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EVALUATION OF PULP BROWNING IN MINIMALLY PROCESSED 'ROYAL GALA' APPLE TREATED WITH ERYTHORBIC ACID

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ABSTRACT
The production and marketing of minimally processed (MP) apple is limited by the rapid and intense browning of the pulp. The application of antioxidants is the main alternative to overcome this problem, but with limited results, mainly due to the lack of information about the most adequate concentration of antioxidant for a specific fruit under certain storage conditions, such as type of packaging, temperature, humidity and storage time. Therefore, the objective of this study was to evaluate the effect of different concentrations of sodium erythorbate on color, weight loss, total soluble solids (TSS), total acidity (TA), ratio (TSS / TA), total phenols, antioxidant activity, and enzymatic activity (peroxidase and polyphenoloxidase) in 'Royal Gala' apple MP during refrigerated storage. Each fruit was cut in four wedge shape pieces of similar size, and immediately immersed (1.0 min) in the antioxidant solutions: (a) distilled water - negative control; (b) L-cysteine chloride 0.6 % [$m.v^{-1}$] - positive control; (c) erythorbic acid 1.0 % [$m.v^{-1}$]; (d) erythorbic acid 2.0 % [$m.v^{-1}$] and (e) erythorbic acid 3.0 % [$m.v^{-1}$]. The activity of the enzymes peroxidase and polyphenoloxidase increased throughout the storage, however, in the apples where the antioxidants L-cysteine (0.6 %) and erythorbic acid (1%, 2% and 3%) were applied the enzymatic activity was lower than the control. The 3 % erythorbic acid, in addition to satisfactorily preserving the color, preserved the phenolic compounds and the antioxidant activity during the nine days of refrigerated storage.

1. Introduction

The demand for minimally processed (MP) fruits and vegetables is growing in the food market. However, consumers look for fresh products with organoleptic characteristics close to *in natura* equivalents (Tappi et al., 2017). The preparation of MP products consists of a series of steps, such as sanitizing, cutting, packaging, storage, marketing, among others (Segura-Ponce et al., 2018). These steps decrease the products shelf life (Putnik et al., 2017) and cause enzymatic browning (EB), especially in apples (*Malus domestica* Borkh). Thus, the control of EB is of great importance, as it occurs in many fruits and vegetables and negatively affect attributes of color, taste and nutritional value (Holderbaum et al., 2010, Ioannou and Ghoul 2013). Dogan and Dogan (2004) observed that colour changes

in apple pulp occur due to the high concentration of phenolic compounds in this fruit. This occurs because cutting and other mechanical damage allow oxygen penetration into tissues, resulting in rapid darkening by the oxidation of phenolic compounds. In this process, quinones are polymerized with other quinones or with phenolic compounds, resulting in the appearance of brown pigments (Kim et al., 2017). The phenolic substrates involved in the darkening reaction (e.g. 5-caffeoylquinic acid) are separated from polyphenoloxidase (PPO) enzyme inside intact organelles (vacuoles and plastids, respectively), thus inhibiting the darkening reaction. The cutting, or other mechanical damage during minimal processing, allow enzymes and substrates to react, causing tissue browning. Genetically modified apple cultivars, resistant to enzymatic darkening, have already been successfully obtained (Espley et al., 2013). However, although it is already in the commercialization phase, these cultivars remain little diffused due to their compatibility with different edaphoclimatic conditions and also the consumer resistance to consuming genetically modified products. In this context, one of the alternatives to inhibit enzymatic darkening in MP apples is the use of antioxidant agents. A commonly used agent is L-cysteine; this amino acid contains a thiol group with reduction properties (Richard-Forget et al., 1992). Several studies have demonstrated the efficiency of L-cysteine in the inhibition of pulp darkening of MP products such as 'Red Delicious' apples (Eissa et al., 2006), Fuji apples (Moreno et al., 2016), 'Stylist' lettuce (Bernardo et al., 2015) and Lychia (Ali et al., 2016). Similarly, the antioxidant erythorbic acid (D-isoascorbic acid) (Kall and Andersen 1999, Sun et al., 2013) is a stereoisomer of ascorbic acid but without the activity of vitamin C. This antioxidant has the same properties of its stereoisomer but five times cheaper (Pineli et al., 2005, Martin-Belloso and Fortuny 2010). The use of EA as an antibrowning agent was evaluated in MP 'Ágata' potatoes (Pineli et al., 2005), canned apple and beer (Andersen 1999). In addition, EA is considered a safe food antioxidant in the European Union and the US, when used according to its legislation (Sun et al., 2013, EFSA 2016). Despite the proven antioxidant properties, there are few reports in the literature evaluating the use of EA as a darkening inhibitor in MP apples.

In this context, the objective of this study was to test different concentrations of EA for the maintenance of physicochemical attributes in MP 'Royal Gala' apples stored in refrigerated environment for up to nine days.

2. Materials and methods

2.1. Apples and maturity indices

Samples of apple (*Malus domestica* Borkh 'Royal Gala') were harvested in the year of 2014, from a commercial orchard located in the municipality of Vacaria, Rio Grande do Sul, Brazil (28º 30' 44" S, 50º 56' 02" O). Apples were harvested considering their commercial mature stage, based on the average starch content (4.80) measured according to Travers et al., (2002), on a scale from 1 (one) to 10 (ten), where 1 and 10 correspond to the maximum and minimum content of starch, respectively; pulp firmness (12.84 N); concentration of total soluble solids (12.03 ºBrix) and titratable acidity (0.22 g of malic acid 100 g^{-1} FW), measured according to the methods described below. Samples were selected according to size, the absence of visible mechanical damage and rot. Fruits were temporarily stored (few weeks) at

1.0 °C, relative humidity of 90.0 % ± 5.0 %, and finally used in the present study at the Postharvest Physiology Laboratory, Food Center, Embrapa Clima Temperado.

2.2. Sanitization, cutting and dipping

Fruits were washed and sanitized by dipping into a sodium hypochlorite solution (100 ppm, pH 6.5, at 6.5 ºC ± 1.5 ºC), for 10 min. Each fruit was cut in four wedge shape pieces of similar size; the central core and seeds were discarded and the fruit epidermis was preserved. After cutting, the apple pieces were dipped for 1.0 min in the following liquids: **(a)** distilled water - control (CT), **(b)** L-cysteine chloride 0.6 % [m v^{-1}] positive control (LC), **(c)** erythorbic acid 1.0 % [m.v^{-1}] (EA 1.0 %), **(d)** erythorbic acid 2.0 % [m.v^{-1}] (EA 2.0 %) and **(e)** erythorbic acid 3.0 % [m.v^{-1}] (EA 3.0 %). After the immersion, the apple pieces were drained for 5 min, placed in trays of expanded polystyrene, wrapped with PVC film (9 μm thick) and stored for different periods (0 d, 3 d, 6 d, 9 d) at 4.0 °C ± 1.0 °C and relative humidity of 90.0 % ± 5.0 %, for simulation of shelf-life.

2.3. Physicochemical analysis

2.3.1. Color measurement: measured on the equatorial region of the wedge-shape pieces of apple, on the pulp, using a Minolta CR-400 colorimeter with a CIE L*a*b* reading system, proposed by the *Commission Internationale de I'Eclairage* (CIE). These parameters were used to calculate the browning index (BI) according Palou et al., (1999).

2.3.2. Mass loss: measured according to Pereira et al. (2006) with the formula *ML* (%) = (*Mi* - *Mf*) / *Mi* x 100, where *Mi* and *Mf* correspond to the apple pieces initial mass and final mass, respectively;

2.3.3. Pulp firmness: measured according to Melo et al., (2009) using a Texture Analyser (TA XT plus 40855, Stable Microsystems, England) with a 2 mm diameter probe, penetration depth of 5 mm, pre-test velocity of 1.0 mm s^{-1}; 2.0 mm s^{-1} test; post-test of 10.0 mm s^{-1} and force of 5 kg. The readings were performed in the middle portion of the pieces and the results were expressed in Newton (N).

2.3.4. Soluble solids: determined by using an Atago refractometer (ATAGO, model PAL^{-1}), with results expressed as °Brix.

2.3.5. Titratable acidity: obtained by titration of 0.1 M NaOH solution into 100 mL sample solution (10 mL mashed pulp + 90 mL distilled H_2O) using a digital burette (Brand®) until pH stabilization at 8.1; the results were expressed in mg of malic acid equivalents (MAE) per 100g of pulp (FW).

2.3.6. Antioxidant activity (DPPH): evaluated using the method described by Brand-Williams et al., (2005), with some modifications. First, 10 mL of methanol was added to 2.5 g of fresh apple and homogenized for 1.0 min (ultra-turrax homogenizer, IKA). Extracts were centrifuged at 4000 rpm, 30 min, 1.0 ºC (Eppendorf – Centrifuge 5810R - Rotor F-45-30-11). The supernatant was collected and stored at -80 ºC until analysis. Apple extract (100 μL) was added to 3900 μL $DPPH^{\bullet}$ solution (in methanol), and the reaction mixture was kept in the dark for 24 h. After this period, the absorbance was spectrophotometrically read at 515 nm. The results are expressed as mg of Trolox equivalent per 100 g of FW.

2.3.7. Total phenolic compounds: measured according to the Folin–Ciocalteu method adapted from Swain and Hillis (1959).

Briefly, 250 µL aliquot of the extracts (the same used for DPPH• analysis) was combined with 250 µL of 0.25 M Folin-Ciocalteu reagent and 4000 µL ultrapure water. After 3 min of reaction, 500 µL of 0.5 M Na_2CO_3 was added, following incubation for 2 h at room temperature and absorbance reading at 725 nm. The results were expressed as grams of chlorogenic acid equivalents (CAE) per 100 g of FW. A chlorogenic acid standard curve (0.0 mg mL^{-1} to 0.5 mg mL^{-1}) was used**.**

2.3.8. Polyphenoloxidase enzyme (PPO) activity: determined according to the adapted methodology described by Cano et al., (1997), based on the increase of the absorbance (420 nm) rate at 25 ºC. Apple extracts were obtained as follows: 10 mL of phosphate buffer (0.2 M, pH 7), containing 0.2 g of polyvinylpyrrolidone (PVP), was added to 5 g of apple and homogenized for 1 min (ultra-turrax homogenizer, IKA), filtered and centrifuged at 16000 *g*. An aliquot of 0.1 mL of supernatant was then mixed with 2.9 mL of catechol (0.11 M in phosphate buffer 0.5 M, pH 7.0). The enzymatic activity was spectrophotometrically monitored at 420 nm for 3 minutes. The results are expressed as ΔA_{420} $min^{-1}g^{-1}$.

2.3.9. Peroxidase enzyme (POD) activity: determined according to the adapted methodology described by Cano et al., (1997), based on the increase of absorbance (485 nm) rate, at 25 ºC, using the same extract obtained for polyphenoloxidase analysis. An aliquot of 50 µL of apple extract was mixed with 2.7 mL of phosphate buffer (0.05 M, pH 7.0), 0.1 µL of hydrogen peroxide (1.5 %, v v^{-1}) and 200 µL of guaiacol. The enzymatic activity was calculated based on the increase of absorbance at 485 nm as a function of time (3 min). The results are expressed as ΔA_{485} $min^{-1}g^{-1}$.

2.3.10. Statistical analysis: The experimental design was completely randomized in a factorial scheme with three biological replicates and three analytical replicates. Treatment factors were the storage time (0 d, 3 d, 6 d and 9 d) and the liquids for dipping the apple pieces: distilled water; 0.6% L-cysteine and erythorbic acid (1 %, 2 % and 3 %). Data normality was determined by Shapiro-Wilk test and variance homoscedasticity by the Hartley test. Later, the results were submitted to analysis of variance (One-Way ANOVA) ($p < 0.05$); when significative, the means were compared by the LSD test ($p < 0.05$).

3. Results and discussions

Enzymatic browning is the main cause of decline in quality and shelf life of minimally processed apples. Therefore, when evaluating the color parameters, there were significant ($p < 0.05$) differences in the variables L *, a *, b * and darkening index. In relation to the L * coordinate (Figure 1a), all treatments with antioxidants, LC and EA (1 %, 2 % and 3 %), remained stable and higher than CT during storage. After 9 d, EA 1 % presented a small decrease and CT a small increase in L* value, but without statistical differences. These results were different from those obtained by Vilas-Boas et al., (2015) when applying antioxidants to MP 'Williams' pears. Regardless of the antioxidant used, these authors observed a decrease in the L* value along the refrigerated storage. The inactivation of POD and PPO enzymes by erythorbic acid acidification of pulp surface may have contributed to the maintenance of luminosity during storage. In addition, Qi et al., (2011) reported that storage at low temperature contributes to delay the loss of luminosity in MP apples.

Regarding a* coordinate (Figure 1b), LC and EA (1 %, 2 % and 3 %) also provided a satisfactory control in the darkening of the pulp until the third day of storage, since negative values of a* indicate pulp with a greenish-yellow coloration. From the sixth day on, only EA 3 % treatment was capable of maintaining the same coloration observed on the first day (0 d) of storage. In contrast, apples with reddish flesh (positive values of a*) were observed under the treatments LC and EA (1 % and 2 %), until the sixth day of storage. The reddish tint observed in apples treated with LC is possibly due to the regeneration of phenolic compounds; this occurs when low concentrations of this antioxidant are used (Martins et al., 2015). However, the satisfactory effect observed with EA 3 % can be attributed to its strong reducing properties (Carocho et al., 2018), since this compound can react with oxygen and thus remove it from a closed system (Lee et al., 2012), avoiding the darkening of the pulp.

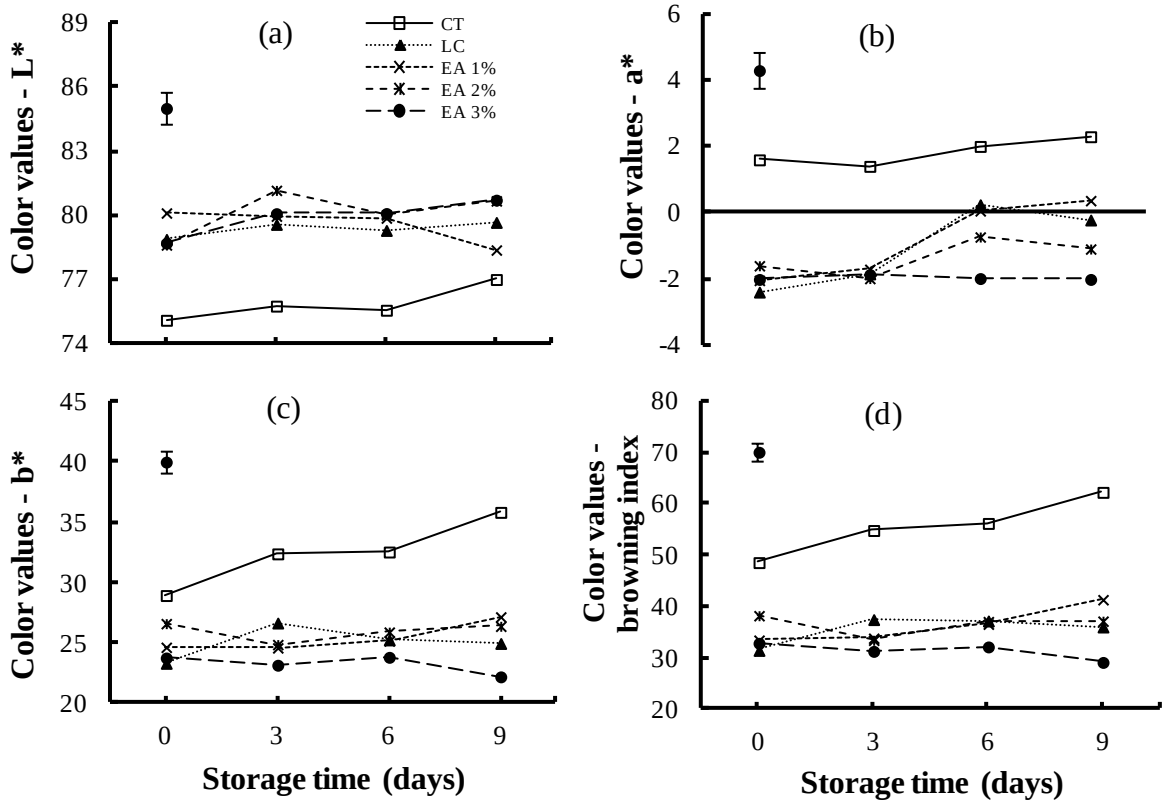

Figure 1. Color values, L* (*LSD = 0.45), a* (*LSD = 0.27), b* (*LSD = 0.78) and browning index (*LSD = 1.67) measured in minimally processed 'Royal Gala' apples stored for 0 d, 3 d, 6 d, and 9 d at 4.0 ºC ± 1.0 ºC, relative humidity of 90.0 % ± 5.0 %, after treatment with distilled water (CT) as negative control; L-cysteine chloride (LC) 0.6 % ($m.v^{-1}$) as a positive control; erythorbic acid (EA): EA 1 % ($m.v^{-1}$), EA 2% ($m.v^{-1}$) and EA 3% ($m.v^{-1}$). *Vertical bars indicate the Least Significant Difference (LSD) at $p \leq 0.05$.

Regarding the b* coordinate (Figure 1.c), although all antioxidants prevented pulp darkening, the EA 3 % treatment resulted in a lighter pulp throughout the storage period. The process of oxidative staining is triggered by the membrane

rupture inside cells (Toivonen 2004), which results in the mixture of substrates (polyphenols) with PPO enzyme (Cortellino et al., 2015). In the presence of oxygen, PPO catalyse the monophenols hydroxylation into diphenols and subsequently the oxidation of diphenols in quinones (Cortellino et al., 2015). The former reaction is relatively slow and results in colorless products; the latter is relatively rapid and results in colored quinones. Subsequent reactions that occur after quinones production lead to the melanin accumulation, a brown pigment that gives the oxidized MP products their characteristic color (Cortellino et al., 2015). Hence, b* values indicate a satisfactory action of the tested antioxidants, with emphasis on the EA 3 %. This result is similar to that observed by Pizato et al., (2013) when working with protein isolates on 'Gala' apples.

As for the browning index (Figure 1d), the different EA concentrations (1 %, 2 % and 3 %) and LC maintained pulp darkening lower than CT during the evaluation period. The EA 3 % kept the color of MP apples very close to that observed on the first day of storage (Figure 2), being the best treatment. It should be noted that the more intense yellow observed near the seed cavity (endocarp), in apples treated with EA 3%, does not indicate ineffectiveness of this antioxidant, since this region is naturally more yellowish than the fleshy pulp (mesocarp).The satisfactory result obtained with EA 3% is important because according to Altisent et al., (2014), the maintenance of MP fruit color indicates freshness and quality. The darkening inhibition process triggered by L-cysteine in apples pulp occurs through conjugation with *o*-quinones to form colorless compounds; another mechanism is the reduction of *o*-quinones to their phenolic compound precursors (Koblitz 2000). According to Richard-Forget et al., 1992, the compounds resulting from cysteine and *o*-quinones conjugation may act as competitive inhibitors of PPO, being another mechanism of darkening inhibition. However, when there is a quinone excess and all of the L-cysteine has been consumed, the former can react with the cysteine-quinone addition compounds, giving rise to violet pigments. This explains the slightly browning observed at the end of the storage in apples treated with LC 0.6 %. In addition, LC contains sulphur in its constitution, giving rise to volatile sulphur compounds during its metabolization; this alters the taste and characteristic odour of the products treated with this antioxidant. On the other hand, the EA provides color maintenance; without compromising the taste, aroma and nutritional quality in MP products (Salata et al., 2014). Because of that, there are advantages in its use in comparison to LC, in addition to its low cost. According to Mishra et al., (2012), the EA prevents the darkening process by inhibiting the PPO enzyme and reducing quinone intermediates back to the diphenols (Mishra et al., 2012). Rojas-Grau et al., (2008), when working with ascorbic acid (AA), an ester of erythorbic acid, reported that although several authors have demonstrated the efficiency of AA in the control of enzymatic darkening, its efficacy is lower than that of L-cysteine. According to the authors, when AA is completely oxidized to dehydroascorbic acid, the quinones may again accumulate and cause browning. In view of this, it is assumed that the good results obtained with EA 3 % are due to the higher concentration of this salt, retarding its oxidation and thus preventing pulp and bundle sheath darkening in MP apples.

