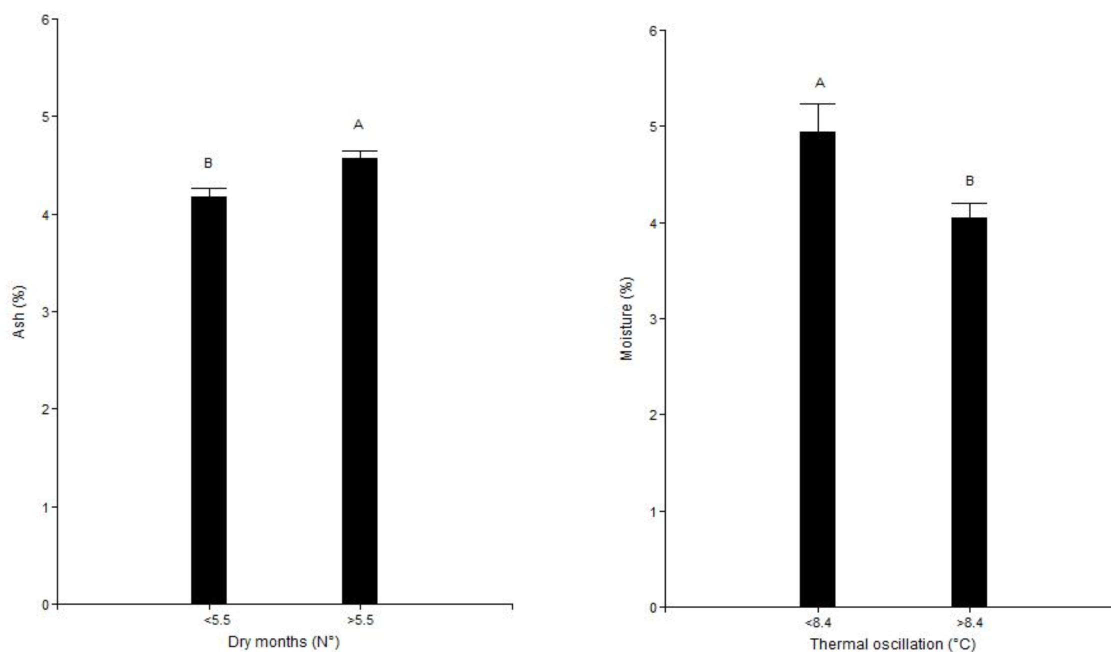


Figure 4. Climatic variables that influence chemical composition across fifteen localities distributed in six countries.
Each threshold was detected by CART analysis. Different letters indicate statistically significant differences ($p<0.05$).
Thermal oscillation: annual average maximum absolute temperature minus annual average minimum absolute temperature.

The soil nutrient content effect has been limitedly studied. In fact, in Turkey, a positive correlation between nitrogen, phosphorus, calcium and manganese depletion was detected in needles and cone loss (KILCI, 2013). In Israel, MALCHI and SHENKER (2011) found that iron deficiency decreased root growth and induced a reduction in chlorophyll concentration on needles in soils with high concentration of calcium carbonate, being a high soil pH the cause for reduced iron absorption. However, no studies have been performed on the relationship among pine nut composition and soil contents. In our study, we observed a similar pine nut protein content when grown in sandy soils, around 33%. Thus, the chemical composition of pine nuts grown in different regions could be explained, at least in part, by the environment and soil type variability between regions, which is in agreement with several authors (GÓMEZ-ARIZA *et al*., 2006; EVARISTO *et al*., 2013; LUTZ *et al*., 2016).
Future studies should also address the use of cultural practices such as fertilization on pine nut quality, considering that BORRERO (2004) reported an increase in pine nuts concentrations of fat, copper, magnesium and sodium in fertilized plots.

4. CONCLUSIONS

The study describes the proximate chemical composition of pine nuts harvested in six countries. The results obtained indicate that from a nutritional quality standpoint, all the analyzed seeds exhibited good nutritional properties, independently of the geographic zone where they were grown, justifying their inclusion in a healthy diet. The major components of pine nuts are lipids, protein and dietary fiber, while their carbohydrates

content is low, which make them a good choice in the prevention of diabetes, metabolic syndrome and other common non-transmissible diseases.
The effect of the climatic conditions, soil quality and other environmental variables are usually not taken into consideration when average values are used in food composition databases.
The results obtained in this study indicate significant differences among countries for protein, TDF, ash and moisture, variability probably related to climate and environmental conditions of the growing areas. Relevant climatic variables were thermal oscillation for moisture, dry months for minerals, and minimum and maximum average temperature for TDF.
The study reveals that *Pinus pinea* L., a traditional ancient tree grown in the Mediterranean basin, may also be successfully grown in South America, contributing to diversify agriculture, as pine nuts represent an opportunity for the global food industry as well. Finally, the composition of the seeds collected from different countries, in various climatic conditions, constitutes relevant information that should be considered when food composition data are included in tables and reference data, shown as mean values.

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PAPER

ENOLOGICAL ELIGIBILITY OF GRAPE CLONES BASED ON THE SIMCA METHOD: THE CASE OF THE SANGIOVESE CULTIVAR FROM TUSCANY

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ABSTRACT

Sangiovese is the most widespread Italian red grape cultivar and it constitutes the basis of internationally known wines. It has a large diversity of clones whose performances vary with environmental conditions due to interaction with the weather and soil. In this study, the performance of grapes from seven Sangiovese clones was evaluated by analyzing grapes from four vineyards in the Chianti Classico Region in Tuscany over the ripening period. In order to assess the enological eligibility of grape clones, a grape reference model was developed using chemical parameters from commercially available Sangiovese wines, by performing a soft independent modeling of class analogy (SIMCA).

Keywords: anthocyanins profile, clone plasticity, ripening, Sangiovese, SIMCA, wine quality

1. INTRODUCTION

Sangiovese is the most widespread Italian red cultivar and, according to the last agricultural census of the Ministry of Agricultural, Food, and Forestry Policies (http://catalogoviti.politicheagricole.it), the total area planted with Sangiovese was 69787 ha, equivalent to 10.3% of the total area of vineyards in Italy. 47% of these vineyards are in Tuscany, producing 92.5% of the Sangiovese world output. Sangiovese constitutes the basis of internationally known wines such as Chianti, Brunello di Montalcino, Nobile di Montepulciano, and, furthermore, its use is allowed in the production of 11 DOCG (Denominazione di Origine Controllata e Garantita), 103 DOC (Denominazione di Origine Controllata) and 99 IGT (Indicazione Geografica Tipica) wines all over Italy.