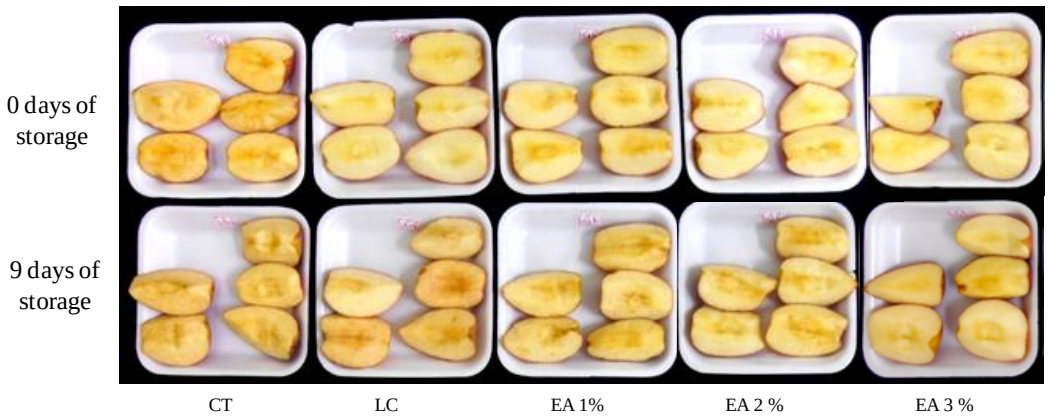

Figure 2. Pulp darkening of minimally processed apples, treated with distilled water, as negative control treatment (CT); L-cysteine chloride (LC) 0.6 % ($m.v^{-1}$), as a positive control; erythorbic acid (EA): EA 1 % ($m.v^{-1}$), EA 2 % ($m.v^{-1}$) and EA 3 % ($m.v^{-1}$); stored for 0 d, 3 d, 6 d, and 9 d at 4.0 ºC ± 1.0 ºC, and relative humidity of 90.0 % ± 5.0 %.

The *ratio* (total soluble solids / titratable acidity) is commonly used to evaluate fruit quality. A change in the *ratio* might affect how the apple taste (Piagentini and Pirovani 2017). This relationship (Figure 3a) had a small decrease in all treatments until the sixth day of storage, with a subsequent increase until the ninth day of storage. These changes can be explained by the metabolism of organic acids in the respiratory pathways and subsequent conversion into non-acidic molecules (Pech et al. 2008). Total soluble solids can also accumulate by protopectin hydrolysis into soluble pectin or by starch hydrolysis into glucose and fructose (Barnes and Anderson 2018; Liu et al. 2018).

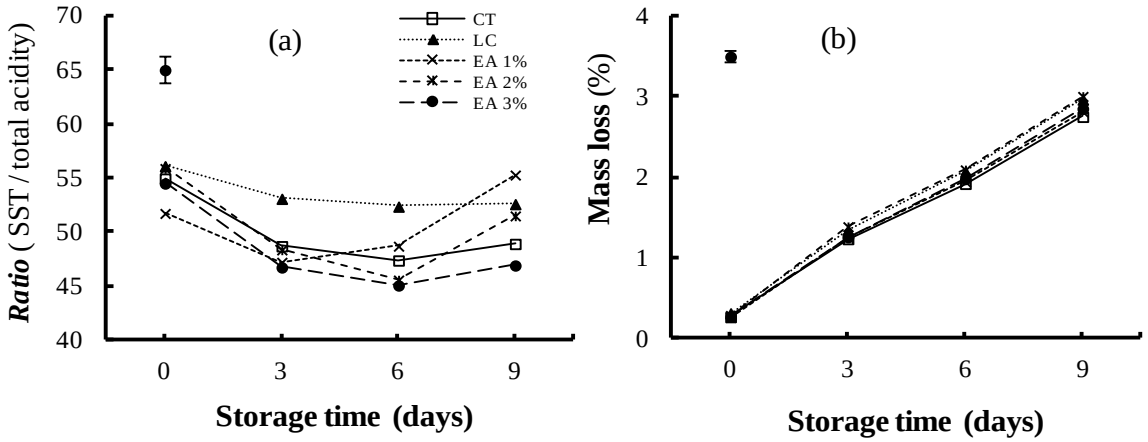

Figure 3. *Ratio* (*LSD = 1.21) and mass loss (*LSD = 0.067); measured in minimally processed 'Royal Gala' apples stored for 0 d, 3 d, 6 d, and 9 d at 4.0 ºC ± 1.0 ºC, relative humidity of 90.0 % ± 5.0 %, after treatment with distilled water as a negative control treatment (CT); L-cysteine chloride (LC) 0.6 % ($m.v^{-1}$) as a positive control; and erythorbic acid (EA): EA 1 % ($m.v^{-1}$), EA 2% ($m.v^{-1}$) and EA 3% ($m.v^{-1}$). *Vertical bars indicate the Least Significant Difference (LSD) at $p \leq 0.05$.

The mass loss, a limiting factor in the shelf life of MP fruits and vegetables, increased during storage; with a mean mass loss (Figure 3b) of 2.6 % at the end of the storage period. This loss of mass was 5 times lower than that observed by Sumonsiri (2017) when working with ascorbic acid and nisin on 'Fuji' MP apples. The gradual mass loss throughout storage is due to pulp exposure to the atmosphere, potentiating the loss of water (Pajak et al., 2017). Despite the significant differences observed between treatments, the mass loss was minimal, not compromising the final quality of the product. The pulp firmness (data not shown) did not differ between treatments and along the storage period, with a mean value of 2.5 N.

Apples contain large amounts of endogenous phenolic compounds with antioxidant properties (Kim et al., 2017), as well as ascorbic acid (up to 0.25 g kg^{-1}). However, the antioxidant loading could be complemented by the immersion of MP fruit in solutions containing exogenous antioxidants (Aguayo et al., 2010). This process aims to interfere in oxidation reactions after MP and preserve endogenous phenolic compounds and ascorbic acid (Aguayo et al., 2010). When evaluating the concentrations of phenolic compounds in apples treated with antioxidant solutions (Figure 4a), it was observed that the EA 3 % maintained phenolic compounds concentrations constant during the nine days. In addition, it promoted the increase of these bioactive compounds throughout storage. The other treatments (LC, EA 1 % and EA 2 %), although resulting a lower amount of phenolics, remained within the values reported in the literature (50 mg.100 g^{-1} FW to 380 mg.100 g^{-1} FW) (Ceymann et al., 2012). The application of EA 3 % maintained the concentrations of phenolic compounds. It occurs because EA, together with its stereoisomers, limits the production of *o*-quinones (Grant-Preece et al., 2013) by eliminating oxygen from the tissue before it reacts with the phenolic compounds (Clark et al., 2009, Bradshaw et al., 2011). In the absence of the antioxidant, PPO catalyses the phenolic compounds oxidation leading to the formation of undesirable pigments in the apple pulp (Zorzella et al., 2003). Oxidation, in addition to causing browning, may also result in loss of nutritional quality and provide flavor modifications. According to Son et al., (2001), the brown color intensity resulting from the PPO activity depends on the type of phenolic compounds involved.

The antioxidant activity was quantified based on the free radical scavenging activity (DPPH) (Figure 4b). The observed behavior for antioxidant activity was similar to that observed for total phenols (Figure 4a). EA 3 % resulted in the highest antioxidant activity, with a tendency to increase throughout storage, corresponding to a significant increase in free radical sequestration, from 444.68 mg Trolox.100 g^{-1} FW (0 d) to 505.50 mg Trolox.100 g^{-1} FW (9 d); an increase of 12 %. Aguayo et al., (2010) when working with the addition of antioxidants in 'Mariri Red' apples reported that to maintain a higher antioxidant activity than in the control treatment, a treatment with at least 6 % of calcium ascorbate was necessary. In the present study, EA 3 % maintained the antioxidant activity higher than in the TC as follows: 12.96 % (0 d), 14.11 % (3 d), 21.73 % (6 d) and 28.12 % (9 d). The increase in antioxidant activity over time can be attributed to the preservation of polyphenols, as shown previously. EA has antioxidant action due to its reducing

properties (Watanabe et al., 2014), acting in similar manner to AA (Clark et al., 2009). EA is considered safe for human consumption, low cost, well accepted by consumers and capable to increase the vitamin C content (Loan; Manzano, 1993), even though it presents only 5 % of the AA vitamin activity. As for LC, its ability to preserve antioxidant activity in apples is attributed to its ability to sequester free radicals due to the presence of a thiol group (Altunkaya and Gökmen, 2008).

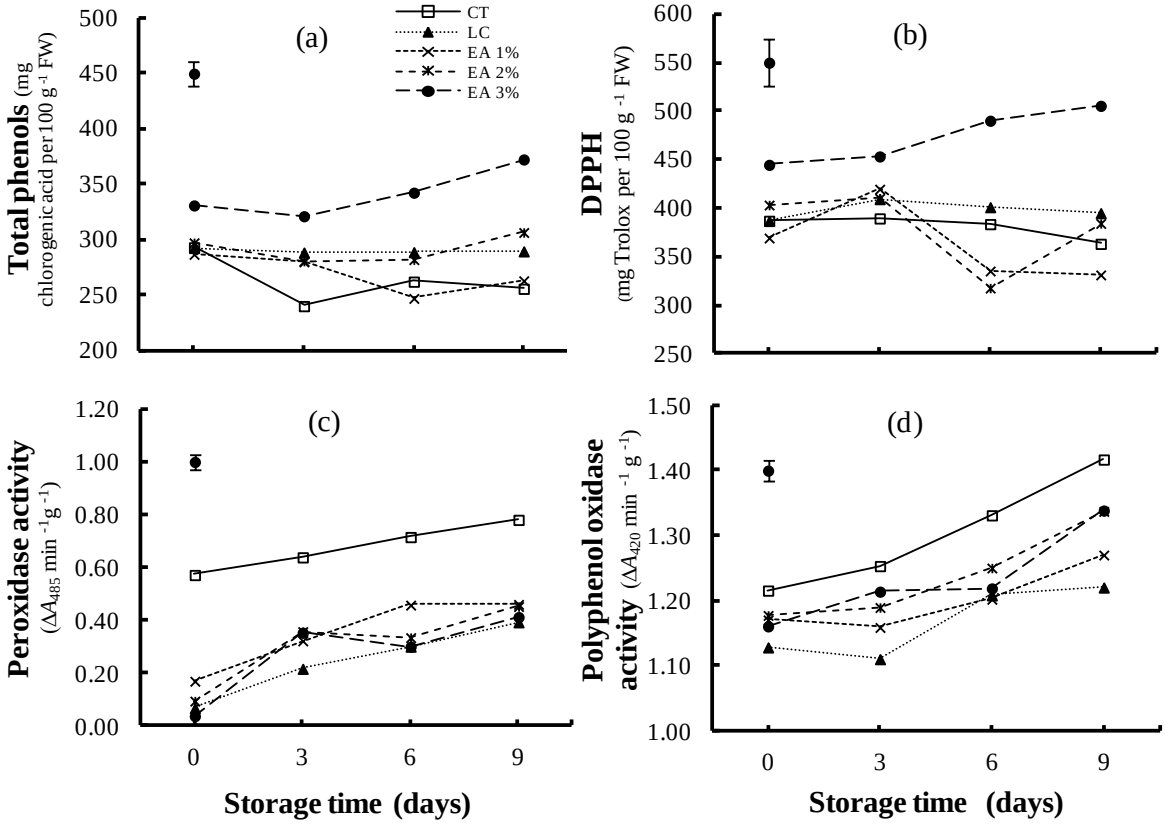

Figure 4. Total phenols (*LSD = 11.37), DPPH (*LSD = 24.20), peroxidase activity (*LSD = 0.026) and polyphenoloxidase activity (*LSD = 0.014) in minimally processed 'Royal Gala' apples stored for 0 d, 3 d, 6 d, and 9 d at 4.0 ºC ± 1.0 ºC, relative humidity of 90.0 % ± 5.0 %, after treatment with distilled water as a negative control treatment (CT); L-cysteine chloride (LC) 0.6 % ($m.v^{-1}$) as a positive control; and erythorbic acid (EA): EA 1 % ($m.v^{-1}$), EA 2% ($m.v^{-1}$) and EA 3% ($m.v^{-1}$). *Vertical bars indicate the Least Significant Difference (LSD) at $p \leq 0.05$.

The control of PPO and POD activity after MP is important because these enzymes are involved in the darkening process; it occurs almost instantly after destruction of the cellular structure (Jang and Moon 2011). There was an increase in POD activity along the storage (Figure 4c), regardless of the application of antioxidants. The remarkable difference was the lower activity of POD under LC and EA (1 %, 2 % and 3%) treatment in comparison to the activity under the CT. Considering that EA is an AA stereoisomer, the reduction in activity is in accordance with the results reported by Jang and Moon (2011), where the presence of AA effectively reduced POD

activity in MP apples. The reduced POD activity in AA treated fruits could be the result of lower oxidative stress on the fruit surface, due to the antioxidant nature of the molecular AA; it could also be a result of the formation of the POD-hydrogen donor complex (Saba and Sogvar, 2016).

The results observed for PPO activity (Figure 4d) are similar to those of POD activity. There was an increase in the PPO activity for all the treatments during the nine days of storage. Although there were oscillations in the enzymatic activity in apples treated with the antioxidant solutions, the PPO activity of these treatments was lower than the activity observed under the CT. Similar results were observed by Mirshekari et al., (2017) when working with MP 'Berangan' bananas treated with calcium propionate and chitosan. Even without major differences in POD and PPO activity among antioxidant treatments, LC and EA (1 %, 2 % and 3 %), the phenolic substrates were preserved as previously shown. This is an important result because one of the main reasons for the darkening of many fresh fruits and vegetables is the oxidation of phenolic substrates by PPO (Nguyen et al., 2003, Xing et al., 2010) and POD (Jang and Moon 2011). In addition to the antioxidant effect of LC and EA, the low pH of these solutions (2.22 and 1.62, respectively) may have contributed to the reduction of enzymatic activity. Tsouvaltzis and Brecht, (2017) when working with MP 'Russet Burbank' potatoes found that immersion of the MP tubers in H_2SO_4 pH 2.39 (< 0.04 %) reduced the PPO activity in comparison to the control treatment. According to these authors, this occurred because the optimum pH for the enzymatic activity (pH 5 to pH 7) was altered.

4. Conclusions

The use of erythorbic acid 3 % ($m.v^{-1}$) efficiently controls enzymatic browning and preserves physicochemical characteristics of minimally processed 'Royal Gala' apples under refrigeration for up to nine days.

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MICROWAVE-ASSISTED EXTRACTION OF PHENOLIC ANTIOXIDANT COMPOUNDS AND ANTIBACTERIAL ACTIVITIES OF *THYMUS TRANSCAPICUS* ESSENTIAL OIL FROM NORTH KHORASAN PROVINCE OF IRAN

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ABSTRACT

Thymus is a traditional pharmaceutic plant which is also used as a spice and perfumed plant in different industries. In present study, Microwave Assisted Hydrodistillation (MAHD) and hydrodistillation in a Clevenger-type apparatus methods. After preparation of essential oils, antioxidant properties were measured by two methods, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). BHT was used as positive control for comparison. Also antibacterial activities were screening against two Gram-positive bacteria (Staphylococus aureus, and Listeria Monocytogenes) and two Gram-negative bacteria (Salmonella enterica, Escherichia coli) by minimum inhibitory and bactericidal concentration (MIC and MBC) and disc and well diffusion method. Comparison between two extraction methods showed that extraction efficiency of antioxidant and antimicrobial activity at, Microwave Assisted Hydrodistillation method is more than hydrodistillation method. Results presented here suggest that the essential oil of Thymus transcapicus possess strong antimicrobial and antioxidant properties, and therefore, they can be used as a natural preservative ingredient in food and/or pharmaceutical industry.

1. Introduction

In the third world and developing countries, due to the increasing consumer demand for more natural foods, the abuse of toxic synthetic food substances and the increasing microbial resistance of pathogenic microorganisms against antibiotics, natural substances isolated from plants are considered as promising sources of food preservatives (Burt, 2004; Peschel et al., 2006; Smith-Palmer et al., 2001). It is clear from these studies that these secondary plant metabolites have potential uses in medical procedures and applications in the cosmetic, pharmaceutical and food industries (Baratta et al., 1998; Baratta et al., 1998).

Among the different groups of plant products, essential oils are especially recommended as one of the most promising groups of natural products for the formulation of safer antimicrobial agents (Varma & Dubey, 2001). Other than antibacterial and antiviral effects, most essential oils investigated possess antiinflammatory, antifungal and antioxidant properties (Sacchetti et al., 2005). Essential oils are also widely used as food flavours and preservatives to prevent growth of food-borne

bacteria and molds, and so extend the shelf life of processed foods (Burt, 2004).

The Thymus genus comprises over 300 species of which, 14 are found in Iran (Rechinger & Hedge, 1982), which grow wiled in many regions and four of them are endemic (Mozaffarian, 1996). Thymus is a well –known medicinal plant which is native to Southern Europe which its essential oil is manufactured commercially for use in cough drops, mouthwashes, liniments, toothpastes, detergents and perfumes. The herb is approved by Commission E in the treatment of bronchitis, whooping cough and upper respiratory inflammation.

In folk medicine, Thymus spp. are used an anthelmintic, antispasmodic, carminative, sedative, diaphoretic usually in form of an infusion, or externally in bath to cure rheumatic and skin disease (Rustaiyan et al., 2000). Thyme oil is also carminative, expectorant and possesses antimicrobial and anthelmintic properties due to concentrated thymol and carvacrol content but it is extremely toxic. Thymus essential oil and extract is a source of aromatic terpens and terpenoids, flavonoids and phenolic acids (Stahl-Biskup & Sáez, 2002). Thymol, which is the main components of many Thymus species is known as an antiseptic agent and is approved for diverse effects like hookworm treatment (Sefidkon et al., 2001; Evans et al., 1998). Also thymol and their salts used about 0.1-1% in formulation of many lotions, creams and ointments. In external used about 0.1-1% in formulation of many lotions, creams and ointments. In external use, thymol is known as a strong antiseptic agent in toothplaste, gargle and mouthwashes (Zargari, 1990).

The other major component of Thymus spp. oil is carvacrol which is used nowadays on a large scale in the food, cosmetic and mouthwashes industries. In addition, it has been shown several activities like antimicrobial, analgesic and antioxidant activities but it is toxic in high concentration (Monzote et al., 2009).

There are many reports of the essential oil composition and biological activity of different Thymus species especially common Thyme (Thymus vulgaris) and wild Thyme (Thymus serpyllum). Hence of the use of Thymus species or their essential oils in the food and traditional medicine of Iran, we were interested in studying on the essential oil contents and chemical composition of all Iranian endemic species. Several studies have been shown that Thymus species have antibacterial (Mehrgana et al. 2008; Figueiredo et al. 2008; Tohidpour et al. 2010), antifungal (Figueiredo et al. 2008. Bonjar 2004; Sokovic et al. 2009), cytotoxic (Goncalvesa et al. 2010), analgesic (Sokovic et al. 2009), antiparasitic (Goncalvesa et al. 2010), topical anti-inflammatory (Ismaili et al. 2002), antispasmodic (Begrow et al. 2009), mosquitocidal (Pavela et al. 2009) and antioxidant (Zamani et al. 2009; Soares et al. 1997) activities.

In the last few decades, various technologies including maceration, mechanical rabbling, heat reflux, ultrasound and ultrahigh pressure have been applied to polysaccharide extraction. However, these techniques have had some drawbacks including low extraction efficiency, high operating costs and abnormal extract quality (Zhao et al., 2013). Recently, microwave-assisted extraction has been widely applied to extract bioactive compounds from various natural resources. The microwave energy penetrates the material structure, producing molecular friction due to the dipolar rotation of polar solvents, and accelerates the mass transfer of target compounds. Compared with traditional extraction processes, microwave-assisted extraction has enhanced the extraction efficiency and is also more environmental friendly in terms of its reduced use of energy and solvents (Chen et al., 2015a).

The aim of this work was to identify of total phenolic and flavonoid contents of extract of T. transcapicus and also antioxidant and antimicrobial activity of the plant. To the best of our knowledge, this is the first report on chemicals and biological activity of T. transcapicus.

2.Materials and methods

2.1. Plant material

The Plant material was collected in May 2016 from North Khorasan Province Mountains in Iran. Then, the plant was identified and confirmed by Natural Products & Medicinal Plants Research Centre, North Khorasan University of Medical Sciences (Iran) and Voucher specimen (No: MP 32/4) was deposited in herbarium of the Natural Products & Medicinal Plants Research Centre.

2.2. Extraction of *Thymus transcapicus* oil through Microwave Assisted Hydrodistillation (MAHD)

A domestically modified microwave oven (Samsung MW71E model) was fitted to the Clevenger-type apparatus as describe previously in literature (Golmakani and Rezaei, 2008). For MAHD extraction, 1 L sized reactor (round bottom flask) containing 25 g of powdered *Thymus transcapicus* matrix (pre-soaked in distilled water at 8:1 w/w of water to dried *Thymus transcapicus* powder) was placed within the microwave oven cavity. A Clevenger apparatus which has been set on top, outside the microwave oven, was used to collect the extracted essential oil. Extraction was continued for about 90 min and at microwave power level of 250 W. The extraction parameters were selected based on previous research (Jeyaratnamet al., 2016a). After extraction, the *Thymus transcapicus* oil was dehydrated over anhydrous sodium sulfate to remove excess water, then the concentrated *Thymus transcapicus* oil was weighed and stored in vial at 4°C for further analysis.

2.3. Hydrodistillation

The plant (80 g of dried material) was submitted to hydrodistillation for 3 h, using a Clevenger-type apparatus, according to the European Pharmacopoeia (1975). The volatile arrack was accumulated over anhydrous sodium sulphate and refrigerated previous to analysis (Yamini et al., 2008).

2.4. Oil extraction yield

The amount of oil calculated by the following formula:

Oil (%v/w) = Volume of essential oil (ml) / Weight of raw materials (g).

2.5. Preparation of plant extract

The aerial parts of the plants was dried under shade at room temperature and then cut into small pieces. About 100 g of sample was macerated in methanol at room temperature for 48 h separately. Each solvent was allowed to remain in contact with plant material for 24 h, and replaced with fresh solvent four times. Removal of the solvents under vacuum at 40 °C gave the crude extract (Boozari et al. 2015).

2.6. Determination of total phenolic and flavonoid contents

The total phenolic content (TPC) in each extract was determined using the Foline–Ciocalteu procedure as described in Ardestani and Yazdanparast (2007) with minor modifications. Briefly, to prepare a sample extract, 10 mL of 80% methanol was added to 250 mg of the dried-milled samples and shaken slowly. The solution thus obtained was filtered, 0.5 mL of the methanolic extract was mixed with 2.5 mL of the Foline-Ciocalteu's reagent (1:10 diluted with distilled water) and 2 mL of 7.5% sodium carbonate solution in a tube test and shaken well. The mixture was maintained at 45 °C in a hot water bath for 15 min. Then, the absorbance of the mixture was measured at 765 nm using a spectrophotometer. A blank sample consisting of water and the reagents was used as the reference. Tannic acid equivalents (TAE) were used as the reference standard and the TPC was expressed as mg of TAE per gram of each extract on a dry basis.

The aluminum chloride colorimetric method was adapted for the determination of total flavonoids (Zhang et al., 2015) with some changes. A volume of 125 µL of the extract was added to 75 µL of a 5% $NaNO_2$ solution. The blend was allowed to remain for 6 min before 150 µL of $AlCl_3$ (10%) was added and incubated

for 5 min. To this was then added 750 µL of NaOH (1 M). The final volume of the solution was made to 2500 µL with distilled water. After 15 min of incubation, the mixture turned pink and the absorbance was measured at 510 nm. The total flavonoid content (TFC) was presented in mg of quercetin equivalents (QE) per gram of the extract.

2.7. Antioxidant Activity Determination

2.7.1. Antioxidant activity by DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of the essential oil was measured on the basis of the scavenging activity of the stable radical DPPH according to the method of Wang (Wang et al., 2003). 100µl from essential oil at different concentration range (2.5- 25 mg/ml) were mixed in the freshly prepared 4 mM DPPH in methanol. Absorbance at 517 nm was specified after 30 min. The scavenging activity was calculated using Eq.1.

$$\% \text{ DPPH scavenging activity} = \frac{\text{A 517 of control} - \text{A 517 of sample}}{\text{A 517 of sample}} \quad (1)$$

The percent of scavenging activity was plotted against the sample concentration to obtain EC_{50} (effectual concentration) defined as the concentration of sample necessary to scavenge 50% of the DPPH radicals and it was computed using graphpad prism (version 5.0) software. BHT was used as reference antioxidants.

2.7.2. Total reduction ability by $Fe3^{+}$-Fe^{2+} transformation

The total reduction ability of essential oil was determined by the method of Oyaizu (1986). The capacity of essential oil to reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) was evaluated by measuring the absorbance at 700 nm. To the different concentrations of the essential oils 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) were added. The mixture was incubated at 50 °C for 20 min. Then 2.5 ml of trichloroacetic acid (10%) were added. The mixtures were revolved at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride. Absorbance was measured at 700 nm on UV spectrophotometer after allowing the solution to stand for 30 min. Butylated hydroxytoluene (BHT) was used as a standard.

2.8. Antimicrobial Activity

Determination of the minimum inhibitory concentrations (MIC) antimicrobial activities of essential oil of the aerial part of the plant was determined against two Gram-positive bacteria*:* *Staphylococus aureus* (ATCC 6538p), and *Listeria monocytogenes* (ATCC 35152), two Gram-negative bacteria: *Salmonella enterica* (ATCC 53648), *Escherichia coli* (ATCC 10536).

2.8.1. Determination of the minimal inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) were determined by broth macro dilution method in 96-well plates by Rios and Duffy methods (Rios et al. 1988; Duffy & Power 2001).

Initial concentration of essential oil was prepared with the aid of bath sonicator with 4 ml solvent and 30% dimethylsulphoxide in sterile distilled water and one drop of Tween 80. 1 ml of diluted extract was infused into macro-plate with 1ml of sterile Mueller-Hinton broth (MHB; HiMedia, India) and then diluted (50% with MHB). 0.5 McFarland standard turbidity for microbial suspension equivalent was prepared by suspensions of the growth from brain-heart infusion medium (HiMedia, India). Suspensions were further diluted to obtain a concentration of 10^7 colony-forming units (CFU) per ml for the bacteria. Then, 10 µl of diluted inoculums was added to each well of macro-plate. The sterility of the medium was also tested in two wells and Gentamicin was used as the positive control for bacterial strains. Plates were incubated for 24 h at 37°C for bacteria. The growth of

microorganisms was assessed by TTC (2, 3, 5-triphenyl tetrazolium chloride, Sigma, USA) assay. Briefly, 0.5 ml of TTC (5 mg. ml^{-1}; dissolved in sterile water) was added to each well and the plates were incubated at 37 °C for bacteria. The results were expressed as the lowest concentration of plant extract that could inhibit any red dye production. MIC values were defined as the lowest concentrations of oil that inhibit bacteria after 24 h. All experiments were done in triplicates.

2.8.2. Determination of minimum bactericidal concentrations (MBC)

The bactericidal effects of essential oil were determined according to the method described by Rios (Rios et al. 1988). 100 µl of clear dilutions in wells of macro-plate were sub cultured on the Mueller- Hinton agar plates and subsequently incubated at 37 °C for 24 h. Minimal bactericidal concentration (MBC) were recorded from the first tube that showed no growth on solid media.

2.8.3. Antimicrobial activity by disc and well diffusion method

The essential oil of the plant was tested for antibacterial activity using the disc and well diffusion methods on solid media Mueller-Hinton agar (MHA) plates. The sterile paper discs and wells of 6 mm diameter were placed on the agar plates with the appropriate media, and the bacteria density was adjusted to approximately 10^7 CFU/ml. Then, 50 µl of the essential oil was applied to test paper disc and well in plates and the agar plates were further incubated for 24 hr at 37°C. Finally, the zones of growth inhibition around the discs were measured. Gentamicin and DMSO were used as positive and negative controls, respectively (Firdaus et al, 2011).

2.9. Statistical analysis

The measurements of antibacterial activity, total phenolic compounds, DPPH radical scavenging activity and FRAP assay were carried out for three replicates. The results are expressed as mean values ± standard deviation (SD).

3. Results and Discussion

3.1. Essential oil yield

Nowadays, microwave treatment is one of the most commonly used methods for solid-liquid extraction due to its power, convenience, and reasonable cost. Many studies have reported about the benefits of microwaves for extracting some active compounds from plant materials such as triterpene, saponins, and antioxidant components etc. (Li et al., 2010; Zhao et al., 2013). These results highlight the ability of microwaves to disrupt hydrogen bond networks. The microwave-induced dipole rotation of molecules, and the migration of ions that enhance the penetration of solvent in to matrix, disrupts the cell wall and releases the intracellular product, allowing for the extraction of different components (Li et al., 2010).

The present study, the extraction yield of essential oil were with microwave assisted hydrodistillation and hydrodistillation, 0.494% and 0.243% (v/w), respectively. Among the samples based on a dry weight. Essential oil content can be highly affected by both environmental factors and plant species (Bahreininejad, Mirza, & Arzani, 2010; Llorens et al., 2014; Yavari et al., 2010). Previous studies reported various ranges for the EO yields in different Thymus species. For instance, *T. fedtschenkoi* had been previously reported to have its maximum EO yield (2.9%) at the flowering stage and its minimum (0.7%) during the seed set stage (Rustaiee et al., 2011). Hazzit, Baaliouamer, Veríssimo, Faleiro, and Miguel (2009) had also reported an EO yield in the range of 4.2–4.6% for T. pallescens in its full flowering stage and a minimum of 0.9–1.3% at the beginning of the vegetative cycle.

3.2.Total phenolics and total flavonoid contents

TPC and TFC are the two key indicators widely employed to represent the overall antioxidant activity in the samples. The results

the amount of phenolic in essential oil were with microwave assisted hydrodistillation and hydrodistillation,11.42 and 9.21 mg tannic acid equivalents (TAE) g^{-1} DW, respectively and flavonoid content 7.65, 4.04 mg quercetin equivalents (QE) g^{-1} DW, respectively.

Furthermore, phenolic compounds were capable of scavenging the reactive oxygen intermediates without invoking further oxidative reactions (Al-Abd et al., 2015). Previous research has shown that TPC takes on variable values depending on the Thymus species (Jabri-Karoui, Bettaieb, Msaada, Hammami, & Marzouk, 2012).

Safaei-Ghomi et al. (2009) found TPC values for *T. caramanicus* were higher than those obtained for the accessions investigated in the present study. High TPC values have also been reported for *T. spathulifolius* (Sokmen et al., 2004) and *T. Serpyllum* (Mata et al., 2007).

The studies conducted so far have established that the Thymus species can be considered not only as rich sources of phenolics and flavonoids but as promising sources of natural antioxidants as well. The phenolic content of each plant, however, is a function of a multitude of factors such as the extraction method employed and the phenological stage (Gharibi et al., 2015).

However, it is likely that different species use different mechanisms to distribute flavonoids among their subcellular parts. From a metabolic point of view, plant polyphenols such as flavonoids and phenolics are biosynthesized through several pathways and form a heterogeneous group (Gharibi et al., 2015). Baharfar et al. (2015) reported that the TFC value of *T. kotschyanus* ranged from 32.04–74.60 mg QE g^{-1} of the dry extract. Jabri-Karoui et al. (2012) reported a TFC value of 10.62 ± 0.24 mg CE/g DW for *T. capitatus*. Furthermore, the use of different solvents can affect the flavonoid content of the plants. Hossain et al. (2013) revealed that methanol as a solvent produced higher amount of flavonoid in comparison with four other solvents in *T. vulgaris*. The mechanism underlying the flavonoid functions is based on the scavenging or the chelating process. The composition of these compounds is highly influenced by the location where the sample is collected as well as the dominant climatic and environmental factors (Rahimmalek et al., 2009).

3.3.Essential oil antioxidant activities

DPPH assay and reducing power assay were used to assess antioxidant potential of *Thymus transcapicus* essential oil. The synthetic antioxidant BHT was used as an equivalence parameter for the antioxidant activity of the essential oil.

Reactive oxygen species (ROS), including oxygen radicals and their reaction products, are known to react with biological molecules, leading to cell and tissue damage. Antioxidant activity is a complex process usually occurring through several mechanisms. Due to its complexity, the evaluation of the antioxidant activity for pure compounds or extracts should be carried out by more than one test method (Aruoma, 2003). The lower IC_{50} value indicates a stronger ability of the extract to act as a DPPH scavenger while the higher IC_{50} value indicates a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction.

The DPPH scavenging activity of the tested oil was found higher than butylated hydroxytoluene (BHT) as is evident from lower IC_{50} value of essential oils. In reducing power assay, *Thymus transcapicus* essential oil showed comparable ferric reducing power to BHT at the tested concentrations of 20–100 mg/ml (Table 1). Microwave assisted hydrodistillation, which led to higher antioxidant activity, is a more efficient technique than hydrodistillation.

Table 1. Antioxidant activity of *Thymus transcapicus* essential oil measured in term of DPPH radical scavenging capacity and Total Ferric Reducing ability.

Technique applied	Test system	Conc. (µg/ml)	*Thymus transcapicus*	BHT	IC_{50} value, µg/ml *Thymus transcapicus*	IC_{50} value, µg/ml BHT
Microwave	DPPH radical scavenging capacity (%)	20	34.6±0.4	31.3±0.9	31.08±0.4	47.28±0.5
		40	59.87±0.5	43.74±0.8		
		60	69.9±0.3	59.9±0.4		
		80	81.9±0.6	75.9±0.5		
		100	89.8±1.2	80.8±0.3		
	Reducing power (absorbance at 700 nm)	20	1.86±0.8	1.13±0.7		
		40	1.92±0.6	1.19±0.5		
		60	1.99±0.3	1.25±0.3		
		80	2.37±0.4	1.53±0.1		
		100	2.87±0.2	1.73±0.5		
Hydrodistillation	DPPH radical scavenging capacity (%)	20	14.9±0.2		49.8±0.2	47.28±0.5
		40	25.87±0.6			
		60	45.3±0.4			
		80	64.9±0.8			
		100	77.1±0.3			
	Reducing power (absorbance at 700 nm)	20	0.65±0.2			
		40	0.79±0.9			
		60	0.91±0.1			
		80	1.19±0.2			
		100	1.22±0.7			

The antioxidant capacity of Thymus species has been well researched. The most relevant chemotypes of Thymus species have been reported to be rich in phenolic monoterpenes such as thymol and carvacrol (Jabri-Karoui et al., 2012). Species such as *T. carmanicus* (Safaei-Ghomi et al., 2009) and *T. spathulifolius* (Sokmen et al., 2004). In most

such studies, phenolics, due to their chemical structures that allow them to donate hydrogen to free radicals, were introduced as the major factor contributing to the antioxidant activity of the species (Ang et al., 2015). Moreover, essential oil consisting of phenolic monoterpenes and/or sesquiterpenes has been recognized for their higher antioxidative capacity (Mancini et al., 2015).

The reducing power of the studied *transcapicus* was observed to increase with increasing essential oil concentration. In this model system, *T. transcapicus* showed a more reducing power than BHT. Previous studies had indicated that the high reducing power of the Thymus species was not directly related to its thymol and carvacrol contents but the substitution of hydroxyl group in the aromatic ring might have contributed to its antioxidant activity (Jabri-Karoui et al., 2012).

3.2. Antimicrobial activity

The results presented in table 2, zones of growth inhibition around MIC and MBC of *Thymus transcapicus* essential oil was evaluated.

Table 2. Determination of MIC, MBC of *Thymus transcapicus*

Technique applied	**Test bacteria**	**MIC (mg/ml)**	**MBC (mg/ml)**
Microwave	*Staphylococus aureus* (ATCC 6538p)	25	25
	Listeria monocytogenes (ATCC35152)	25	25
	Salmonella enterica (ATCC 53648)	50	50
	Escherichia coli (ATCC 10536)	50	50
Hydrodistillation	*Staphylococus aureus* (ATCC 6538p)	50	50
	Listeria monocytogenes (ATCC35152)	100	>100
	Salmonella enterica (ATCC 53648)	100	>100
	Escherichia coli (ATCC 10536)	100	>100

Table 3. Antibacterial activity of *Thymus transcapicus* was assessed by disc and well-diffusion methods.

Technique applied	Test system		Microorganism			
Microwave	Diameter of inhibition zones (mm), Disc-diffusion method*		*S. aureus*	*L. monocytogenes*	*S. enterica*	*E. coli*
		Essential oil	30 mm±0.02	31 mm±0.03	29 mm±0.01	28 mm±0.05
		Gentamicin	27 mm±0.05	28 mm±0.02	26 mm±0.04	25 mm±0.02
	Diameter of inhibition zones (mm), Well-diffusion method*	Essential oil	32 mm±0.02	34 mm±0.03	31 mm±0.04	30 mm±0.03
		Gentamicin	32 mm±0.01	33 mm±0.04	29 mm±0.01	28 mm±0.05
Hydrodistillation	Diameter of inhibition zones (mm), Disc-diffusion method*	Essential oil	25 mm±0.07	26 mm±0.01	23 mm±0.04	21 mm±0.02
		Gentamicin	27 mm±0.05	28 mm±0.02	26 mm±0.04	25 mm±0.02
	Diameter of inhibition zones (mm), Well-diffusion method*	Essential oil	26 mm±0.07	28 mm±0.05	27 mm±0.06	25 mm±0.01
		Gentamicin	32 mm±0.01	33 mm±0.04	29 mm±0.01	28 mm±0.05

*Expressed as the size of the growth inhibition zones (mm) as the average of triplicates.