The selective pressure carried out by mankind over the centuries in different growing conditions has induced the diversification of Sangiovese into many clones (CAMPOSTRINI *et al.*, 1995).

Nowadays, 116 clones of Sangiovese are listed in the Italian National Registry of Grapevine Varieties. Clone performances vary with environmental conditions according to the interaction between the grapevine, weather and soil (BARBEAU *et al.*, 1999) and they can influence wine quality and typicality. Some authors (TONIETTO and CARBONNEAU, 2004) consider that climate is the most dominant factor in determining grape quality and that is responsible for the *terroir* effect.

A few studies have been conducted on the physiological and productive response of Sangiovese clones to environmental and pedoclimatic factors. On the other hand, while some authors have focused on the relationship between the Sangiovese grape composition and different grapevine growth conditions, they only monitored standard parameters such as pH, titratable acidity, and sugar content (DI COLLALTO *et al.*, 2000).

However, some studies on other cultivars, such as Tempranillo, Pinot Noir, Merlot, and Cabernet Sauvignon, have shown that, within the same grape variety, different clones can be distinguished by comparing their field performances including yield components (number and weight of clusters and berries, pruning weight) and the chemical compositions of grapes including anthocyanin content (REVILLA *et al.*, 2009, FIDELIBUS *et al.*, 2006, FIDELIBUS *et al.*, 2007, CASTAGNOLI *et al.*, 2006, AROZARENA *et al.*, 2002, RANKOVIĆ-VASIĆ *et al.*, 2015) and anthocyanin profiles (GUIDONI *et al.* 2002, RYAN and REVILLA, 2003, DOWNEY *et al.*, 2006, GONZÁLEZ-NEVES *et al.*, 2004, ORTEGA-REGULES *et al.*, 2006).

These studies demonstrated that climate and soil are crucial factors in determining the final composition of grapes. However, it is not easy to predict how these changes could affect the final characteristics of wines. Integrated approaches can identify the relationship between grape chemical features, wine chemical composition, and wine sensory attributes, in order to predict wine flavor from grape composition and provide a practical tool for guiding the winemaking process (ZANONI *et al.*, 2010, FORDE *et al.*, 2011).

Many studies (CADOT *et al.*, 2012, KONTOUDAKIS *et al.*, 2011, KOUNDOURAS *et al.*, 2006, RISTIC *et al.*, 2010) have proposed different methodological approaches to measure the qualitative characteristics of grapes and the related wines, but a complete methodological approach to assess the eligibility of grapes in order to obtain a wine with well-defined characteristics has not been taken into account by these authors. A recent study proposed a multivariate statistical approach to relate grape features, and enological and agronomical practices with the composition of wines, in order to provide a practical tool to manage the vineyard and the winemaking process and preserve or enhance wine typicality (CANUTI *et al.*, 2017).

The aim of this study was to create a tool capable of discriminating grapes, depending on their chemical composition, according to a given enological objective. For this purpose, a

grape eligibility model (GEM) was developed by computing the chemical parameters of commercially available Sangiovese wines, grapes, and the relevant wines from previous experiments by performing a soft independent modeling of class analogy (SIMCA). The model was then applied to assess the enological eligibility of seven Sangiovese clones grown in four vineyards in different growing areas of the Chianti Classico region in Tuscany.

2. MATERIALS AND METHODS

2.1. Grape samples

The present study was carried out using seven *Vitis vinifera* cv. Sangiovese clones listed in the Italian National Registry of Grapevine Varieties as reported in Table 1.

Table 1. Sangiovese clones studied for performance evaluation.

Registered clones	Code	Area of origin
SS-F9-A5-48	F9	Lamole (Florence - Tuscany, Italy)
Rauscedo 24	R24	Predappio (Forlì Cesena - Emilia Romagna, Italy)
Montalcino 42	M42	Montalcino (Siena - Tuscany, Italy)
AP-SG-1	AP1	Cossignano (Ascoli Piceno - Marche, Italy)
Peccioli 1	PEC	Peccioli (Pisa - Tuscany, Italy)
Rauscedo 10	R10	Lamole (Florence - Tuscany, Italy)
SG-12T	12T	Predappio (Forlì Cesena - Emilia Romagna, Italy)

The vineyards were located in four different estates in the Chianti Classico DOCG area in Tuscany. These were situated in the provinces of Florence (Panzano and Greve in Chianti) and Siena (Castellina in Chianti and Castelnuovo in Berardenga) and coded with the letters A, B, C, and D respectively. Each vineyard was planted in 1990 and has an average surface area of 1.7 hectares. The vines were spaced 2.8 m (between rows) × 1.0 m (between vines in the row), resulting in a density of 3571 vines/ha, in a randomized block design with a minimum of three replicates. Each clone was grafted on to 420 A (*Vitis berlandieri* x *Vitis riparia*) rootstock. The vines, with a permanent unilateral cordon, were spur-pruned and trained to a vertical shoot-position. The same soil management techniques were applied in all the vineyards, in which the rows were alternately covered with a spontaneous permanent grass and tilled. No irrigation system was installed.
The meteorological indices, based on the data recorded by the nearest climate stations to the experimental vineyards and the characteristics of the soils, are available at the reader's request.
Grapes were harvested for sampling at three different ripening stages during the 2005 vintage (September 10th, 20th and 30th). In 2005, rain events were quite intense at the beginning of September, as indicated by the data collected at the local meteorological stations. On each sampling date, 2 kg-samples of grapes were collected from portions of different clusters picked randomly from all the randomized blocks and analyzed.

2.2. Chemicals

The acetonitrile and *O*-phosphoric acid (HPLC grade) were purchased from Panreac (Barcelona, Spain). The malvidin-3-monoglucoside (M3MG) (HPLC grade) was purchased from Extrasynthèse (Genay, France). All of the other chemicals were of the highest purity available and were purchased from Sigma-Aldrich (Milan, Italy).