Antimicrobial activity of the plants of different areas of the world has been reported (Janovska et al, 2003). The disc and well-diffusion methods are dependent on the diffusion ability of the substances and in these methods; antibacterial property is expressed as diameter (mm) of the zone of inhibition (He et al, 2010).

Table 3 shows the antibacterial activity of the essential oil of this plant was markedly higher than gentamicin against gram-positive bacteria (*Staphylococus aureus*, and *Listeria Monocytogenes)* and two gram-negative bacteria (*Salmonella enterica*, *Escherichia coli*). Antimicrobial activities of some Thymus species have been shown in other previous

studies. *Thymus pubescens* and *Thymus vulgaris* extract demonstrated good antibacterial activity against some drug resistant Gram-positive bacteria (Mehrgana et al., 2008; Tohidpour et al., 2010). The essential oil of the Thymus caramanicus showed high inhibitory activity against Helicobacter pylori (Eftekhar et al., 2009). *T. transcaspicus* essential oil was tested for its antibacterial activity against various Gram-positive and Gram-negative bacteria Standard strains. All the bacteria were inhibited by the essential oil but in variable degrees. Inhibition of *Staphylococcus aureus* (de Oliveira et al., 2010) and antibacterial effects against E. coli (Pei et al., 2009) by thymol and carvacrol have been reported. Carvacrol also has been reported to exhibit a dose dependent inhibitory effect on Vibrio cholerae in food (Rattanachaikunsopon et al., 2010). Thymol, which is the main component of many Thymus spp. and also in the oil of *T. transcaspicus* (64%), is known as an antiseptic agent (Miri et al., 2002). The antimicrobial activity of *T. transcaspicus* EO was, therefore, attributed to the presence of Thymol. Other constituents of the essential oil such as gammaterpinene and p-cymene, could be also taken into account for their possible synergistic or antagonistic effects.

The effectiveness of essential oil is demonstrated by the size of the microorganism growth inhibition zone around the filter paper disc, which is typically expressed as the diameter of the zone in mm.

4.Conclusions

The results showed that the activity of the oil can be attributed, to a considerable degree. MAE, which led to higher antioxidant and antimicrobial activity, is a more efficient technique than hydrodistillation. The results of this study suggest the possibility of using *Thymus transcapicus* essential oil as a natural food preservative, because the oil found to possess strong antibacterial activity.

5.References

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EFFECT OF VARIATION IN REGION AND SEASONS ON SENSORY, CHEMICAL AND MICROBIA CHARACTERISTICS OF LABNEH MANUFACTURED BY TRADITIONAL METHODS

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Sensory analysis

ABSTRACT

This work investigated the chemical, physical, microbiological, and sensory characteristics of labneh, made from fresh cow's milk in the northern, middle and southern regions of Jordan, in the spring and summer seasons. The chemical composition (%) fell within the limits specified for labneh by the Jordanian standards. . where the data shows that the mean moisture levels ranged from 72.40 - 73.22 % , fat between 8.73 - 9.38 and protein between 12.66 – 13.30,for the samples from the northern, middle and southern regions, respectively. also the results for all chemical composition values, showed no significant ($p>0.05$) differences between both the seasons and the regions .

Palmitic acid (33.32–36.22%) was the predominant fatty acid, followed by oleic acid (19.68–23.34%). fifty-six samples contained coliforms, *Escherichia coli* and yeast, due to the production method. However, all samples were free of *Salmonella and Staphylococcus aureus.* The sensory evaluation results showed that all labneh samples met the panellists' satisfaction, Where the data showed that the scores for all the samples ranged between 3.0 - 4.03 of 5 scores. for flavour , texture , appearance and colour.

1.Introduction

Labneh (concentrated yogurt) is a traditional fermented milk product manufactured by draining away a proportion of the yogurt water, using a cheese cloth bag at 6 °C, until reaching the desired total solids (23–25%). Under these conditions, the titratable acidity of labneh reaches 1.8 – 2.0% (as lactic acid), while the fat content is around 9 – 11% (KÖse and Ocak, 2011). Labneh has a cream or milky white appearance, a soft and smooth body, and good spread ability, with minor syneresis and a slightly acidic flavour (Nsabimana *et al*., 2005).

Labneh is regarded as one of the most popular foods, in various parts of the world. It plays a significant role in the family diet, particularly in the Middle Eastern countries and the Balkans (Haddad *et al*., 2007; El-Salam, 2011; KÖse and Ocak, 2011). Moreover, it is considered an intermediate product between sour cream and unripe cheeses, with a characteristic sharp acidic flavour that is modulated by diacetyl, produced during fermentation (Haddad *et al*., 2007).

The popularity of labneh has increased around the world due to the rise in consumer awareness. Its nutritional benefits and storage characteristics have increased its economic importance and nutritional value. Labneh is valued more than yogurt because it has 2.5-fold higher protein content and 50% more minerals (Nsabimana *et al*., 2005). In addition, the lactose content in labneh is low (approximately 6%) due to its fermentation into lactic acid by lactic acid bacteria. Thus, it can be consumed by lactose intolerant patients (Özer and Robinson, 1999; Nsabimana *et al*., 2005). Furthermore; there is a global interest in probiotic microorganisms, which can enhance the health benefits of dairy products (e.g. labneh). Consequently, these products can be considered functional foods (Rocha, et.al. 2014; Songtummin and Leenanon, 2016).

Labneh that is prepared by the traditional method, with total solids around 22%, has a short shelf life of about 2 weeks, even if refrigerated (Tamime and Crawford, 1984; Shamsia and El-Ghannam, 2012). This may be due to the sanitary problems that are usually associated with the cloth bags used in its production and the unhygienic handling of the product, which increases the microbial contamination (El-Samragy, 1997). The high microbial load of labneh coupled with the packaging and storage conditions result in the formation of off-flavours and undesirable physicochemical changes that eventually lead to rejection of the product (Otaibi and Demerdash, 2008). Burt, 2004 mentioned that there is a possibility to extend the shelf life of perishable food products, such as labneh, through the use of bio-preservatives such as essential oils. In this regard, the most common way to extend labneh shelf life is to increase its solid concentration up to 40%, then, store it in vegetable oil at room temperature. This would extend the expiry date up to 2 years (Kecel*i et. al*., 1999; Otaibi and Demerdash, 2008).

This study focuses on the chemical, microbiological and sensory characteristics of Jordanian labneh, made from cow's milk from three different Jordanian regions (northern, middle and southern) in spring and summer seasons.

2. Materials and methods

2.1.Materials

2.2.1.Samples collection

One-hundred-and-eighty samples of labneh, made from fresh cow's milk, were collected from northern, middle and southern regions of Jordan (60 samples from each region). Labneh samples were collected in two seasons (30 samples in spring and 30 samples in summer). The collection process always took place on the same day of production. Chemical and microbiological analysis, in addition to sensory evaluation, were conducted on the collection day.

2.2. Chemical analysis

Water content, fat and crude protein (N × 6.38) were determined according to AOAC, 2000 methods. Titratable acidity (expressed as a percentage of lactic acid), was determined as described by Marshall (1992).

All determinations were carried out on duplicate samples.

Fat from Labneh was extracted using chloroform-methanol (2: 1; v/v) according to the Modified Folch's technique (Prandini et al., 2007, and then converted into fatty acids methyl esters (FAME) according to IUPAC (International Union of Applied and Pure Chemistry) method (Commission Regulation (EC) No 796/2002 of 6 May 2002.

Fatty acids profiles were determined using a gas chromatography (variancp -3800, Japan) equipped with a flame ionization detector and restek capillary column(50m×0.25mm×0.25µm thickness). The column oven temperature was programmed: the initial temperature was165 °C and held at this temperature for 10 min, then temperature was increased to 180 ° C at a rate of 10 ° C /minute and remained at this degree for 30 minutes and then rose to 240 ° C, then sample injection volume 1 µl (injector temperature was 240 °C) and the flame ionisation detector temperature rises to 260 °C. High purity nitrogen gas was used as the

mobile phase and flow rate was 1.5 mL min–1. The FAMEs were identified using a chromatogram of fatty acid standards (Sigma Chemical Co.). Peak identification was achieved by retention time and comparing with reference standards. Peak areas were measured with an HP computing integrator. Data were expressed as percentages of total FAME content.

2.3. Microbial analysis

Total Coliform numbers were enumerated by the pour plate technique from the diluted samples (dilution of 1 g of sample in 9 mL of sterile distilled water with 0.8% NaCl), then, cultured on violet red yellow agar (Scharlau Chemie, Spain) and incubated at 37 °C for 24 h.

Salmonella spp. were isolated, after enrichment in selenite cystine broth (SC), at 37 °C for 24 h, then plated on xylose lysine deoxycholate agar and incubated at 37 °C for 24 – 48 h.

Staphylococcus aureus was isolated from the diluted samples on Baird-Parker agar, with egg yolk tellurite emulsion (Scharlau Chemie, Spain) and then incubated at 37 °C for 48 h.

Yeasts and moulds were also isolated by the spread plate technique, using potato dextrose agar at 25°C for 5 days. Furthermore, *Escherichia coli* bacteria were isolated by the most probable number method, using three tubes per dilution (10^{-1}, 10^{-2}, and 10^{-3}), containing 9 mL of lauryl tryptose broth (Scharlau Chemie, Spain). All tests were duplicated for each isolated bacterial species (Yousef and Carlstrom, 2003).

2.4. Sensory evaluation

Sensory evaluation was performed by 12 specialists in the field of food, using a 5-point hedonic scale (1 = dislike too much, 2 = dislike moderately, 3 = neither like nor dislike, 4 = like and 5 = like very much) (Meilgaard et al., 2006). The sensory characteristics of the samples that were evaluated included the appearance, flavour, colour and texture.

3. Results and discussion

3.1. Chemical composition of labneh

Table 1 shows the chemical composition of the labneh samples. The data shows that the mean moisture levels were 72.40 - 73.22, 73.00, and 72.90 – 73.00 % for the samples from the northern, middle and southern regions, respectively. In the same order of the regions, the samples contained between 9.25 - 9.38, 8.73 - 8.83, and 8.98 -9.05% fat, and correspondingly, 12.74 – 12.68, 12.66 – 13.30, and 12.90 – 13.03% protein. Also, the acidity value of the samples from the northern, middle and southern regions ranged from 1.51 - 1.58, 1.48 - 1.58, and 1.54 - 1.87%, respectively, and the corresponding total solids, as dry matter, ranged from 26.48 -27.59, 26.93 - 27.00, and 27.10 - 27.85%.

The aforementioned results, for all chemical composition values, showed no significant ($p>0.05$) differences between both the seasons and the regions. Furthermore, the chemical values were within the acceptable limits of the Jordanian legislation standard 108/2003 (JS, 2003), which states that labneh content should not contain less than 9%, 12%, 23% and 2.5% fat, protein, total solids and titratable acidity, respectively.

The results of this study were in agreement with results reported by previous researchers. For instance, the values of fat, protein, total solids and acidity found by Thabet et. al. (2014), were 8%, 11%, 23% and 1.4%, respectively, while Atamian et. al. (2014) reported corresponding values of 9%, 8%, 26% and 1%, respectively. For the same parameters, Tamime et al.(1991) reported 11.0%, 8.23%, 24.23% and 1.46%, respectively. The differences in the mentioned values might be due to many factors, such as the animal breed and its feeding and processing conditions.

There were no significant differences among the samples collected from the middle region, except for the fat percentage, which was less than required by the Jordanian standards (JS, 2003).

Also, previous studies have found

similar fat contents, reporting values ranging from 5.51% 11.61% (KÖse and Ocak, 2011; SÖMER and KiliÇ, 2012).

3.2. Fatty Acids content of labneh

The fatty acids in milk are derived from two sources, the feed and the microbial activity in the rumen of the cow (Parodi, 2004).

There are many factors that are related to variations in the fatty acid profile of cow milk lipids, such as animal origin, genetics, stage of lactation, mastitis and ruminal fermentation, or they may be feed-related factors including fibre and energy intake, dietary fats, and seasonal and regional effects (Jensen, 2002).

Table 1. Chemical composition of labneh produced in various regions of Jordan, in two seasons.

Region	Season	Moisture (%)	Fat (%)	Protein (%)	Acidity (%)	Total solids (%)
North	Spring	72.40±4.31^a	9.25±1.32^a	12.74±2.34^a	1.51±0.31^b	27.59±3.11^a
	Summer	73.22±3.11^a	9.38±1.67^a	12.68±3.10^a	1.58±0.21^b	26.48±2.98^a
Middle	Spring	73.00±4.92^a	8.73±1.85^a	13.03±2.98^a	1.47±0.32^b	27.00±3.24^a
	Summer	73.00±3.33^a	8.83±1.93^a	12.66±2. 65^a	1.58±0.22^b	26.93±3.33^a
South	Spring	73.00±4.21^a	9.05±1.56^a	13.03±2.87^a	1.87±0.42^a	26.85±2.67^a
	Summer	72.90±3.98^a	8.98±1.94^a	12.90±2.32^a	1.94±0.41^a	27.10±2.45^a

Values are averages of two repetitions ± standard deviation values in the same columns in the same season followed by different letters are significantly different ($p<0.05$).

Table 2 displays the fatty acid profile of labneh. The fatty acid content of labneh varied according to the region and season. The results revealed that the most abundant fatty acid in labneh was palmitic acid (C16:0), which ranged from 33.32 – 36.22 g/100 g lipid, followed by oleic, myristic, stearic, linoleic, capric,palmitoleic, caprylic, caproic, and, finally, linolenic. Generally, the results of this study were in agreement with Mirelle and Mattar (2015). Also, the fatty acid profiles of all the labneh samples were within the acceptable limits of the Jordanian food legislation for labneh (JS, 108/2003) which indicated that the fat in the labneh is of animal origin.

The minor differences between the fatty acids content of labneh samples were due to the region from which the samples were collected and the season in which the sample were collected. Published research has shown that milk obtained from cows fed on fresh green forage, particularly those that graze grass, had a significantly higher level of unsaturated fatty acids(Alqaisi*et al*. 2013)

In Jordan, cows rely on the use of concentrated feed. Therefore, the absence of grassland has led to the dominance of concentrated feed in cow's diet (Jordanian Ministry of Agriculture, 2016).

3.2. Microbial analysis

As stated in the Jordanian standard 108/2003, labneh products should be free of pathogenic microorganisms, and the total coliforms count should not be more than 10 CFU/g. The existence of coliforms in labneh, indicates the substandard hygiene conditions during conventional processing, which lead to recontamination at one or more stages of processing, together with the possibility of contaminates in the water used during labneh processing, by the same microorganisms. Moreover, these contaminations have a negative effect on the consumer's health, as well as the general public health (SÖmer and KiliÇ, 2012).

The mean values of the general microbial counts of labneh, collected from the various regions and in different seasons, are presented in Table 3. The data show that *Salmonella* spp. and *S. Aureus* ,were not detected in all the labneh samples collected from the three

regions. However, the results show that some samples (in all regions and for both seasons) contained total coliforms, and *E. coli*, in addition to yeasts.

The numbers of samples containing total coliform from the northern, middle and southern regions were 6, 5, and 9, respectively, of which 4, 4, and 7 contained *E. coli*. The coliform load ranged from $1.0 – 1.5 \times 10^3$ CFU/g in the northern region in spring, up to $7.0 – 7.3 \times 10^3$CFU/ g in the southern region in the summer. AL-kadamany *et al.* (2002) reported that the coliforms were detected at a level of 1.5×10^2 CFU/g These results showed that the coliform contaminations were high exceeded the limit reported in the Jordanian standard specification108/2003(JS,2003), particularly in summer .

Table 3. Microbiological characteristics of labneh produced in different region of Jordan, in two different seasons.

Types of microbes	**North region *No. of total sample 58/n**		**Middle region *No. of total sample 58/n**		**South region *No. of total sample 58/n**	
	1	2	1	2	1	2
Salmonella	ND	ND	ND	ND	ND	ND
E.colicfug^{-1}	2-3 x 10^2 1n	3 -4 x 10^2 3 n	1-1.2 x 10^2 2 n	1.5-2 x10^2 2 n	3-4 x 10^2 3n	4-5 x 10^2 4n
Total coliform cfug^{-1}	1-1.5x10^3 2 n	2-3 x10^3 4n	2.2-x10^3 2n	2.8-.5x10^3 3n	5.6-6 x10^3 4n	7-7.3 x10^3 5n
Staphylococcus *aureus*	ND	ND	ND	ND	ND	ND
Yeast cfug^{-1}	3-4 x10^3 5n	5-6 x10^3 6n	4-5 x10^3 5n	4.5-5.2x10^3 7 n	5.2-5.6x10^3 6n	6-6.5 x10^3 8n

*No. of samples from north, n: no. of the samples detected *Season 1=spring (29 samples), 2= summer(29 samples)

Table 4. Sensory attribute scores of labneh produced in various regions of Jordan, in two different seasons

Region	**Season [a]**	**Attributes**			
		Appearance	**Color**	**Flavor**	**Texture**
North	1	3.90±0.32^a	3.98±0.41^a	3.72±0.31^{ab}	3.65±0.41^a
	2	3.82±0.25^a	3.87±0.37^a	3.71±0.33^a	3.58±0.26^a
Middle	1	3.71±0.37^a	3.60±0.33^a	4.03±0.25^a	3.25±0.12^a
	2	3.87±0.42^a	3.78±0.40^a	3.75±0.43^a	3.37±0.33^{ab}
South	1	3.00±0.30b	3.16±0.26^b	3.50±0.21^b	3.45±0.22^a
	2	3.90±0.32^a	3.00±0.21^b	3.00±0.23^b	3.00±0.14^b

Values are averages of two repetitions of 12 panelists ±standard deviation.

Values in the same columns in the same season followed by different letters are significantly different ($p<0.05$), according to Duncan's multiple range test.

[a]1 = spring, 2 = summer.

Table 2. Fatty acid profile (g/100 g lipid) of labneh produced in various regions of Jordan, in two seasons.

Region	Season[a]	Caproic C6:0	Caprylic C8:0	Capric C10:0	Lauric C12:0	Myristic C14:0	Palmitic C16:0	Palmitoleic C16:1	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3
North	1	1.91±0.21^{ab}	1.21±0.03^{b}	3.05±0.65^{a}	3.72±0.76^{a}	11.61±2.31^{ab}	34.77±4.24^{ab}	1.69±0.12^{ab}	9.64±1.23^{b}	20.29±3.53^{a}	3.70±1.03^{a}	0.36±0.01^{b}
	2	1.98±0.13^{a}	1.27±0.06^{a}	3.08±0.72^{a}	3.74±0.64^{a}	11.84±1.87^{a}	34.95±3.45^{a}	1.93±0.08^{a}	8.73±1.11^{b}	19.77±2.33^{ab}	3.05±0.98^{b}	0.33±0.01^{b}
Middle	1	1.93±0.15^{a}	1.27±0.11^{a}	2.64±0.57^{b}	3.57±0.85^{ab}	11.18±2.32^{b}	33.32±4.26^{b}	2.06±0.42^{a}	8.23±1.31^{b}	21.34±2.89^{a}	3.39±1.05^{ab}	0.35±0.01^{b}
	2	1.98±0.33^{a}	1.14±0.05^{b}	2.95±0.98^{ab}	3.75±0.74^{a}	11.63±2.12^{ab}	35.08±3.66^{a}	1.62±0.26^{ab}	7.51±1.02^{bc}	18.91±2.64^{b}	3.28±0.89^{ab}	0.48±0.01^{ab}
South	1	1.89±0.26^{ab}	1.20±0.03^{b}	2.58±0.99^{b}	3.37±0.59^{b}	11.55±1.87^{ab}	36.22±4.21^{a}	1.67±0.22^{ab}	9.85±1.37^{b}	19.68±3.22^{ab}	3.59±0.96^{a}	0.44±0.01^{ab}
	2	1.86±0.32^{b}	1.17±0.04^{b}	2.73±0.89^{b}	3.23±0.73^{b}	11.17±1.95^{b}	34.19±2.78^{a}	1.41±0.12^{b}	11.57±2.34^{a}	20.60±2.45^{a}	3.56±1.11^{a}	0.56±0.01^{a}

Values are averages of two repetitions ± standard deviation.