2.3. Instrumentation

The HPLC analyses were carried out on a Perkin-Elmer 200 LC Series equipped with an autosampler and a diode-array detector (Perkin Elmer, Shelton, CT, USA). The ultraviolet-visible (UV/vis) absorbance of the samples was measured on a Perkin Elmer Lambda 35 UV/Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA).

2.4. Grape analysis

The technological ripeness of the grapes was measured following official OIV methods (Compendium of International Methods of Analysis – OIV – Oeno 21/2004). Two hundred berries were pressed to extract their juice. The juice sugar contents (Brix), titratable acidity (g/L), and pH were measured after centrifugation of the juice at 3000 rpm for 10 min. The berry weight was determined as the ratio between the total weight and the number of berries. Phenolic maturity was measured as described by SAINT-CRIQ *et al*. (1998).
The anthocyanin profiles (expressed as relative abundance of the different anthocyanins) of the same grape extracts at pH 3.2 were determined by HPLC, according to a previously published method (PENG *et al*., 2002) used to determine the phenolic maturity by acquiring a chromatogram at 520 nm. At the same time, the tannin contents (expressed as peak height) were determined by acquiring a chromatogram at 280 nm, as described in CANUTI *et al*. (2012). Chromatograms were acquired, recorded, and processed using Total Chrome Navigator software (Perkin Elmer).

2.5. Analysis of commercial and experimental wines

Standard parameters were measured in the wines following official EU methods (Official Methods of Wine Analysis, Reg. 440/2003). Color intensity (CI) and hue (Hue) were measured according to GLORIES (1984) and the total phenols index (TPI) was measured as described by RIBEREAU-GAYON (1970).
Monomer anthocyanin contents, expressed as mg/L of malvidin-3-monoglucoside (M3MG), colored polymeric pigments (CPP) expressed as mg/L of M3MG, and tannins (expressed as peak height) were determined by HPLC (PENG *et al*., 2002). Chromatograms were acquired at 520 and 280 nm respectively, using the same HPLC parameters reported in the grape analysis section.

2.6. Statistical analysis

The analysis of variance (ANOVA) was performed using Statgraphics Centurion (Ver. XV, StatPoint Technologies, Warrenton, VA), considering clones, growing area, and sampling date as factors. Principal Component Analysis (PCA) and the Soft Independent Modelling of Class Analogy (SIMCA) were performed using Unscrambler (V10.3, CAMO Process AS, Oslo, Norway).
The SIMCA analysis enables the assessment of which factors are decisive in determining the classification. Indeed, in this classification method, each class is described by an

independent principal component analysis model. New samples are classified on the basis of their fit with the different PCA models. The optimal number of PCs for each model is chosen independently since the classes may exhibit different shapes and structures. For new samples the residuals and scores are calculated for each PCA model. The residuals provide information on the ability of each model to describe the new data, like a sort of object-to-model distance, while the scores can be combined to measure the distance between the object and the model center.

2.7. Grape quality model and evaluation of clone performance

A model evaluating the grape quality, and the consequent performance of the clones, was built in order to provide an objective tool to assess the grapes' eligibility for winemaking. The classification method (SIMCA) consisted of describing each class of samples (wines and grapes), identified by their chemical composition, in independent Principal Component Analysis (PCA) models. Grape and wine samples were classified on the basis of their membership limit within the different PCA models (WOLD and SJOSTROM, 1977). In particular, the methodology to build the model and evaluate the clone performances consisted of three phases as follows:

Phase 1 – Sangiovese wine eligibility model (WEM)

In Phase 1, a Sangiovese wine eligibility model was built which described the Chianti Classico region wines. The aim of this phase was to establish a definition of Sangiovese wines from the region. For this purpose, 37 commercial Chianti Classico DOCG wines (coded with numbers from 101 to 137) were analyzed to determine their alcohol contents, titratable acidity, pH, total phenol index, total anthocyanins, tannins, color intensity, and hue. Later, a global PCA was run considering all the parameters for the 37 commercial wines. All the wines used to build the WEM were produced with 100% Sangiovese grapes without oak contact and analyzed one year after the harvest.

Phase 2 – Sangiovese grape eligibility model (GEM)

The aim of Phase 2 was to create a grape eligibility model (GEM) starting from the WEM built in Phase 1. For this purpose, 30 Sangiovese grape samples from the Chianti Classico region were analyzed to determine their sugar content, pH, titratable acidity, total and extractable anthocyanins, cellular maturity index, phenolic richness, and tannins. The grapes were then vinified, on an industrial scale, to obtain 30 experimental Sangiovese wines (coded with numbers from 1 to 30) that were analyzed, after aging for one year, to determine their alcohol contents, titratable acidity, pH, total phenol index, total anthocyanins, tannins, color intensity, and hue.
Finally, the chemical composition parameters of 30 Sangiovese grape samples and the related experimental wines were statistically analyzed in order to correlate the grapes to wine composition. A global PCA of the experimental wines was performed and the resulting model was compared with a SIMCA analysis to assess which wines fitted the WEM.
The grape samples whose wines fitted the WEM obtained in *Phase 1* were used to create the Grape Eligibility Model (GEM) by running a global PCA which considered all of the grape parameters.

Phase 3 – Evaluation of clone performance

In order to evaluate the clones' performances, the grape samples were classified according to their composition using a SIMCA analysis to assess which grapes fitted the GEM. Outliers were detected during the exploratory analysis by calculating the Hotelling's T^2 distance diagnostic tool for each class of samples.

3. RESULTS

3.1. Grape clones' characteristics

The data collected from grape sample analyses at three different ripening stages were statistically analyzed and the results are reported in Table 2.

Table 2. F-values and interactions for chemical parameters analyzed in the grape samples.

Variable	SD	C	GA	C x GA	GA x SD	C x SD
Sugar	13.97***	2.86*	70.43***	4.46***	4.25***	3.79***
pH	15.45***	5.65***	21.08***	1.23 ns	1.48 ns	1.53 ns
Titratable acidity	65.61***	14.35***	112.52***	6.43***	4.11***	9.24***
Berry weight	4.02*	13.78***	93.69***	11.52***	5.76***	5.01***
Total potential in anthocyanins	14.37***	23.41***	54.05***	13.06***	13.57***	5.00***
Extractable anthocyanins	10.46***	20.47***	70.42***	10.50***	9.89***	5.21***
Cellular maturity index	22.66***	6.09***	18.27***	7.10***	6.65***	6.26***
Phenolic richness	6.48**	5.15***	7.67***	2.87***	2.58*	2.08*
Di/Tri substituted anthocyanins ratio	21.82***	15.93***	92.03***	27.48***	7.90***	2.07*
Tannins	5.05*	46.30***	186.47***	46.18***	4.48***	5.39***

*$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.001$; ns, not significant; C, clone; GA, growing area; SD, sampling date.

All the variables measured have been significantly affected by the factors clone (C), growing area (GA), sampling date (SD) factors and their interactions, with the exception of pH (Table 2). Furthermore, the ANOVA results indicate that GA was the most influential factor; in fact, the F-values of GA were the highest for all the considered grape parameters. The C factor was found to be a determining factor for the concentrations of phenolic compounds (tannins, potential and total anthocyanins). As expected, the SD factor did not only influence the cellular maturity index but also the quality of the anthocyanins, modifying the di-substituted and tri-substituted pigment ratios.
With regard to the interactions between the various factors, the results suggest that the interaction between clone and growing area (C x GA) was the most significant, in relation to tannin contents ($F = 46.18$) and the Di/Tri index ($F = 27.48$).
The mean plots for the standard and phenolic parameters of the grape samples are shown in Figs. 1 and 2, respectively.
The berry weight (BW) provides a qualitative and quantitative indication about the berry size and the skin to pulp ratio. The most important factor that influenced BW (Table 2) was GA, which showed the highest F - value (93.69). According to Fig. 1, BW remained fairly constant during the ripening stage, indicating that the SD factor had less influence on BW (F – value = 4.02) if compared to the GA and C factors which had a higher statistically

significant influence (Table 2). In particular, clone F09 showed a higher BW in comparison to clone 12T, which produced smaller berries. Growing areas A and D seemed to stimulate the growth of the berry more than growing area B. Clone R24 had the highest berry weight variability, producing the lightest berries (1.24 g) in area B and the heaviest (2.37 g) in D. The clone PEC stood out in area C with an average weight of 2.35 g. Clones F09, AP1, and R10 produced grapes with a more constant berry weight in all the growing areas.

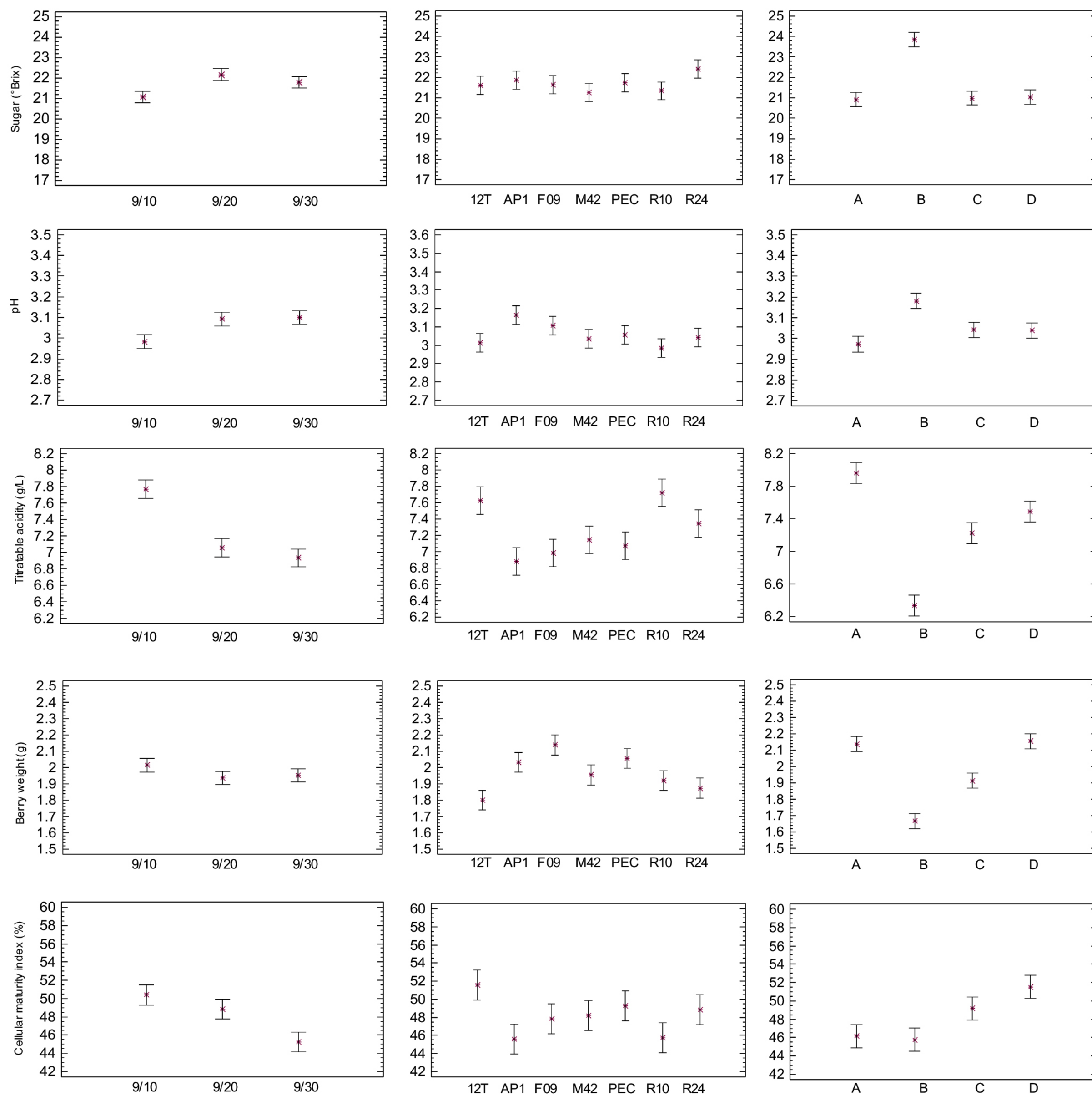

Figure 1. Mean plots of standard parameters of grape samples (sugar, pH, titratable acidity, berry weight, and cellular maturity index); significance at 95% confidence level according to Fisher's least significant difference (LSD) procedure. Bars represent LSD; total number of grape samples for each plot = 252.