Values in the same columns in the same season followed by different letters are significantly different (p 0.05) Duncan's multiple range test.

[a] 1 = spring, 2 = summer.

There were 11, 12, and 14 samples containing yeast, in the northern, middle and southern region, respectively, and the yeast load ranged from 3.0 - 4.0 × 10^3CFU in the northern region in spring to 6.0 – 6.5 × 10^3CFU in the southern region in summer. The values in this study revealed were lower than the values detected by Muir and Banks (2000), which were ≥ 10^6CFU/ml, as well as Yamani and Abu-Jaber (1994) of >10^6CFU. Yamani and Abu-Jaber (1994) documented 2.6 × 106 and 4.4 × 10^6CFU, in the northern and southern regions, respectively, whereas, in the current study, the yeast loads were comparatively lower, ranging from 1.0 - 1.5 × 10^3CFU in the northern region and up to 7.0 - 7.3 × 10^3CFU in the southern region. Furthermore, Yamani and Abu-Jaber (1994) claimed that yeasts were the main cause of labneh spoilage since they provided ideal conditions for the growth of yeast, such as high concentration of lactic acid and little of air during refrigerated storage.

It is known that Middle Eastern consumers prefer labneh that is prepared by traditional methods, even though the yeast flavour may be present. Somer and KiliÇ (2012) mentioned that the transfer of yoghurt from cloth bags, which are used for straining, to the packaging materials, may contribute to contamination of labneh with fungi and coliforms. In addition, the hygienic conditions of the packaging material, storage, manufacturing place, practices of the workers and market places would increase microbial contamination which lead to rejection of the product(Otaibi and El.demerdash,2008).

3.4. Sensory evaluation

The sensory attributes studied were appearance, flavor , colour and texture.

A 5-point hedonic scale was adopted, to obtain the scores. The mean sensory attribute scores for the samples obtained from the various regions and both seasons are shown in Table 4. The data showed that the appearance and colour of the samples collected from northern and middle regions in both seasons, scored higher, and were significantly different ($p \leq 0.05$) than the samples obtained from the southern region.

The scores for all the samples ranged between 3.0 - 3.9. The flavour scores of samples for all the regions and both seasons ranged between from 3.00 to 4.03. However, the labneh samples collected from the northern and middle regions of the two seasons were significantly different ($p \leq 0.05$) than the southern region. This may be due to the higher acidity value of the labneh from the southern region. The texture of all labneh samples scored 3.00 - 3.65.

The scores also showed that there was no significant difference detected in the texture, among the samples from all regions in spring. However, the northern region scored a higher value in the summer, matching the results in Table 1, which showed these samples had the highest total soluble solids. This result concurred with Mahdian and Tehrani (2007), who reported that the texture acceptability increased significantly with increasing total solids. 4.

4.Conclusions

The labneh that was produced in various regions was within the acceptable limits of the Jordanian standards. The predominant fatty acid in labneh was palmitic acid (C16:0), ranging from 33.32 – 36.22 g/100 g lipid, followed by oleic acid. Also, no significant variations ($p > 0.05$) in the average values of caproic acid (C6:0) and palmitic acid (C16:0) were observed, whereas the remaining fatty acids in labneh showed only minor variations. Moreover, the microbiological analysis revealed that some samples of labneh contained coliforms, E. coli and yeast, indicating poor hygienic conditions. However, all the samples were free of pathogenic bacteria (*Salmonella* spp. and *S. aureus*). Regarding the sensory evaluation, the labneh was considered acceptable by all the panellists.

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EFFECT OF FORTIFICATION ON TEXTURAL, MICROBIOLOGICAL AND PHYSICO-CHEMICAL PROPERTIES OF BREAD

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ABSTRACT
The effect of fortification with *Spinacia oleracea* L. on the physico-chemical, sensory value, textural properties and microbiological analysis of bread was carried out. Breads were made by substituting wheat flour with spinach powder (SP) at 0, 1, 2.5, 5% and Ferrous Sulphate powder (FSP) ($FeSO_4$. 7 H_2O) at 0.025, 0.05, and 0.10% levels. Addition of different levels of fortificants showed a marginal effect on the textural and physico-chemical properties. Significant increase in iron content was observed even with low levels of fortification. The hardness, chewiness and gumminess of bread samples increased during storage. Total plate count, yeast and mold count showed slight decreasing trend within treatments and an increasing trend during the storage from 0.14-1.45×10^2cfu/g and 1.4 to 2.7 x 10^1cfu/g respectively.

1. Introduction

Cereal based diet is primarily the basic diet in India besides other Asian countries. This type of diet has basically less bioavailability of micronutrients including deficiency of iron, vitamin A and anaemia. Iron deficiency anaemia (IDA) is a general nutritional disorder among the young children and women in developing and under developed countries. In developing world this nutritional disorder affects almost 3.6 billion people (ACC/SCN, 2000) and 30-60 % of women and children. Green leafy vegetables grow abundantly in India and are a precursor of vitamin A and a very good source of β-carotene (Premavalli et al., 2001). Leafy vegetables include fenugreek, spinach, coriander and mint etc which grows in abundance and have a short life span, in order to use this diet during off seasons preservation should be done properly to avoid wastage. There are so many techniques by which micronutrients in food can be enhanced. Fortification of food is one among such approaches by which the production of food and dietary diversification of micronutrients in foodstuff can be improved (Allen et al., 2006). According to USA Food and Drug (FDA) ferrous sulphate is regarded as a safe fortificant because of its bioavailability and is also used in bakery products as a fortificant. For baking and food processing industry ferrous sulphate is an excellent choice and can be mixed up with the flour to increase the bioavailability of micronutrients (Hurrell, 2000).

Spinach (*Spinacia oleracea* L.) is among the family of Amaranthaceae, an edible flowering plant (Le et al., 1999). Spinach are known to possess high levels of minerals that supports formation of nutrients for

bones like calcium and magnesium; phosphorus; iron and a free radical-scavenger i.e it possess vitamin K; vitamin A; vitamin B; vitamin C and vitamin E. It also contains vitamin B6, vitamin B2, potassium, copper, protein, manganese, selenium, omega-3 fatty acids and naicin. It also contains antioxidants and adjusts blood pressure (Kuriyamaa et al., 2005).

About 90% of wheat is produced in India and is used for manufacturing of bakery products like bread, cake, muffins and biscuits (Bedekar 2001). Bread is a fermented bakery product and is mainly prepared by using a mixture of wheat flour, yeast, salt and water through a step wise procedure of mixing, kneading, proofing, shaping and baking (Dewettinck et al., 2008). World's half of the population consumes wheat flour as it is a staple food but still is an incomplete diet as it lacks micronutrients. For a large group of people in the world, the balanced diet is not easily available especially for those in developing countries. Green leafy vegetables are the only source of plant food that are cheap and are locally available. These green leafy vegetables are rich in micronutrients and can be incorporated during the development of breads that enhances the nutritive value of food. Therefore, attempts were made to prepare bread by incorporating wheat flour with ferrous sulphate powder (FeSO4) and dehydrated spinach powder to evaluate their effect on physico-chemical, microbiological, textural and sensory characteristics.

2. Materials and methods

2.1. Raw Materials

Fresh spinach (*Spinacia oleracea* L.) and food grade ferrous sulphate powder $(FeSO_4)^2.7H_2O$ were purchased from a local market in Srinagar, Kashmir. Samples were collected and stored at 4^{o}C till further use.

2.2. Preparation of the raw material

Spinach were cleaned manually, washed under tap water and oven dried at 55^{o}C. The dried spinach was ground into powder by using an electrical grinder (National brand, MX-895M model) and sifted through 125µm screen to obtain uniform size. The spinach powder (SP) was then stored in hermetically sealed packs at 4^{o}C for further use.

2.3. Bread Preparation

Bread was formulated according to Adeleke and Odedeji (2010) with slight modification. Wheat flour and FSP were used in the ratio of 0.025, 0.05, 0.10% and SP in the ratio of 1, 2.5 and 5% designated as T_0, T_1, T_2, T_3, T_4, T_5 and T_6 respectively. All ingredients were put together and mixed in a planetary mixer (Model SM-25, SINMAG Japan) for 2 min at 214 rpm and different substitution levels of ferrous sulphate powder and spinach powder were added and the content were mixed to obtain an elastic and smooth dough. The dough was then kept in a proofer (incubator) at a temperature of 37^{o}C for 1 hour. Afterwards dough was manually moulded and proofed for another 30 minutes and then knocked back to remove excess gas, rolled and moulded into pans. The pans loaded with breads were then placed in pre-heated baking oven at 225^{o}C for 30-35 min. After baking, the bread loafs were cooled for 1 hour at ambient temperature and then sliced and were packed in air tight polythene bags (LDPE) prior to analysis.

Table no: 1 Bread formulations

Whole wheat	150g
Yeast	3.0g
Sugar	6.0g
Salt	1.5g
Baking powder	2%
Ferrous sulphate	0.025, 0.05, 0.10%
Spinach powder	1,2.5,5 %

2.4. Compositional Analysis of SP and FSP

Moisture, fiber, ash, protein and crude fat content were determined by AOAC (2000) methods. Carbohydrate content of spinach powder and ferrous sulphate powder were determined by difference method that is by subtracting the sum of percentage of protein, crude fat, fiber, ash, and moisture content from 100.

2.5. Determination of Iron Content

The iron content in the samples was measured by using an atomic absorption spectrophotometer (AA700, Shimazdu) according to AOAC (1990) method. 1g sample was digested with 20 ml Di-acid (nitric acid and perchloric acid in the ratio of 3:1. The resulting digested sample was diluted with distilled water in a 50 ml calibrated flask. The solution was then used to determine iron.

Iron (mg/100g) = AAS reading (mg/L) × Dilution Factor (1)

2.6. Physical Evaluation of Bread

Loaf volume was calculated using rapeseed displacement method. Density and specific volume were determined by using the method of Feili et al., (2013) while as loaf height was measured using a meter rule.

Loaf volume (mL) = V_1-V_2 (2)

Where V_1 represent the volume of rapeseeds in the empty container (ml)

V_2 represents volume of the rapeseeds in the container containing sample (ml)

Specific volume was calculated as per the formula below

$$\text{Specific volume} = \frac{\text{loaf volume of bread (cm}^3\text{)}}{\text{weight of bread (g)}} \quad (3)$$

The density of bread was calculated as

$$\text{Density of bread} = \frac{\text{weight of bread (g)}}{\text{loaf volume of bread (cm}^3\text{)}} \quad (4)$$

2.7. Texture Profile Analysis (TPA).

Texture Analyser TA.HD.Plus (Stable Micro Systems, Godalming, Surrey, UK) was used to analyze the texture of bread crumb. The analysis was performed by using a cylinder probe (p/36) of 36mm radius. The force deformation curve was recorded when a compression cell of 5g and a compression distance of 25% was used. The speed of matrix was set at 1 mm s^{-1}, post-test speed of 10.0mm/s, test speed at 1.7mm/s. The test was determined after 24 hours of baking, 48 hours, 72 hours and 90 hours after storage at 22 ±1 °C and relative humidity of 45 ±1% (Xie et al. 2003).

2.8. Sensory Analysis

Sensory evaluation of bread samples fortified with different percentage of ferrous sulphate powder and spinach powder was carried by using 5 point hedonic scale. Consequently the bread samples were sliced (about 1.5cm thick), coded and served to semi-trained panel. Sensory panel recognized the attributes as sensory properties of bread like taste, color, texture and overall acceptability.

2.9. Microbial Evaluation

Total plate count (TPC), yeast and mold count in the bread samples were determined as per APHA (1976) method.

2.10. Statistical Analysis

One-way analysis of variance was used to analyze data in a randomized design with three replications and mean comparisons by using commercial statistical package SPSS (16.0, Chicago,IL).

Flow chart for the preparation of Bread

Raw materials
↓
Addition of water containing yeast
↓
Kneading (20 minutes)
↓
Proofing (1 hour at 37ºC)
↓
Knock back
↓
Proofing (30 min)
↓
Baking (220 ºC for 30-35 min)
↓
Cooling at ambient temperature (1 hour at 25±2°C)
↓
Slicing
↓
Packing in LDPE pouches
↓
Storage at ambient temperature (25±2°C)

3. Results and discussions

3.1. Compositional Analysis of Whole wheat flour and SP

The chemical analysis of wheat flour and spinach powder (SP) is depicted in Table 2. In order to get information about the overall proximate composition of any ingredients, analysis of chemical composition is important in determining the nutritional quality of the components being used in food. It doesn't only determine the chemical or nutritional status of food but also determines the shelf life and the production of designer foods for specific target groups. The shelf life of flour can be determined by its moisture content (9.50%) which is an important factor to define the quality parameter. At ambient temperature and at low moisture content of flour the storage stability is enhanced and thus increases its shelf life. Ash content of spinach powder (19.82%) measures the amount of mineral matter present in the powder. Protein, crude fiber, fat content of Whole Wheat flour and SP was found to be 11.20%, 2.50%, 1.43%, 19.82%, 4.92%, 3.32% respectively. Carbohydrate and Iron content in Whole Wheat flour and SP was also found to be 69.70%, 2.75 mg/100g iron, 12.01%, 11.75 mg/100g iron respectively (Riaz et al., 2007 and Chaturvedi et al., 2013).

Table 2. Compositional analysis of whole wheat flour (WWF) and Spinach Powder (SP)

Parameter	WWF	SP
Moisture (%)	9.50±0.02^{a}	5.68±0.08 ^{b}
Protein (%)	11.20±0.04^{b}	19.82±0.05^{a}
Fat (%)	1.43±0.02^{b}	3.32±0.14^{a}
Ash (%)	1.67±0.01^{b}	19.82±0.05^{a}
Fiber (%)	2.50±0.01^{b}	4.92±0.09^{a}
Carbohydrate (%)	69.70±0.04 ^{a}	12.01±0.29 ^{b}
Iron (mg/100 g)	2.75±0.06^{b}	11.75±0.21^{a}

Values are mean of three replications ± standard deviation. Values followed by same small letter superscripts in a column do not differ significantly ($P< 0.05$)

3.2. Chemical Properties

Table 3 shows the effect of different substituents on the chemical composition of bread. Moisture and carbohydrate contents decreased with increased levels of SP. However, an increase in ash, protein, fibre and fat content was observed. This could be attributed due to the substitution effects caused by the fortificants. In case of bread fortified with FSP, moisture and fat contents decreased slightly with the increasing levels. Ash and protein content increased significantly ($p\leq0.05$) (Akhtar et al. 2008).

The iron content significantly ($p \leq 0.05$) increased in both SP and FSP due to high levels of iron present in the fortificants.

Table 3. Effect of different levels of SP and FSP on the chemical composition of bread

Parameters	T_0	T_1	T_2	T_3	T_4	T_5	T_6
Moisture (%)	35.20±0.02^a	34.91±0.01^a	34.46^b± 0.12^b	33.46± 0.04^b	35.18± 0.11^a	35.16±0.02^a	35.04±0.04^b
Protein (%)	9.07±0.13^d	9.10±0.03^c	9.33±0.15^c	9.66±0.11^c	9.11±0.06^c	9.18±0.04^d	9.24±0.14^d
Fat (%)	1.51±0.07^d	1.62±0.04^c	1.73±0.05^b	1.91±0.01^a	1.49±0.08^a	1.47±0.06^a	1.44±0.07^a
Ash (%)	1.10±0.05^d	1.28±0.06^c	1.51±0.03^a	2.01±0.07^a	1.95±0.14^b	2.03±0.06^a	2.08±0.05^a
CHO (%)	51.02^a±0.03	50.52^b±0.07	50.04^c±0.06	49.00^d±0.03	51.08^a±0.08	51.15^a±0.12	51.22^a±0.02
Iron (mg%)	2.72±0.13^b	2.75±0.03^a	2.79±0.07^a	2.86±0.05^a	2.73±0.07^a	2.76±0.05^a	2.78±0.12^a

Values are mean of three replications ± standard deviation. Values followed by same small letter superscripts in a column do not differ significantly

T_0: whole wheat bread, T_1: Bread substituted with 1% SP, T_2: bread substituted with 2.5% SP, T_3: Bread substituted with 5% SP.T_4: Bread substituted with 0.025% FSP, T_5: bread substituted with 0.05%FSP, T_6: Bread substituted with 0.10% FSP.

3.3. Physical Characteristics

Physical parameters of fortified breads are listed in Table-4. The fortification of breads with SP and FSP significantly resulted in increased weight and density. The weight of FSP incorporated bread increased from 410.29 to 418.22g and the density increased from 0.63 to 0.65g/cm^3(Keetels et al., 1996). The effectiveness of gluten network decreases as the fortificant level increases, reducing the bread volume, height while increasing density (Feili et al., 2013).

Specific volume and loaf volume of breads fortified with SP and FSP decreased with increase in fortificant levels. SP and FSP cause dilution of gluten and subsequently affect the formation of optimal matrix of gluten during mixing, fermentation and baking process (Ragaee et al., 2011). Lower bread volume results due to partial replacement of wheat flour.

Table 4. Effects of spinach powder and ferrous sulphate powder incorporated on physical characteristics of bread

Physical characteristics	T_0	T_1	T_2	T_3	T_4	T_5	T_6
Bread weight (g)	407.15±0.07^a	420.51±0.03^e	453.47±0.13^f	480.54±0.04^g	410.29±0.03^b	413.83±0.11^c	418.22±0.02^d
Bread height (cm)	10.21±0.04^f	9.64±0.07^e	9.00±0.06^b	8.91±0.15^a	9.72±0.08^e	9.56±0.14^d	9.40±0.11^c
Bread volume (cm^3)	650.25±0.05^g	639.33±0.03^c	630.01±0.08^b	601.03±0.05^a	645.37±0.01^f	642.16±0.06^e	640.25±0.03^d
Density (g/cm^3)	0.62±0.14^a	0.65±0.02^a	0.71±0.04^b	0.80±0.08^c	0.63±0.04^a	0.64±0.02^a	0.65±0.05^a
Specific volume (cm^3/g)	1.59±0.04^c	1.52±0.05^b	1.39±0.08^b	1.25±0.04^a	1.57±0.09^c	1.55±0.13^c	1.53±0.02^c

Mean± SD with different superscripts in a column differ significantly ($P < 0.05$) (n= 3)

T_0: whole wheat bread, T_1: Bread substituted with 1% SP, T_2: bread substituted with 2.5% SP, T_3: Bread substituted with 5% SP.T_4: Bread substituted with 0.025% FSP, T_5: bread substituted with 0.05%FSP, T_6: Bread substituted with 0.10% FSP.