All of the factors had a statistically significant influence in relation to the grapes' sugar content, and GA was the one with the greatest effect (F-value = 70.43). There were also significant effects when considering the interactions between factors. For example, Fig. 1

shows that the sugar content increased, as expected, during ripening, with clone R24 having the highest sugar content, and growing area B promoting the accumulation of sugars. The sugar content of the grapes produced by clones 12T and AP1 showed the most unstable and extreme values, which varied between 25.0 Brix in B and 20.5 in the other zones. R10 showed constant concentrations (21.3 Brix) in all the growing areas.

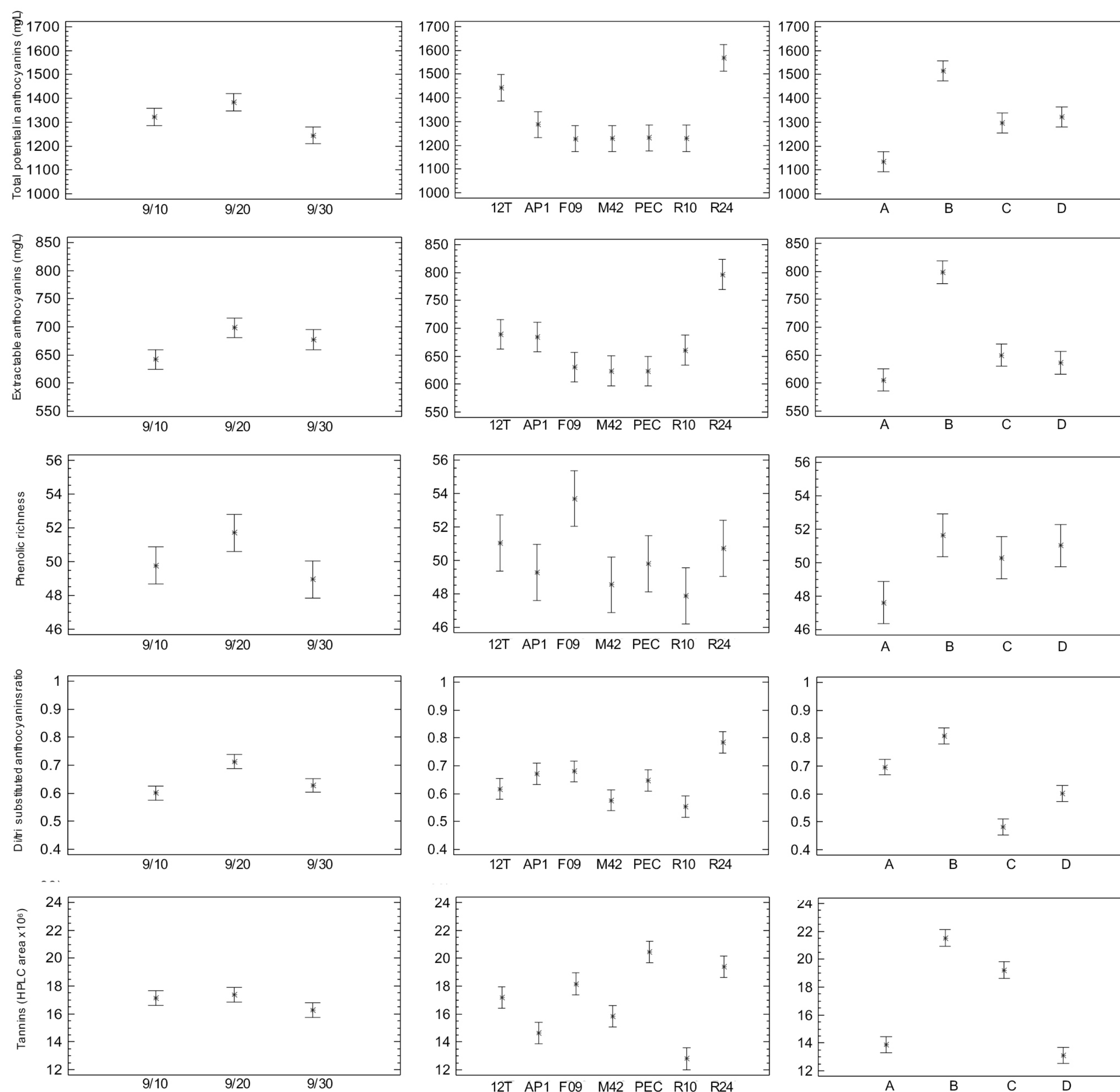

Figure 2. Mean plots of phenolic parameters of grape samples (total potential in anthocyanins, extractable anthocyanins, phenolic richness, di/tri substituted anthocyanins ratio, and tannins); significance at 95% confidence level according to Fisher's least significant difference (LSD) procedure. Bars represent LSD; total number of grape samples for each plot = 252.

Concerning the titratable acidity and pH (Table 2), the most determining factor was again GA (F - value = 112.52 and 21.58 respectively). Moreover, the titratable acidity decreased as the pH increased at the three different sampling dates (F = 65.61). Clone AP1 showed the highest pH and clones 12T and R10 the highest titratable acidity (Fig. 1).

Growing area B produced grapes with the highest pH and the lowest titratable acidity in comparison to the other GAs. The grapes grown in area A showed the highest titratable acidity and the lowest pH. The titratable acidity was similar for all the clones in growing area B, while the results showed a high variability in the other zones. F09 reached the lowest value of titratable acidity (6.65 g/L) in area D. Clone R10 displayed the highest values in zones A (8.59 g/L) and C (8.08 g/L). Moreover, in growing area A, clones 12T (8.72 g/L) and R24 (8.57 g/L) stood out for their high titratable acidity.
The cellular maturity index (EA%) measures the berries' ability to release anthocyanins: the lower the EA index value, the higher the extractable potential of the anthocyanins (SAINT CRIQ *et al.*, 1998). The evolution of this factor at the different SDs (Fig. 1) highlighted a progressive cellular maturity that should result in a better release of the phenolic compounds during winemaking. Statistical analysis (Table 2) pointed out that EA% mostly depended on the SD (F = 22.66) and GA factors (F = 18.27) and that growing areas A and B promoted the ripening of the grape skin cells the most. Moreover, clone 12T showed the highest EA% (Fig. 1). The cellular maturity index was stable for clone PEC (49) while it was inconstant for F09 (38 in B and 51 in C) and M42 (40 in A and 56 in C). The EA% was the highest for all the clones in growing area D.
During the ripening of the grapes, there was a constant and significant increase in phenolic richness and total potential in anthocyanins from the first to the second SD and a significant decrease at the third SD (Fig. 2). Instead, the extractable anthocyanins increased over time, reaching the maximum at the second sampling date and remaining constant between the second and third SDs.
The tannin contents remained constant throughout the ripening stages; this result could indicate that the increase in the total amount of phenolic compounds was due to the accumulation of monomeric phenols (Fig. 2). However, clones PEC and R24 showed the highest tannin content, and there were significant differences induced by the GA factor, with growing areas A and D having the lowest values and areas B and C showing the highest value for tannin contents.
The largest differences in phenolic composition between the grapes emerged when considering GA as the main factor. In fact, the content of total potential (F = 54.05), extractable anthocyanins (F = 70.42) and tannins (F = 186.47) was higher in zone B and lower in all the other growing areas. Moreover, the differences in the levels of phenolic richness were less remarkable but still significant for growing areas B, C, and D, which showed higher values than area A.
Upon examining the chemical profile of the different Sangiovese clones, it is clear that there were differences among them due to the content of total anthocyanins but not at the level of phenolic richness. Clone R24 showed a different behavior when compared to all the other clones, resulting in significantly higher contents of total potential and extractable anthocyanins and tannins.
Regarding the clone x growing area interactions, clone F09 always showed similar levels of phenolic richness (54), while R24, with values that ranged between 47 in A and 56 in D, was the clone most influenced by the GA factor. The tannin content was similar for all the clones in areas A and B; a higher variability was observed in the other zones where clones PEC, R24 (in B), and F09 (in C) stood out with higher values.
The anthocyanin content, both potential and extractable, showed the highest variability in zone B where clones 12T (1879 and 946 mg/kg) and R24 (2011 and 983 mg/kg) stood out with the highest values. In area B, the values were higher for all the clones with the exception of F09 (1043 and 642 mg/kg) which, on the contrary, was the richest in anthocyanins in growing area C (1609 and 744 mg/kg).
Different considerations should be made regarding the ratio between the di- and tri-substituted anthocyanins (Di/Tri) and the anthocyanin profiles of the different clones.

Clone R24 showed the highest Di/Tri value and growing areas A and B showed larger levels of Di/Tri in comparison to areas C and D (Fig. 2).
Regarding the relative abundance of anthocyanins, it was seen that the pigments were mainly represented by delphinidin-3-O-glucoside (DEL), cyanidin-3-O-glucoside (CYA), peonidin-3-O-glucoside (PEO), petunidin-3-O-glucoside (PET), and malvidin-3-O-glucoside (MAL). In agreement with results reported elsewhere (MATTIVI *et al*., 2006, ARAPITSAS *et al*., 2012), the acylated anthocyanins were found in very low amounts (sum of total relative area below 2% of the total anthocyanin content, data not shown).
Due to the low amounts of acylated pigments in the Sangiovese cultivar, only glucoside anthocyanins were taken into consideration for the statistical analysis (DEL, CYA, PEO, PET and MAL).
The variability in Di/Tri levels, according to the multifactor ANOVA, was affected by all the factors taken into consideration (Table 2). All the clones had similar Di/Tri values with the exception of clone R24 (F = 15.93), which showed a higher value (Fig. 2). Small variations occurred during the ripening period (F = 21.82). In this case too, the growing area was the most influential factor on this parameter (F = 92.03), with areas A and B showing the highest values of the index. The Di/Tri ratio was similar for all the clones in growing areas A and C while in area B the values varied between 1.37 for clone R24 and 0.49 for R10.
Upon analyzing the anthocyanin profile (Table 3), MAL resulted the major component of the anthocyanin pool present in the Sangiovese grapes from all the different clones; while DEL was the smallest component.

Table 3. Grape clone anthocyanin percentages and significance[1].

	Anthocyanins					
Factors	**Del**	**Cya**	**Pet**	**Peo**	**Mal**	**Σ tri-sub**
Clone						
12T	9.5^{c}	20.5^{a}	13.3^{cd}	16.9^{b}	39.7^{bc}	62.5^{ab}
AP1	9.1^{b}	19.5^{a}	12.8^{b}	19.8^{d}	38.7^{b}	60.6^{ab}
F09	6.8^{a}	19.9^{a}	10.4^{a}	18.6^{c}	44.1^{d}	61.3^{ab}
M42	9.7^{c}	19.5^{a}	13.4^{d}	16.5^{b}	41.0^{c}	64.1^{b}
PEC	9.4^{c}	23.7^{b}	13.7^{d}	14.8^{a}	38.1^{b}	61.2^{ab}
R10	10.2^{d}	19.9^{a}	14.1^{c}	14.3^{a}	41.2^{c}	65.5^{b}
R24	10.3^{d}	24.5^{a}	12.9^{bc}	17.4^{b}	34.6^{a}	57.8^{a}
Growing area						
A	9.9^{c}	24.2^{c}	13.7^{b}	16.2^{b}	35.8^{a}	59.4^{a}
B	8.9^{b}	24.7^{c}	12.3^{a}	18.7^{d}	35.2^{a}	56.4^{a}
C	9.9^{c}	16.6^{a}	13.8^{b}	15.2^{a}	44.4^{b}	68.1^{c}
D	8.5^{a}	18.9^{b}	12.0^{a}	17.5^{c}	43.0^{b}	63.5^{b}
Sampling date						
9/10	9.4^{a}	19.6^{a}	13.0^{a}	16.5^{a}	41.3^{c}	63.7^{b}
9/20	9.3^{a}	22.8^{a}	12.8^{a}	17.3^{b}	37.6^{a}	59.7^{a}
9/30	9.3^{a}	20.8^{a}	12.9^{a}	16.9^{ab}	39.9^{b}	62.1^{ab}

[1]Different letters within the same row mean significant differences (significance at 95% confidence level according to Fisher's least significant difference (LSD) procedure). DEL: delphinidin-3-O-glucoside; CYA: cyanidin-3-O-glucoside; PEO: peonidin-3-O-glucoside; PET: petunidin-3-O-glucoside; MAL: malvidin-3-O-glucoside; Σ tri-sub: sum of tri-substituted anthocyanins.