3.4 Sensory Evaluation

Table 5 evinced the result of hedonic test on different samples of breads. The bread enriched with SP was dark green in color than the control bread having slightly green color and has a negative approach on the acceptability of bread. This significant decrease in the colour of bread fortified with spinach powder ($T_1$to T_3) could be due to the chlorophyll present in the leaves that gives green color to bread (Abraham et al., 2013). Breads supplemented with FSP had slight blackish colour (Garby 1985) due to the precipitation caused by iron compounds when added to food and thus produces unacceptable changes in color and flavor of the product.

The taste, texture and overall acceptability of breads made by using different substitution levels of SP and FSP were non-significant with respect to the control. Higher levels of SP and FSP resulted in an unpleasant taste attributed by the herbal flavour of leaf powder (Abraham et al., 2013) and due to the slight metallic taste of iron (Saeed 2007). Higher levels of SP had negative influence on the texture. This decrease in texture might be due to slight coarser texture of spinach powder and reduction in the gluten contents of the bread (Sharma and Chauhan 2000). The results for sensory characteristics indicated that wheat flour bread incorporated with SP upto 1% (T_1) gives satisfactory overall consumer acceptability (Alam et al., 2013).

Table 5. Effect of spinach powder and ferrous sulphate powder on the sensory evaluation of breads

Attribute	T_0	T_1	T_2	T_3	T_4	T_5	T_6
Colour	4.0	3.5	2.5	2.0	2.9	2.5	2.0
Taste	4.0	3.0	2.5	2.0	2.5	2.4	2.0
Texture	3.9	3.0	2.0	2.0	3.5	3.5	3.5
Overall acceptability	4	3.0	2.5	2.0	2.9	2.5	2.0

Mean± SDwith different superscripts in a column differ significantly ($P< 0.05$) (n= 3)
T_0: whole wheat bread, T_1: Bread substituted with 1% SP, T_2: bread substituted with 2.5% SP, T_3: Bread substituted with 5% SP.T_4: Bread substituted with 0.025% $FeSO_4$, T_5: bread substituted with 0.05%$FeSO_4$, T_6: Bread substituted with 0.10% $FeSO_4$.

3.5 Texture

The results for the texture analysis of the breads fortified with both SP and ferrous sulphate powder are presented in Table 6. Breads supplemented with spinach powder were significantly ($p\leq0.05$) harder than ferrous sulphate powder. This could be due to interaction between gluten and fibrous material (Gomez et al., 2003). Among treatment samples showed decreasing trend whereas increasing trend was followed throughout the period of storage. This increase in hardness could be due to effect of starch retrogradation (Paulina et al., 2012)

Results of statistical analysis of each sample for springiness and cohesiveness during storage and among treatments at same storage time can be found in Table 6. Springiness and cohesiveness significantly ($p\leq0.05$) decreased in all samples during storage. Intermolecular attractions are lost in between ingredients causing crumbling of crumb, and loss of water (Sharoba et al., 2013).Springiness was significantly higher in the spinach powder enriched samples as compared to ferrous sulphate incorporated bread. In composite breads the dilution of gluten structure minimizes the amount of

gluten and thus reduces the elasticity in breads (Hoseney, 1994).

Gumminess and chewiness significantly increased during the entire storage period. However an increasing trend was observed among the treatments. Increase in hardness during storage results in retrogradation properties of wheat flour as reported previously by Sharoba et al., (2013) Wang et al., (2002).

Table 6. Texture profile analysis (TPA) of iron fortified bread during storage

Treatments	**Storage (days)**				**Treatment Mean**
	0	**2**	**4**	**6**	
Hardness					
T_0	8.20^{aD}	8.75^{bC}	9.20^{cC}	9.42^{cD}	**8.89^{C}**b
T_1	7.83^{aC}	8.20^{bC}	9.01^{dC}	9.36^{eD}	**8.60^{Cc}**
T_2	6.00^{a}	6.41^{bA}	6.98^{cA}	7.20^{cB}	**6.63^{Ab}**
T_3	5.94^{Aa}	6.56^{bA}	7.62^{dB}	8.01^{eC}	**7.03^{Bc}**
T_4	6.20^{Aa}	6.57^{bA}	6.81^{bA}	6.84^{bA}	**6.60^{Ab}**
T_5	6.15^{Aa}	6.52^{bA}	$6.^{73Ab}$	6.82^{bA}	**6.58^{Ab}**
T_6	6.09^{Aa}	6.45^{bA}	$6.^{68Abc}$	6.78^{Aa}	**6.51^{Ab}**
Storage Mean	**6.63^{Ba}**	**7.07^{Bb}**	**7.57^{Bc}**	**7.77^{Cc}**	
Springiness					
T_0	0.94^{Ba}	0.92^{Ba}	0.90^{Ca}	0.88^{Ba}	**0.90^{Aa}**
T_1	0.91^{Ba}	0.87^{Ba}	0.85^{BCa}	0.84^{Ca}	**0.86^{Aa}**
T_2	0.90^{Bb}	0.88^{Bab}	0.86^{Ba}	0.81^{ABC}	**0.85^{Aa}**
T_3	0.86^{Ac}	0.81^{Abc}	0.77^{Ab}	0.68^{Aa}	**0.79^{Ab}**
T_4	0.92^{Bb}	0.91^{Bb}	0.85^{Ba}	0.80^{ABa}	**0.86^{Aab}**
T_5	0.87^{ABb}	0.85^{Ab}	0.79^{Aa}	0.73^{ABa}	**0.81^{Aab}**
T_6	0.80^{Ab}	0.77^{Aab}	0.71^{Aa}	0.69^{Aa}	**0.74^{Aa}**
Storage Mean	**0.88^{Bb}**	**0.85^{Ba}**	**0.81^{Ba}**	**0.76^{Aa}**	
Cohesiveness					
T_0	0.72^{Bb}	0.70^{Cb}	0.62^{Ca}	0.59^{Ca}	**0.66^{Bab}**
T_1	0.67^{ABb}	0.63^{BCb}	0.55^{BCa}	0.51^{BCa}	**0.59^{Bab}**
T_2	0.65^{Ac}	0.54^{ABb}	0.48^{Bab}	0.43^{ABa}	**0.52^{Ab}**
T_3	0.61^{Ac}	0.47^{Ab}	0.39^{Aa}	0.38^{Aa}	**0.46^{Aab}**
T_4	0.69^{Bc}	0.54^{ABb}	0.45^{Aa}	0.48^{Ba}	**0.51^{Aab}**
T_5	0.62^{Ac}	0.60^{Bb}	0.58^{Cb}	0.39^{Aa}	**0.52^{Ab}**
T_6	0.59^{Ac}	0.52^{Abc}	0.47^{ABb}	0.36^{Aa}	**0.48^{Ab}**
Storage Mean	**0.65^{Ac}**	**0.56^{Bb}**	**0.49^{Ba}**	**0.43^{ABa}**	
Chewiness					
T_0	3.62^{DEa}	4.25^{Eb}	5.05^{Ed}	5.92^{Fe}	**4.71^{Dc}**
T_1	2.89^{BCa}	3.32^{Bb}	4.45^{Dd}	4.08^{Bcd}	**3.97^{Cc}**
T_2	2.03^{Aa}	3.27^{Bb}	3.92^{BCc}	4.20^{Bd}	**3.35^{Bb}**
T_3	1.98^{Aa}	2.61^{Ab}	3.03^{Acd}	3.36^{Ad}	**2.67^{Abc}**
T_4	3.61^{Da}	3.82^{Cab}	4.45^{Dcd}	5.14^{Ed}	**4.06^{Dbc}**
T_5	3.04^{Ca}	3.98^{Cb}	4.39^{Dc}	4.84^{Dd}	**4.25^{Dbc}**
T_6	3.72^{Ea}	3.91^{Ca}	4.27^{CDb}	4.39^{BCb}	**4.07^{Dab}**
Storage	**3.04^{Ca}**	**3.59^{BCb}**	**4.18^{Cc}**	**4.66^{CDc}**	

Mean					
Gumminess					
T_0	3.15^{Da}	3.62^{Dbc}	4.66^{Fc}	5.58^{Ed}	**4.25^{Dc}**
T_1	2.52^{Ca}	3.63^{Db}	3.95^{DEc}	4.36^{Cd}	**3.61^{BCb}**
T_2	1.92^{Aa}	2.18^{Aa}	2.86^{Ac}	3.08^{Ac}	**2.51^{Ab}**
T_3	2.04^{Aa}	2.92^{Bc}	3.21^{Bc}	3.62^{Bd}	**2.39^{Ab}**
T_4	2.82^{Ca}	3.52^{Db}	4.15^{Ec}	4.87^{Dd}	**3.76^{Cb}**
T_5	2.64^{Ca}	2.82^{Ba}	3.69^{CDc}	4.53^{Cd}	**3.67^{Bbc}**
T_6	2.45^{Ca}	3.19^{Cb}	3.62^{Cc}	4.32^{Cd}	**3.39^{Bbc}**
Storage Mean	**2.50^{Ca}**	**3.12^{Cb}**	**3.73^{Dc}**	**4.33^{Cd}**	

Means with different superscripts in a column differ significantly (P< 0.05) (n= 3)

T_0: whole wheat bread, T_1: Bread substituted with 1% SP, T_2: bread substituted with 2.5% SP, T_3: Bread substituted with 5% SP.T_4: Bread substituted with 0.025% $FeSO_4$, T_5: bread substituted with 0.05%$FeSO_4$, T_6: Bread substituted with 0.10% $FeSO_4$.

4.6 Microbiological studies

Total plate count, yeast and mould count of breads fortified with SP and FSP were performed at 0, 2, 4 and 6th day of storage and are presented in Table-7. There was an increase in total plate count (0.14 to1.45× 10^2cfu/g) and yeast and mould count (1.4 × 10^1 to 2.7×10^1cfu/g) with increase in storage. However, with increase in the levels of fortification there was a decrease in total plate count and Yeast and mold count. Increasing the levels of fortification, there was a decrease in water activity which in turn decreases the microbial load. During storage an increase in the water activity of bread favors the growth of bacteria. However, microbial load remained within the permissible limits (Smith et al., 2004).

Breads fortified with FSP showed lower microbial count than that of spinach incorporated breads. This could be due to the high mineral content which can possibly inhibit the growth of microbes. As the minerals are hygroscopic in nature, they can stop the microbial growth by decreasing the available moisture (Akhtar et al. 2008).

Table 7. Effect of ambient storage (21-25 °C) on microbiological characters of wheat bread supplemented with various levels of spinach powder and ferrous sulphate

Treatments	**Storage (days)**					
		0	**2**	**4**	**6**	**Treatment mean**
Total plate count (cfu/g)	T_0	TLTC	$0.22^{Aa}\times10^2$	$0.30^{Aa}\times10^2$	$1.35^{Ac}\times10^2$	$0.46^{Ab}\times10^2$
	T_1	TLTC	$0.19^{Aa}\times10^2$	$0.26^{Aa}\times10^2$	$1.33^{Ac}\times10^2$	$0.44^{Ab}\times10^2$
	T_2	TLTC	$0.16^{Aa}\times10^2$	$0.22^{Aa}\times10^2$	$1.32^{Ab}\times10^2$	$0.42^{Aa}\times10^2$
	T_3	TLTC	$0.13^{Aa}\times10^2$	$0.20^{Aa}\times10^3$	$1.29^{Ab}\times10^2$	$0.40^{Aa}\times10^2$
	T_4	TLTC	$0.14^{Aa}\times10^2$	$0.18^{Aa}\times10^2$	$1.20^{c}\times10^2$	$0.38^{Ab}\times10^2$
	T_5	TLTC	$0.11^{Aa}\times10^2$	$0.15^{Aa}\times10^2$	$1.17^{Ac}\times10^2$	$0.35^{Ab}\times10^2$
	T_6	TLTC	$0.10^{Aa}\times10^2$	$0.12^{Aa}\times10^2$	$1.14^{Ac}\times10^2$	$0.34^{Ab}\times10^2$
Storage Mean	**TLTC**		**$0.14^{Aa}\times10^2$**	**$0.20^{Aa}\times10^2$**	**$1.45^{Ab}\times10^2$**	
Yeast and mold count (cfu/g	T_0	TLTC	$2.2^{Ba}\times10^1$	$2.7^{Db}\times10^1$	$3.2^{Cc}\times10^1$	$2.05^{Ba}\times10^1$
	T_1	TLTC	$2.0^{Ba}\times10^1$	$2.4^{CDb}\times10^1$	$3.0^{Bc}\times10^1$	$1.85^{Ba}\times10^1$

	T_2	TLTC	$1.19^{Aa}\times10^1$	$2.3^{Cc}\times10^1$	$2.8^{Bd}\times10^1$	$1.57^{Ab}\times10^1$
	T_3	TLTC	$1.16^{Aa}\times10^1$	$2.0^{BCc}\times101$	$2.8^{Bd}\times10^1$	$1.49^{Ab}\times10^1$
	T_4	TLTC	$1.14^{Aa}\times10^1$	$1.9^{Ac}\times10^1$	$2.9^{Bd}\times10^1$	$1.55^{Ab}\times10^1$
	T_5	TLTC	$1.11^{Aa}\times10^1$	$1.7^{Ab}\times10^1$	$2.6^{ABc}\times10^1$	$1.35^{Aa}\times10^1$
	T_6	TLTC	$1.10^{Aa}\times10^1$	$1.7^{Ac}\times10^1$	$2.2^{Ad}\times10^1$	$1.25^{Ab}\times10^1$
Storage Mean		**TLTC**	$\mathbf{1.4^{Aa}\times10^1}$	$\mathbf{2.1^{Cb}\times10^1}$	$\mathbf{2.7^{Bc}\times10^1}$	

Means with different superscripts in a column differ significantly (P< 0.05) (n= 3)

T_0: whole wheat bread, T_1: Bread substituted with 1% SP, T_2: bread substituted with 2.5% SP, T_3: Bread substituted with 5% SP.T_4: Bread substituted with 0.025% $FeSO_4$, T_5: bread substituted with 0.05%$FeSO_4$, T_6: Bread substituted with 0.10% $FeSO_4$.

4. Conclusions

This study was carried out to investigate the effect of fortification of spinach powder on physicochemical, textural and microbiological properties of bread. Six different treatments of the product with substitution of *Spinacea oleracea* powder, and $FeSO_4$ at levels of 1, 2.5, 5 and 0.025, 0.05, 0.10 percent respectively were used in the development of iron fortified breads. The study also revealed that ash content increased significantly with the increasing level of ferrous sulphate while as moisture, protein, fat and fiber decreased with increase in supplementation of ferrous sulphate. The studies revealed that the ash, protein, fiber and fat increased significantly with the increasing level of *Spinacea oleracea* powder, while as moisture and carbohydrate decreased significantly with the increase in spinach powder. The physical characteristics showed significant increase in weight and density of bread while as decreasing trend in bread volume, bread height and specific volume was observed. The scores for overall acceptability of the fortified bread samples decreased by increasing the levels of fortificants. The result obtained revealed that the Bread prepared by using 1% level of Spinach powder showed better composition in terms of physicochemical properties. Prepared products were stored at ambient temperature (25^{o}C) in LDPE pouches. The storage depicted significant increase in textural characteristics while a decreasing trend in the mean values between the treatments was observed. Hardness, chewiness and gumminess of bread samples increased during the storage from 6.63-7.77 N, 2.49-4.14 mm and 3.04-4.66 respectively while the springiness and cohesiveness of bread samples decreased from 0.88-0.76 mm and 0.65-0.43 mm respectively. Total plate count and yeast and mould count showed slightly decreasing trend within treatments while an increasing trend was observed during the storage from 0-1.14×10^2 cfu/g and 0 to 2.2×10^1 cfu/grespectively. This decreasing trend during the storage was found within the permissible limits.

Deficiency of iron is relatively the most common nutritional problem throughout world that affects mainly all the age groups especially women, infants between 9 and 12 months and children under 5 years. In developing countries iron deficiency can affect 30-40% of growing children and women having premenopausal and it can be corrected successfully by population basis. Deficiency of iron can be prevented by increasing the bioavailability and taking iron rich diet.

Current study suggests the possibility of using spinach powder and ferrous sulphate powder as an interventional study to control iron deficiency anemia. The nutritional content of the breads increased but got poor appearance with increasing levels of fortificants. The breads can be improved by

using some bleaching and improving agents to increase their preference/acceptability.

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HEAVY METAL AND LEAD-STRONTIUM ISOTOPE CHARACTERIZATION OF BUJORU, OANCEA AND SMULTI WINE CENTRES

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ABSTRACT

Contamination of vineyard soils with heavy metals has been a worldwide problem, determination of these metals it is important for the wine industry. The aim of this research was to determine the concentration of different heavy metals (Cd, U, Hg, As, Co, Cu, Ni, Cr, Mn, Pb and Sr) isotopic signature of lead ($^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$ and $^{204}Pb/^{206}Pb$) and strontium ($^{87}Sr/^{86}Sr$) from Bujoru, Smulti and Oancea wine-growing centers from Dealu Bujorului vineyard and to assess their ability to discriminate between geographical origin of wines. In this study 180 soils samples from Dealu Bujorului vineyard were investigated. The determination of metals from soil samples was performed on mass spectrometer with inductively coupled plasma, (ICP-MS) iCAP Q Thermo scientific model. In case of Cd (1 mg/kg), Pb (20 mg/kg), Hg (0.1 mg/kg), As (5 mg/L), Co (15 mg/kg), Ni (20 mg/kg) and Cr (30 mg/kg) metals in analysed in soil samples were under Maximum Permissible Limits (MPL). Cu concentration in the soil exceeds the maximum admissible limit (20 mg/kg) having the average value of 415.40 mg/kg, this value is a common one for vineyards soils. Our results confirm that the $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ isotope ratio can be used to track the geographical origin of wine.

1. Introduction

The globalization of food and drink markets has raised consumer concerns regarding product origin and quality. The place and origin of food and drink stuff is regarded as value-added information and as a guarantee of authenticity and quality. For the wine market in particular, geographical origin has a direct effect on its commercial and quality value, being one of the most studied products in terms of food authentication (Barbaste *et al.*, 2002; Almeida *et al.*, 2003).

The control of the geographical origin of wine based on its chemical composition is one of the most challenging issues in relation to wine authenticity. Many efforts have been made to identify potential markers and develop reliable analytical methods to determine the wines authenticity.

The inorganic chemical pattern of wine is a reflection of regional soil geochemistry, ecoclimatic and processing. Elements can be considered as good markers of the geographical origin of wine since they are neither metabolized nor modified during the wine making process. Recently, several studies deal with determination of geographical origin of wines based on their composition. It is assumed that the chemical composition of wines reflects the composition of the provenance soil, at least

for certain elements (Coetzee *et al.*, 2014). Studies in different wine-producing European countries, such as Portugal (Almeida *et al.*, 2003), Italy (Galgano *et al.*, 2008), Croatia (Fiket *et al.*, 2011), Czech Republic (Kment *et al.*, 2005), Spain (García-Rodríguez *et al.*, 2011), Slovakia (Koreňovská *et al.*, 2005), Romania (Geana *et al.*, 2013), Macedonia (Ivanova-Petropulos *et al.*, 2013), Cyprus (Kokkinofta *et al.*, 2014), Slovenia (Šelih *et al.*, 2014), Ukraine (Vystavna *et al.*, 2014), Turkey (Alkiș *et al.*, 2014) provided data demonstrating the potential fingerprinting techniques to identify the geographical origin of wines.