CYA was the second most abundant anthocyanin in Sangiovese grapes. The sum of the tri-substituted anthocyanins (DEL, PET and MAL) ranged between 57.8-65.5%. The results showed that the average anthocyanin profile of Sangiovese corresponded to the one described in the literature (MATTIVI *et al*., 2006, ARAPITSAS *et al*., 2012). However, significant differences in the anthocyanin composition of the grapes were related to C, GA, and SD (Table 3).

3.2. Grape quality model and evaluation of clone performance

Phase 1 – Sangiovese wine eligibility model (WEM)

The model was built by running a PCA which considered the chemical parameters of 37 commercial Sangiovese wines from the Chianti Classico region.
The scores of the wines relative to the two first PCs and the Hotelling T^2 Ellipse (95% confidence level) are represented in Fig. 3.

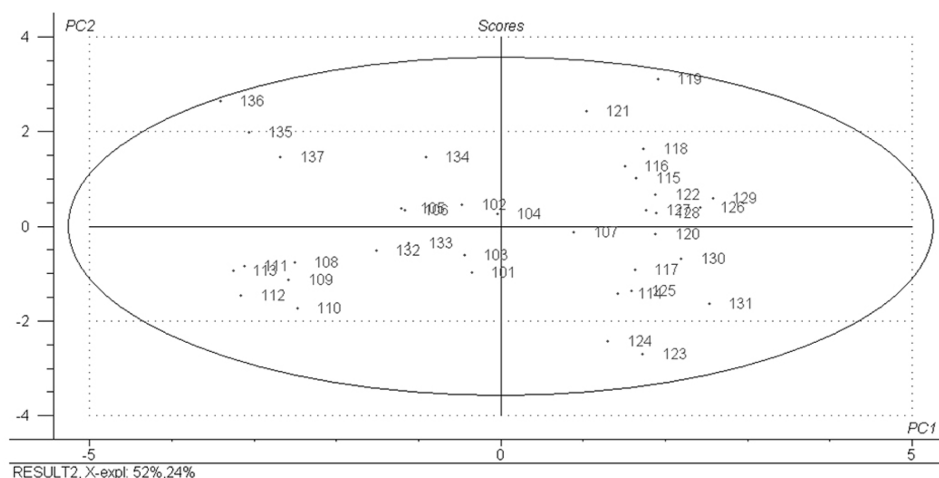

Figure 3. Principal Component Analysis (PCA) and Hotelling T^2 Ellipse of the commercial wines, numbered 101 to 137.

By encircling all the samples, the Hotelling statistic evidenced the absence of outliers among the wines. The statistical analysis confirmed, moreover, a homogeneity between the samples, stating that they reasonably belong to the same class. For the construction of the model, the number of PCs was chosen to reach a level of explained variance between 80 and 90%. For the WEM, three principal components that accounted for 84.11% of the total variance were considered adequate.

Phase 2 – Sangiovese grape eligibility model (GEM)

The WEM built in Phase 1 was used to classify 30 experimental wines according to their analytical profiles. The SIMCA analysis allowed the identification of 23 wines that fitted the model criteria, while rejecting wine samples 6, 13, 20, 22, 23, 27, and 28 (Table 4).
The grapes used in the experimental wines that resulted eligible by applying the WEM were considered suitable for the construction of a grape eligibility model (GEM). The

analytical parameters of the 23 grape samples were then used to calculate a PCA in order to set up the GEM.

Table 4. Classification of the experimental wines (SIMCA). Numbers 1 to 30 represent the wine samples. (●) wine which fitted the WEM, (-) wine which did not fit the WEM.

Experimental wine		Experimental wine	
1	●	16	●
2	●	17	●
3	●	18	●
4	●	19	●
5	●	20	-
6	-	21	●
7	●	22	-
8	●	23	-
9	●	24	●
10	●	25	●
11	●	26	●
12	●	27	-
13	-	28	-
14	●	29	●
15	●	30	●

Fig. 4 reports the PCA performed with the chemical parameters of the eligible grapes previously selected using the SIMCA analysis. In this case too, no outliers were found according to the Hotelling T^2 Test, confirming the homogeneity among the samples and indicating that they reasonably belong to the same class.

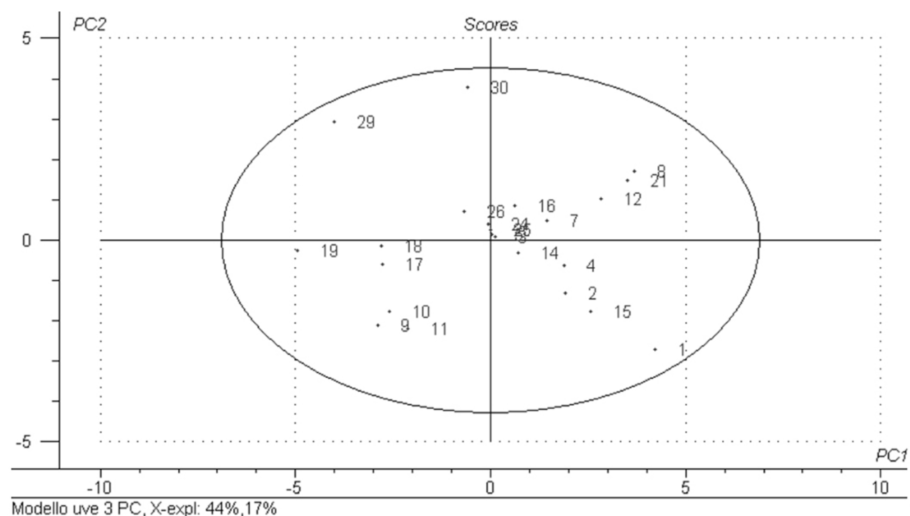

Figure 4. Principal Component Analysis (PCA) and Hotelling T^2 Ellipse of the eligible grapes. The numbers inside the ellipse represent the grape samples whose wines fitted the WEM.

Similarly, as was done for the construction of the WEM, to build the GEM the number of PCs was chosen to reach a level of explained variance between 80 and 90%. For the

purpose of this study, four PCs, which accounted for 81.72% of the total variance, were considered adequate.
The SIMCA analysis also allowed us to assess which factors are decisive in determining the classification of wines. The modeling power represents the contribution of each factor to building the model expressed as the variance of each variable. Any variable having a modeling power higher than 0.3 was considered relevant in the model. In our study all the considered variables were relevant and in particular, hue (0.709), color intensity (0.704), monomer anthocyanins (0.699), and titratable acidity (0.628) were the variables with the greatest modeling power for the wines, followed by total phenol index (0.584), alcohol content (0.554), tannins (0.504), and pH (0.498).

Phase 3 – Evaluation of clone performance

Lastly, the chemical profile of the grape clones was compared with the GEM to classify their performance as a function of the clone, the sampling date, and the different growing areas. The results indicating which grapes had the chemical characteristics to fit the GEM are reported in Table 5.

Table 5. Classification of the grapes (SIMCA) as a function of the clone (12T, AP1, F09, M42, PEC, R10, R24), the sampling date (9/10, 9/20, 9/30) and the different growing area (A, B, C, D); (●) grapes fitting the GEM, (-) grapes not fitting the GEM.

	A			B			C			D		
	9/10	9/20	9/30	9/10	9/20	9/30	9/10	9/20	9/30	9/10	9/20	9/30
12T	-	-	●	-	-	-	-	●	●	-	-	-
AP1	-	-	-	●	-	-	●	●	-	●	●	-
F09	-	●	●	●	-	-	●	-	-	-	-	●
M42	-	-	-	●	●	-	-	-	●	-	-	-
PEC	-	●	-	-	-	-	-	●	-	-	●	●
R10	-	-	-	●	●	●	-	-	-	-	-	●
R24	-	-	-	●	-	●	-	-	●	-	●	-

The clones showed a different performance according to the different growing areas and their ripening stage. Considering the growing area as a factor, it can be seen that in zone A the ideal ripeness was reached in only four cases. In zone A, furthermore, only three clones were in accord with the parameters defined by the model: 12T, F09, and PEC. Area C, on the contrary, showed the highest number of clones that produced grapes with the desired characteristics.
With regard to the performances of the individual clones, F09 resulted the only clone able to fit the model in all the growing areas on at least one of the sampling dates.
The grapes from clones 12T, M42 and R10 fitted the model in two distinct zones: clone 12T in areas A and C; clone M42 in areas B and C; and clone R10 in areas B and D; the other clones (AP1 and R24) reached the maturity required by the model in three growing areas (areas B, C and D for clones AP1 and R24, and areas A, C, and D for clone PEC).
Total potential in anthocyanins (0.797), extractable anthocyanins (0.699), and tannins (0.684) were the variables with the greatest modeling power for the grapes (expressed as the variance of each variable) followed by cellular maturity index EA% (0.674), phenolic richness (0.607), titratable acidity (0.562), pH (0.505), and sugar (0.497).

These results show the strong influence of the soil and climate on the ripeness parameters of Sangiovese grapes. In growing area B five clones already fitted the model in the first sampling period, producing the fastest ripening. In this case, the grapes tended to exit the eligibility model over time. This highlights how the GEM determined the unsuitability of grapes due to over-ripening, and therefore identified precise periods within which the quality of the grapes reaches its peak in relation to a particular enological target (WEM).
Table 5 also shows how the model determined not only thresholds of acceptability but a real eligibility criterion. Indeed, when all these variables are taken into account together, throughout the model, it creates a "space of acceptability where grapes transit over time," with some of the grapes entering the space as they ripen while others, which were initially acceptable, are later expelled from the space of suitability by the model, because of over-ripening.
It should be emphasized that the study was carried out in a single year, in order to propose a methodological approach. On the other hand, an adequate number of replicates over the years, taking into account the seasonal trends, should be analyzed when studying the performance of clones in a given area.

4. CONCLUSIONS

In the present study, grapes from seven Sangiovese grape clones cultivated in the Chianti Classico region in Tuscany were chemically characterized and compared, over the ripening period, to understand if non-genetic factors could affect the performances of different clones.
In the first part of the study, the results showed that grape characteristics were influenced by all the factors considered: clone, growing area, and sampling date. The largest differences between the grapes, according to the phenolic composition, emerged when considering the growing area as a factor and the total anthocyanin content as a variable. The anthocyanin profiles were also affected by the different growing conditions and clones; the most abundant anthocyanins were malvidin-3-O-glucoside and cyanidin-3-O-glucoside while acylated anthocyanins were detected in a very low amount (less than 2%), confirming the results for Sangiovese wines reported elsewhere.
In the second part of the study, a statistical model was developed to evaluate the impact of Sangiovese variability on grape eligibility referring to a given enological target.
For this purpose, a Chianti Classico Sangiovese wine reference model (WEM) was developed with the chemical characteristics of commercial wines from the same area. By comparing the composition of the experimental wines that fitted the WEM with the relevant grapes, a model (GEM) was defined that allowed us to discriminate the Sangiovese grapes on the basis of their suitability to produce wines with the desired properties. The model could be expanded by inserting additional features such as aroma compounds or by using quick analysis methods such as FT-NIR that can easily predict some chemical grape and wine parameters.
With an adequate number of replicates, the proposed approach could be useful in zoning studies or in determining the performance of different varieties or clones with the goal of producing a typical Sangiovese wine of the Chianti Classico region. Moreover, it could be implemented as a more rational use of available analytical data both to monitor grape maturation trends and to improve the management of winemaking processes by transforming chemical analysis databases into active decision-making tools.

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