According to earlier works, the most frequently quantified and cited elements used for wine authentication are, Na, K, Rb, Fe, Ca, Cu, Cr, Co, Sb, Cs, Br, As, Ag, Li, Ba, Sr, Mg, Al and Mn (Arvanitoyannis *et al.*, 1999). For Romania, according to studies performed to date, the main elements allowing a differentiation between wines are Mn and Sr (Suhaj *et al.*, 2005).

More recently, the study of isotopic ratios of heavy elements such as Pb and Sr came into use in this field application, providing additional information on the geographical origin, since plants inherit the isotopic signature of these elements from geological and pedological environment (Rummel *et al.*, 2010).

Lead isotope rations have for different Pb sources in various environmental comportments such as lake sediments (Zhang *et al.*, 2008; Sun *et al.*, 2011), marine (Zillén *et al.*, 2012), atmosphere (Kylander *et al.*, 2010; Tomasevic *et al.*, 2013), and soils (Walraven *et al.*, 2014; Wen *et al.*, 2015). Also, the isotopic composition of lead is not affected to any measurable extent by chemical or physical processes (Bollhöfer *et al.*, 2001).The Pb element has four natural, stable isotopes (^{204}Pb, ^{206}Pb, ^{207}Pb and ^{208}Pb). The latter three are products of the radioactive decay of ^{235}U, ^{238}U and ^{232}Th, respectively (Komárek *et al.*, 2008).The isotopic composition of Pb is commonly expressed by ^{206}Pb/^{207}Pb, ^{208}Pb/^{204}Pb, ^{207}Pb/^{204}Pb and ^{206}Pb/^{204}Pb ratio (Cheng and Hu, 2010). The ^{206}Pb/^{207}Pb is the most commonly used ratio in environmental studies (Komárek *et al.*, 2008). The variations in ^{206}Pb/^{207}Pb in different anthropogenic and lithogenic sources are produced because this ratio decreases with the age of the source and increases with its U/Pb ratio (Tyszka *et al.*, 2012). Although Pb isotopes have been routinely used as environmental traces, global, their use in studies on soil contamination in Brazil are still scarce (Schucknecht *et al.*, 2011).

Other researchers have drawn on a combination of lead and lead isotope ratios (^{206}Pb/^{207}Pb, ^{206}Pb/^{208}Pb) as geochemical tracers to recognize the succession of atmospheric and pedogeochemical changes related to industrial activities in the mining areas (Komárek *et al.*, 2008). Pb is widely considered immobile and persistent in soils, thought its mobility in soil can increase with a high concentration of organic matter complex or chelate, which can play a key role in the movement of Fe and other elements in the soil profile (Bäckström *et al.*, 2006). Stable lead isotope ratios can be used as a complementary tool for dating contamination events and for the evaluation of sedimentation rates, including the use of ^{210}Pb (Haack *et al.*, 2003).

Natural strontium (Sr) isotopes have been proved to be useful tool for tracing the past and for monitoring present-day environmental processes (Shand *et al.*, 2009). The geochemical behaviour of Sr is generally similar to that of Ca (Aubert *et al.*, 2002), but also is different from that of the alkali elements Na and K. The Sr isotopic rations (^{86}Sr/^{87}Sr) of natural materials reflect the sources of Sr available during their formation (Capo *et al.*, 1998). Additionally, ^{86}Sr/^{87}Sr ratios are ideal source tracers because these

isotopes have quite small fractionation during biogeochemical cycling (Shand *et al.*, 2009).

Strontium shows variations in isotope composition due to radioactive decay. Sr has four stable isotopes, ^{84}Sr, ^{86}Sr, ^{87}Sr and ^{88}Sr, ^{87}Sr is radiogenic (Vanhaecke *et al.*, 1999) due to the β-decay of the long-lived radio nuclide ^{87}Rb (half-life $t_{½}$= 48.8 x $10^{9}y$) to generate ^{87}Sr. The mean natural abundances of ^{84}Sr, ^{86}Sr and ^{88}Sr remain constant, but ^{87}Sr gradually increases if ^{87}Rd are present in soils, which is generally the case (Chassery *et al.*, 1998).The $^{87}Sr/^{86}Sr$ ratio was shown not be affected by biological isotope fractionation processes (Kawasaki *et al.*, 2002). Elements are absorbed by the plants in the same isotopic proportions in which they occur in soil. Thus, plants reflect the growth environment, such as bedrock, soil water content and soil, including all sources of Sr: natural (pecipitation, bedrock weathering) and anthropogenic (e.g., fertilizers) (Stewart *et al.*, 1998). For establish correlations between the $^{87}Sr/^{86}Sr$ ratio of wines and soils, a deep knowledge of the regions pedological and geological features are a prerequisite. Horn *et al.*, 1997 demonstrated that the $^{87}Sr/^{86}Sr$ values of several wines were within the respective rages for soils and rocks.

The goal of this study is to determine the elemental composition (Cd, U, Hg, As, Co, Cu, Ni, Cr and Mn) from vineyard soil in Bujoru, Smulti and Oancea wine-growing centers from Dealu Bujorului vineyard and to assess their ability to discriminate between geographical origin of wines. Also, the study enhances the knowledge of the large-scale distribution of strontium ($^{87}Sr/^{86}Sr$) and lead ($^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$, $^{208}Pb/^{204}Pb$) isotope ratios in wine from Dealu Bujorului vineyard. The vineyard areas from Smulti and Oancea wine-growing centers have not been analyzed yet regarding concentration of the elemental composition and distribution of strontium ($^{87}Sr/^{86}Sr$) and lead ($^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$, $^{208}Pb/^{204}Pb$) isotope ratio.

2. Materials and methods

2.1. Experimental section

2.1.1. Study area

A total of 180 soil samples were analysed, samples originated from Bujoru, Smulti and Oancea wine-growing centers part of Dealu Bujorului vineyard (45°52′10″ N, 27°55′8″E). The Dealu Bujorului vineyard is characterized by an alternate landscape, from flat to hilly areas, with altitude between 100 and 230 m and also the predominant soil is levigated chernozem having a clayey sand texture with pH between values 7.4 and 8.1. Although they have a precipitation deficit, natural conditions (ecoclimatic and ecopedological) offer viable ecosystem for the development of vineyard. The vineyard is intersected by the 28° longitude meridian and crossed by the parallel 46° latitude north. Dealu Bujorului vineyard, together with Bujoru, Smulti and Oancea viticultural centers belongs to Galați country. Versants were made from clay deposits and sandy sands. The specificity of the transition area is highlighted by the predominance of deposits of clays and sands.

2.1.2. Soil sample collection

Soil sampling was performed on the depth of the soil profile (0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm, 50-60 cm, 60-70 cm, 70-80 cm, 80-90 cm, 90-100 cm, 100-110 cm and 110-120 cm). Soil samples wasdone using stainless steel shovels and were stored in individual black plastic bags. All soils samples were taken in triplicates from the defined experimental areas. Soil samples have been brought first to sand-size material using a jaw crusher (< 2 mm) then mechanically split to obtain a representative samples and eventually pulverized to powder-size, grain-size smaller than 100 μ (< 400 mesh), using a ball mill. Agate ball mill was used in place of any other pulverization

metal device to avoid accidently trace element contamination

2.2. Materials and methods used

2.2.1. Reagents and solutions

Eleven elements (Cd, U, Hg, As, Co, Cu, Ni, Cr, Mn, Pb and Sr) were determined in order to assess their ability to discriminate soil by geographical origin from Bujoru, Smulti and Oancea wine centers. The soils samples analysis was made using multielement analysis of ICP-MS technique, after an appropriate dilution, using external standard calibration method. The calibration was performed using XXICertiPUR multielement standard, and from individual standard solution of Hg and Cr. The standards and the control sample were prepared daily from the intermediate standards that were prepared from the stock solution. The intermediate solutions stored in polyethylene bottles and glassware was cleaned by soaking in 10% v/v HNO_3 for 12 hours and rinsing at least seven rimes with ultrapure water (18.2 MΩ cm^{-1} ultrapure water-Types 1). The accuracy of the methods was evaluated by replicate analyses of fortified samples (10 µL-10 mL concentrations) and the obtained values ranged between 0.7-12.8 percent, depending on the element. The global recovery for each element was estimated and the obtained values were between 82.4-100.3 %.

Limit of detection (LoD) and Limit of quantification (LoQ) limits were calculated according to the next mathematical formulas: LoD = 3SD/s and LoQ = 10 SD/s (SD = estimation of the standard deviation of the regression line; s = slope of the calibration curve) (Table 1). For quality control blanks and triplicates samples (n = 3) were analyzed during the procedure. The variation coefficient was under 5% and detection limits (ppb) were determined by the calibration curve method.

Table 1. Instrumental conditions for the determination of each element (ICP-MS technique)

Element	Correlation coefficient	LoD* (µg/L)	LoQ*** (µg/L)	BEC** (µg/L)
Cd	0.9999	0.0207	0.0678	0.026
U	0.9999	0.0251	0.0847	0.007
As	0.9999	0.2338	0.7772	0.543
Co	0.9999	0.0363	0.1210	0.149
Ni	0.9999	0.0595	0.1969	0.093
Pb	0.9999	0.0007	0.0018	0.003
Hg	0.9999	0.0412	0.1374	0.136
Sr	0.9999	0.1431	0.4777	0.949
Cu	0.9999	0.0409	0.1334	0.239
Cr	0.9999	1.6637	5.5375	0.635
Mn	0.9999	0.0105	0.0311	0.091

*Detection limit; **Background equivalent concentration; ***Quantification limit.

For calibration and to verify the achieved accuracy and precision, ten NIST-SRM 982 and NIST-SRM 987 analysis results were pooled together with the calculated relative standard deviation presented in Table 2. Based on the obtained results, it was verified that, applying quadrupole ICP-MS, relative standard deviation and reproducibility of approximately 0.5% for, $^{206}Pb/^{207}Pb$ $^{208}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ are feasible. The results were in agreement with those reported by Barbaste *et al.*, 2002 and Geana *et al.*, 2017.

Table 2. Lead isotopic ration and Lead isotopic ration determination precision and accuracy based on the NIST SRM 982 (Lead) NIST SRM 987 (Strontium) (n=10)

Replicate	$^{207}Pb/^{206}Pb$ (a)	RSD (%)	$^{208}Pb/^{206}Pb$ (b)	RSD (%)	$^{204}Pb/^{206}Pb$ (c)	RSD (%)	$^{87}Sr/^{86}Sr$ (d)	RSD (%)
1	0.46484	0.57	0.99897	0.65	0.00272	0.37	0.70485	0.35
2	0.47893	0.43	0.99454	0.63	0.00275	0.44	0.72045	0.47
3	0.46977	0.38	0.99795	0.58	0.00274	0.25	0.70327	0.62
4	0.47127	0.64	0.99689	0.64	0.00274	0.54	0.70635	0.42
5	0.46980	0.55	0.99727	0.46	0.00247	0.19	0.71473	0.37
6	0.46152	0.39	0.99646	0.54	0.00255	0.35	0.71240	0.55
7	0.47367	0.73	0.99964	0.33	0.00276	0.47	0.70984	0.45
8	0.45649	0.44	0.99740	0.57	0.00277	0.55	0.72325	0.45
9	0.41560	0.34	0.99577	0.56	0.00275	0.47	0.70843	0.63
10	0.45612	0.49	0.99877	0.62	0.00277	0.35	0.70787	0.48
Average	0.46180	0.49	0.99737	0.56	0.00270	0.40	0.71114	0.48

[a]Certified value = $^{207}Pb/^{206}Pb$ (0.46707±0.00020); [b]Certified value = $^{208}Pb/^{206}Pb$ (1.00016±0.00036); [c]Certified value = $^{204}Pb/^{206}Pb$ (0.027219±0.00027); [d]Certified value = $^{87}Sr/^{86}Sr$ (0.71034±0.00026); RSD (%) = relative standard deviation.

2.2.2. Sample preparation for determination of isotopic ration and heavy metals from soil using ICP-MS

For the determination of elements from soil samples were used an amount of 0.5 mL soil and adjust 8 mL (7 mL HNO_3 65%+1 mL H_2O_2) were placed in a clean Teflon digestion vessel, after 15-30 minutes the mineralization was performed using a microwave system Milestone START D Microwave Digestion System set in three steps: step I (time 10 min., temperature 220ºC), step II (time 15 min., temperature 220ºC) and step III (time 40 min., ventilation - temperature 43ºC). After mineralization, soil samples were filtered through a 0.45 mm filter and brought to a volume of 50 mL. The Pb ($^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$) and Sr ($^{87}Sr/^{86}Sr$) isotope ration in the analysed wine samples were determined according to the methodology indicated by Mihaljevič *et al*., 2006; Geana *et al.*, 2017; Bora *et al.*, 2018.

In order to confirm the best chosen conditions for soil digestion standard, additions for checking accuracy of the microwave digestion and recoveries were calculated (Table 3). The soil samples digestion seemed visually completed in all of the combinations, but the spiked recoveries showed significant differences for total elements content (*p* - Value = *0.005*).

Table 3. Standard additions for checking accuracy of the microwave digestion ICP-MS method (n = 3) (SRM 1643e)

Element	Certified Concentration (mg/L)	Measured Concentration (mg/L)
Cd	6.568±0.073	6.482±0.107
U	9.994±0.016	9.982±0.036
As	56.85±0.37	53.88±0.74
Co	27.06±0.28	26.84±0.07
Ni	62.41±0.69	61.48±0.27
Pb	19.63±0.21	19.12±0.03
Hg	0.1016±0.0017	0.1107±0.0032
Sr	314.00±19.00	314.07±19.04
Cu	21.44±0.70	21.77±0.32
Cr	18.32±0.10	19.14±0.22
Mn	38.02±0.44	33.07±0.05

2.2.3. Instrumentation

The determination of metals from soil samples was performed on mass spectrometer with inductively coupled plasma, (ICP-MS) iCAP Q Thermo scientific model, based polyatomic species before they reach the quadrupole mass spectrometer, using a PFA micro flow concentric nebulizer. The argon used for analysis was of 99.99% purity (Messer, Austria). The instrument was daily optimized to give maximum sensitivity for M^+ ions and the double ionization and oxides monitored by the means of the rations between Ba^{2+}/Ba^+ and Ce^{2+}/CeO^+, respectively, these always being less than 3%. The experimental conditions were: argon flow on nebulizer (0.85 L/min.), auxiliary gas flow 0.83 L/min., argon flow in plasma 15 L/min., lens voltage 7.30 V; RF power in plasma 1100 W, spray chamber temperature

(2.32±1.00ºC). Accuracy was calculated for the elements taken into consideration (0.5-5.0%).

2.2.4. Statistical analysis

The statistical processing of the results was primarily performed in order to calculate the following statistical parameters: average and standard deviation. The statistical interpretation of the results was performed using the Duncan test, SPSS Version 24 (SPSS Inc., Chicago, IL., USA). This data was interpreted with the analysis of variance (ANOVA) and the average separation was performed with the DUNCAN test at $p \leq 0.05$. Heat map, translocation factor and mobility ratio was performed using Microsoft Excel 2016 and XLSTAT Addinsoft version 15.5.03.3707.

3. Results and discussions

3.1. Heavy metals content in soil samples from Bujoru, Smulti and Oancea Wine Center

In Table 4 are summarized the total contents of Cd, Pb, U, Hg, As, Sr, Co, Cu, Ni, Cr and Mn on the depth of the soil profile. The mean contents of Cd and U were 0.39±0.08 mg/kg and 0.37±0.09 mg/kg, in case of Cd the lowest concentrations were recorded on the depth of the soil profile [0.56±0.06 mg/kg (70-80 cm Bujoru); 0.53±0.11 mg/kg (20-30 cm Smulti); 0.54±0.07 mg/kg (50-60 cm and 0.53±0.09 mg/kg (60-70 cm) in Oancea wine center)]. The U concentration from soil was between [0.22±0.05 mg/kg (90-100 cm) Bujoru] and [0.63±0.08 mg/kg (100-100 cm) Smulti] with an average of 0.37±0.09 mg/kg. The results agree with other scientific papers Mahmoudabadi *et al.*, 2015 (0.30 mg/kg (Cd)), Moragues-Quiroga *et al.*, 2017(0.03 mg/kg (U)) and significantly lower than those obtained by Saat *et al.*, 2015 (2.21 mg/kg (U)). Regarding the distribution of Cd concentration on the depth of the soil profile, it can be observed that with increase of the depth the Cd concentration from soil decreases, and in the case of U it can be observed that with increase of the depth the U concentration from soil it remains the same or even increase.

The content of Hg, As and Co found in Bujoru, Smulti and Oancea wine center agreed with literature data Ottesen *et al.*, 2013, Kailing *et al.*, 2017, Moragues-Quiroga *et al.*, 2017, Laghlimi *et al.*, 2015.The average values of these metals 0.070±0.010 mg/kg (Hg), 0.84±0.11 mg/kg (As), and 3.52±0.48 mg/kg (Co) do not indicate soil pollution in wine centers analyzed (Table 4). In the Bujoru and Smulti wine-growing center the distribution of Hg, As and Co concentration on the depth of the soil profile, it can be observed that with increase of the depth the Hg, As and Co concentration from soil increase and in Oancea wine-growing center it can be observed that with increase of the depth Hg and Co concentration from soil increase, exception does As, with increase of the depth As concentration from soil decreases.

In terms of Ni, Cr and Mn the highest concentration was recorded in the surface of the soil profile for all three metals, Ni [10.38±2.20 mg/kg (0-10 cm) in Bujoru; 8.67±1.02 mg/kg (0-10 cm) in Smulti; 8.17±0.89 mg/kg (0-10 cm) in Oancea wine-growing center] Cr [13.66±1.56 mg/kg (0-10 cm) in Bujoru; 13.42±1.22 mg/kg (20-30 cm) in Smulti; 13.72±0.28 mg/kg (20-30 cm) in Oancea wine-growing center] and Mn [527.66±3.77 mg/kg (10-20 cm) in Bujoru; 429.10±2.79 mg/kg (20-30 cm) in Smulti; 335.80±6.25 mg/kg (20-30 cm) in Oancea wine-growing center] with an average value of 7.01±0.92 mg/kg (Ni); 10.38±0.95 mg/kg (Cr) and 302.72±4.92mg/kg (Mn) (Table 4). The results agree with literature data (Ottesen *et al.*, 2013, Mahmoudabadi *et al.*, 2015). Regarding the distribution of Ni, Cr and Mn concentration on the depth of the soil profile,

it can be observed that with increase of the depth the Ni, Cr and Mn concentration from soil decreases in all areas.

Concerning Cu concentration in soil, at the surface of the soil profile were recorded the highest concentration [591.63±5.56 mg/kg (10-20 cm) in Bujoru; 728.45±7.39 mg/kg (20-30 cm) in Smulti; 539.23±6.28 mg/kg (0-10 cm) in Oancea wine-growing center], these concentrations from far exceed the maximum allowed by the legislation (20 mg/kg). The data demonstrate strong pollution of vineyard soil by copper. Copper concentrations in the topsoil in Bujoru were between 591.63±5.56 mg/kg (10-20 cm) and 114.83±3.02 mg/kg (110-120 cm), in Smulti wine-growing center concentrations was between 728.45±7.39 mg/kg (20-30 cm) and 326.93±5.84 mg/kg (110-120 cm), in Oancea concentrations was between 539.23±6.28 mg/kg (0-10 cm) and 169.76±7.20 mg/kg (110-120 cm) with an average value of 415.40±5.70 mg/kg. The copper enrichment in different vineyard soil types reflects the period of copper-based pesticide application (Çolak, 2012).We consider the ecoclimatic conditions and application of elevated volumes of fungicide as the circumstantial factor for the high copper contamination in vineyards soil. Copper concentration in soil was significantly lower than the maximum value reported in literature (1500 mg/kg) (Mirlean *et al.*, 2007). Concerning the distribution of copper concentration on the depth of the soil profile, it can be observed that with increase of the depth the copper concentration from soil decreases. We assume that copper distribution between vine lines mainly depends on certain technical operations, such as implemented spraying technical operations, foliage removing from midway zones or simply foliage and other organic debris redistribution in horizontal directions by winds, machine wheels, animals and/or humans (Mirlean *et al.*, 2007).

The copper sulphate actually are used in all wine counties and also in Bordeaux, according to Mirlean *et al.*, 2007 mixture preparation contains: Cd 1.4 mg/kg, Pb 95 mg/kg, Ni 10 mg/kg, Cr 19 mg/kg and Zn 1309 mg/kg. Therefore, we considered that copper-based pesticide is the principal source of established soil enrichment by heavy metals. Similar conclusions have reached also some authors in Serbia (Alagić *et al.*, 2014), Brasil (Mirlean *et al.*, 2007), Italy (Pinamonti *et al.*, 1999), Romania (Bora *et al*., 2018), France (Chopin *et al*., 2008) and Spain (Bravo *et al.*, 2017). The results agree with Alagić *et al.*, 2014 (315.00 mg/kg (Cu)), Couto *et al.*, 2015 (602.23 mg/kg (Cu)), Romić *et al.*, 2004 (586 mg/kg (Cu)), Chaignon *et al.*, 2003(398 586 mg/kg (Cu)) and significantly higher than those obtained by Rusjan *et al.*, 2006 (88.00 mg/kg (Cu)).

Reporting the obtained results [Cd average 0.39±0.08 mg/kg (1 mg/kg M.A.L = (maximum limit allowed); Pb average 5.63±0.92 mg/kg (20 mg/kg M.A.L); Hg average 0.070±0.010 mg/kg (0.1 mg/kg M.A.L); As average 0.84±0.11 mg/kg (1 mg/kg M.A.L); Co average 3.52±0.48 mg/kg (15 mg/kg M.A.L); Ni average 7.01±0.92 mg/kg (1 mg/kg M.A.L); Cr average 10.38±0.95 mg/kg (30 mg/kg M.A.L) and Mn average 302.72±4.49 mg/kg (900 mg/kg M.A.L)] to national and international legislation we can say that the soil from Bujoru, Smulti and Oancea wine-growing center falls within the limits set by the law, except the Cu average 356.03±4.36 mg/kg concentration which exceeds this limit (20 mg/kg) (Table 4).

Regarding Pb and Sr concentration in soil, at the surface of the soil profile were recorded the highest concentration for both elements with an average value of 5.63±0.92 mg/kg (Pb) and 42.05±1.86 mg/kg (Sr) these concentrations from far exceed the maximum allowed by the legislation (20 mg/kg for Pb) (Table 5).

3.2. $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio in soil samples from Bujoru, Smulti and Oancea Wine Center

The Pb isotope ratio from soil (Table 5) varies in range between 1.12992-1.18523 ($^{206}Pb/^{207}Pb$), 2.09397-2.14084 ($^{208}Pb/^{206}Pb$) and 17.21204-17.6966 ($^{206}Pb/^{204}Pb$) with average 1.15245 ($^{206}Pb/^{207}Pb$), 2.111041 ($^{208}Pb/^{206}Pb$) and 17.41469 ($^{206}Pb/^{204}Pb$). The wide range of isotope ratio obtained suggest that Pb content in the soil is a product of the combination of different sources and not from a sole origin, natural or anthropogenic, and that it has also been accumulating over time since the massive use of Pb in fuel (Galušková *et al.*, 2014). The $^{206}Pb/^{207}Pb$ ratio is most commonly used in environmental studies because it can be determined precisely, and the abundances of these isotopes are relatively important (Komárek *et al.*, 2008). The isotope ration $^{206}Pb/^{207}Pb$ revealed a different behaviour between soil profiles at each sampling site.

Regarding $^{206}Pb/^{207}Pb$ isotope ration based on analyses it can be concluded that the vineyard soil from Dealu Bujorului with an average value of 1.15241 $^{206}Pb/^{207}Pb$ it does come from automobile emissions, if average value was between 1.1000 and 1.1400 $^{206}Pb/^{207}Pb$ then it could come from the pollution of cars. The values of $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ isotope ratio are between the ranges from 2.09397 to 2.14084 ($^{208}Pb/^{206}Pb$) and 17.21204 to 17.69665 ($^{206}Pb/^{204}Pb$) with an average value of 2.11041 ($^{208}Pb/^{206}Pb$) and 17.41469 ($^{206}Pb/^{204}Pb$). The highest values of $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ isotope ratio were registered on the depth of the soil profile from 0-10 cm [1.18523 ($^{206}Pb/^{207}Pb$) Smulti wine centre] followed by 70-80 cm [2.13819 ($^{208}Pb/^{206}Pb$) Bujoru wine centre] and values recorded at 20-30 cm [17.69665 ($^{206}Pb/^{204}Pb$) Oancea wine centre]. The lowest values of $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ isotope ratio were recorded also on the depth of the soil profile 60-70 cm [1.13150 ($^{208}Pb/^{206}Pb$) Bujoru wine centre] followed by 40-50 cm [2.09397 ($^{208}Pb/^{206}Pb$) Smulti wine centre] and values recorded at 110-120 cm [17.21204 ($^{206}Pb/^{204}Pb$) Smulti wine centre].

Regardless of Pb sources (lithogenic or anthropogenic), the average $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ ration in soil profile horizons followed the order: 1.11492 (0-10 cm $^{206}Pb/^{207}Pb$) > 1.13337 (110-120 cm $^{206}Pb/^{207}Pb$) Bujoru wince center, 1.18523 (0-10 cm $^{206}Pb/^{207}Pb$) > 1.18349 (0-10 cm $^{206}Pb/^{207}Pb$) Smulti wince center, 1.14543 (90-100 cm $^{206}Pb/^{207}Pb$) > 1.14398 (0-10 cm $^{206}Pb/^{207}Pb$) Oancea wince center; 2.12959 (0-10 cm $^{208}Pb/^{206}Pb$) > 2.12311 (110-120 $^{208}Pb/^{206}Pb$) Bujoru wince center, 2.11805 (60-70 cm $^{208}Pb/^{206}Pb$) > 2.10286 (0-10 cm $^{208}Pb/^{206}Pb$) Smulti wince center, 2.10644 (110-120 cm $^{208}Pb/^{206}Pb$) > 2.09668 (0-10 cm $^{208}Pb/^{206}Pb$) Oancea wince center and 17.45429 (110-120 cm $^{206}Pb/^{204}Pb$) > 17.36218 (0-10 cm $^{206}Pb/^{204}Pb$) Bujoru wince center, 17.22465 (30-40 cm $^{206}Pb/^{204}Pb$) > 17.21204 (110-120 cm $^{206}Pb/^{204}Pb$) Smulti wince center, 17.69665 (20-30 cm $^{206}Pb/^{204}Pb$) > 17.64762 (110-120 cm $^{206}Pb/^{204}Pb$) Oancea wince center. Statistically in the case of $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ there are significant differences between the analyzed variants isotope ratio.

These results demonstrate that Pb isotopic ratios were derived mainly from weathered parent material, except the $^{206}Pb/^{207}Pb$ uppermost horizons of soil profiles which come from automobile emissions. The more radiogenic $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{207}Pb$ ratio in soil probably reflects the Pb derived from weathered bedrock, and the isotopic composition of Pb is mostly influenced by the decay of U and Th content in the soil, weathering processes and original rock age, which provide a fingerprint used for

different forensic and archeological purposes (Almeida *et al.*, 2003).

Concerning $^{87}Sr/^{86}Sr$, isotope ratio the values are between the ranges from 0.70178 to 0.73441, with an average value of 0.72821. The highest values were registered on the depth of the soil surface profile 90-100 cm [0.73441($^{87}Sr/^{86}Sr$) Smulti wine centre] followed by values recorded at 50-60 cm [0.73431($^{87}Sr/^{86}Sr$) Smulti wine centre], the lowest value of $^{87}Sr/^{86}Sr$ isotope ratio was registered on the depth of the soil at 90-100 cm [0.70178 ($^{87}Sr/^{86}Sr$) Oancea wine centre] (Table 5). In this case, these are no significant differences between the $^{87}Sr/^{86}Sr$ isotope ratio. These obtained values of $^{87}Sr/^{86}Sr$ isotope ratio can be attributed to a larger proportion of radiogenic (K and Rb rich) mineral due to the weathering of the most weather able mineral, i.e. Ca-plagioclase, which is promoted by a strong decrease of the soil pH (Drouet *et al.*, 2007).

Heat maps were used to discover samples groups, discover feature groups and also to discover related sample/feature groups. In case of heavy metals content and $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio on the depth of the soil profile (horizontal dendrogram), the dendrogram clearly show two cluster, the first cluster is formed from the depth of the soil profile between10-20 cm, 0-10 cm, 40-50 cm, 50-60 cm, (Smulti wine centre), 10-20 cm (Oancea wine centre), 10-20 cm, 30-40 cm, 40-50 cm (Bujoru wine centre) and second cluster was formed from the depth of the soil profile between 60-10 cm, 30-40 cm, 80-90 cm, 70-80 cm, 90-100 cm, 100-110 cm (Oancea wine centre), 80-90 cm, 110-120 cm, 90-100 cm (Smulti wine centre), 110-120 cm (Bujoru wine centre).

Based on this distribution we can say that the heavy metal concentration recorded the highest concentration on the surface and medium soils and the lowest concentration were recorded on the depth of the soil. The vertical dendrogram show also two clusters. The first cluster is formed from Cu and Mn and the second cluster was formed from Sr, Hg, Cd, U, As, Co, Cr, Ni, Pb and $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratio. Based on these it can be said that in all research areas the Cu and Mn concentration in vineyard soils was higher, and lowest at the rest of the elements (Figure 1).

Table 4. Variation of the metal content in soil samples on Dealu Bujorului Vineyard (Bujoru, Smulti and Oancea Wine Centre)

Area	Soil type	Depth (cm)	Cd	U	Hg	As	Co	Cu	Ni	Cr	Mn
			M.A.L.*	M.A.L.	M.A.L.	M.A.L.	M.A.L.	M.A.L.	M.A.L.	M.A.L.	M.A.L.
***Normal Values**			**1 mg/kg**	**-**	**0.1 mg/kg**	**5 mg/kg**	**15 mg/kg**	**20 mg/kg**	**20 mg/kg**	**30 mg/kg**	**900 mg/kg**
Alert threshold	**Susceptible**		**3 mg/kg**	**-**	**1 mg/kg**	**15 mg/kg**	**30 mg/kg**	**100 mg/kg**	**75 mg/kg**	**100 mg/kg**	**1500 mg/kg**
	Less Susceptible		**5 mg/kg**	**-**	**4 mg/kg**	**25 mg/kg**	**100 mg/kg**	**250 mg/kg**	**200 mg/kg**	**300 mg/kg**	**2000 mg/kg**
Intervention threshold	**Susceptible**		**5 mg/kg**	**-**	**2 mg/kg**	**25 mg/kg**	**50 mg/kg**	**200 mg/kg**	**150 mg/kg**	**300 mg/kg**	**2500 mg/kg**
	Less Susceptible		**10 mg/kg**	**-**	**10 mg/kg**	**50 mg/kg**	**250 mg/kg**	**500 mg/kg**	**500 mg/kg**	**600 mg/kg**	**4000 mg/kg**
Bujoru Wine Centre	**Chernozem**	**0-10**	$0.39\pm0.02^{abcdefgh}\beta\gamma\delta$	$0.27\pm0.08^{ef}\beta\gamma$	$0.050\pm0.005^{ghijk}\beta\gamma$	$0.39\pm0.09^{mno}\varepsilon$	$2.97\pm0.53^{fghijk}\beta\gamma\delta$	$584.49\pm5.51^{d}\alpha$	$10.38\pm2.20^{ab}\alpha\beta$	$13.66\pm1.56^{abc}\alpha\beta$	$423.21\pm3.24^{cd}\gamma$
		10-20	$0.33\pm0.04^{cdefgh}\beta\gamma\delta$	$0.38\pm0.11^{bcdef}\alpha\beta$	$0.068\pm0.023^{abcdefghi}\alpha\beta\gamma$	$0.52\pm0.08^{klmnop}\delta\varepsilon$	$3.25\pm0.01^{fghijk}\alpha\beta\gamma$	$591.63\pm5.56^{d}\alpha$	$9.46\pm2.46^{bc}\alpha\beta\gamma$	$13.35\pm0.84^{abc}\alpha\beta$	$427.39\pm5.33^{c}\gamma$
		20-30	$0.43\pm0.09^{abcdefgh}\alpha\beta\gamma$	$0.29\pm0.02^{def}\beta\gamma$	$0.063\pm0.013^{bcdefghijk}\alpha\beta\gamma$	$0.82\pm0.05^{fghijk}\gamma\delta$	$3.17\pm0.15^{fghijk}\alpha\beta\gamma$	$571.55\pm9.48^{e}\beta$	$11.49\pm2.11^{a}\alpha$	$12.46\pm1.91^{cdef}\beta\gamma$	$329.33\pm7.87^{ef}\delta$
		30-40	$0.43\pm0.04^{abcdefgh}\alpha\beta\gamma$	$0.44\pm0.09^{abcdef}\alpha$	$0.049\pm0.010^{hijk}\beta\gamma$	$1.28\pm0.24^{bcd}\beta$	$2.84\pm1.15^{fghijk}\gamma\delta$	$554.68\pm7.36^{f}\gamma$	$8.90\pm0.29^{bcde}\beta\gamma\delta$	$14.80\pm0.40^{ab}\alpha$	$527.66\pm3.77^{a}\alpha$
		40-50	$0.27\pm0.10^{gh}\gamma$	$0.34\pm0.06^{bcdef}\alpha\beta\gamma$	$0.046\pm0.015^{ijk}\gamma$	$1.36\pm0.11^{bc}\beta$	$3.72\pm0.51^{defghi}\alpha\beta$	$435.39\pm8.14^{j}\delta$	$7.49\pm1.43^{cdefghij}\gamma\delta\varepsilon$	$11.73\pm1.32^{cdef}\beta\gamma$	$481.77\pm5.11^{b}\beta$
		50-60	$0.26\pm0.04^{h}\gamma$	$0.34\pm0.06^{bcdef}\alpha\beta\gamma$	$0.084\pm0.013^{abc}\alpha$	$1.41\pm0.13^{b}\beta$	$2.54\pm0.37^{ijk}\gamma\delta$	$411.36\pm4.10^{k}\delta\varepsilon$	$6.03\pm0.77^{ijklmn}\varepsilon$	$9.18\pm0.86^{hijk}\delta\varepsilon$	$477.74\pm9.51^{b}\beta$
		60-70	$0.48\pm0.11^{abcd}\alpha\beta$	$0.28\pm0.03^{def}\beta\gamma$	$0.072\pm0.013^{abcdefg}\alpha\beta$	$1.04\pm0.08^{def}\beta\gamma$	$3.10\pm0.05^{fghijk}\alpha\beta\gamma$	$405.23\pm5.93^{k}\delta\varepsilon$	$8.02\pm0.22^{cdefghi}\gamma\delta\varepsilon$	$9.13\pm0.86^{hijk}\delta\varepsilon$	$216.53\pm0.85^{k}\eta$
		70-80	$0.56\pm0.06^{a}\alpha$	$0.29\pm0.06^{def}\beta\gamma$	$0.061\pm0.026^{cdefghijk}\alpha\beta\gamma$	$2.04\pm0.59^{a}\alpha$	$2.11\pm0.06^{k}\delta$	$325.44\pm4.07^{t}\zeta$	$6.38\pm0.55^{ghijklmn}\varepsilon$	$7.84\pm0.59^{jklm}\varepsilon$	$231.10\pm4.50^{j}\zeta$
		80-90	$0.41\pm0.10^{abcdefg}\alpha\beta\gamma\delta$	$0.30\pm0.05^{cdef}\beta\gamma$	$0.081\pm0.006^{abcd}\alpha$	$2.13\pm0.10^{a}\alpha$	$4.00\pm0.14^{bcdef}\alpha$	$332.32\pm1.41^{pr}\zeta$	$6.24\pm0.07^{ghijklmn}\varepsilon$	$10.69\pm0.45^{efgh}\gamma\delta$	$328.70\pm3.33^{ef}\delta$
		90-100	$0.34\pm0.14^{cdefgh}\beta\gamma\delta$	$0.22\pm0.05^{f}\gamma$	$0.076\pm0.006^{abcdef}\alpha$	$1.28\pm0.06^{bcd}\beta$	$3.43\pm0.32^{defghi}\alpha\beta\gamma$	$130.96\pm3.63^{v}\eta$	$6.56\pm0.51^{fghijklmn}\delta\varepsilon$	$8.49\pm1.06^{ijkl}\varepsilon$	$262.74\pm6.31^{h}\varepsilon$
		100-110	$0.32\pm0.04^{cdefgh}\beta\gamma$	$0.35\pm0.11^{bcdef}\alpha\beta\gamma$	$0.080\pm0.004^{abcd}\alpha$	$1.24\pm0.13^{bcd}\beta$	$2.74\pm0.82^{fghijk}\gamma\delta$	$127.10\pm6.42^{v}\eta$	$5.72\pm1.01^{klmn}\varepsilon$	$7.98\pm1.07^{jklm}\varepsilon$	$327.25\pm6.44^{ef}\delta$
		110-120	$0.34\pm0.08^{cdefgh}\beta\gamma\delta$	$0.30\pm0.05^{cdef}\beta\gamma$	$0.083\pm0.002^{abc}\alpha$	$1.10\pm0.13^{cde}\beta\gamma$	$3.36\pm0.20^{efghij}\alpha\beta\gamma$	$114.83\pm3.02^{x}\theta$	$5.55\pm0.54^{klmn}\varepsilon$	$7.82\pm0.84^{jklm}\varepsilon$	$265.16\pm7.94^{h}\varepsilon$
Smulti Wine Centre	**Chernozem**	**0-10**	$0.49\pm0.16^{abc}\alpha\beta$	$0.40\pm0.04^{bcdef}\alpha\beta$	$0.043\pm0.012^{k}\gamma$	$0.83\pm0.08^{efghij}\alpha\beta\gamma$	$3.20\pm0.12^{fghijk}\delta\varepsilon$	$653.14\pm5.31^{c}\gamma$	$8.67\pm1.02^{bcde}\alpha\beta$	$11.40\pm0.86^{defg}\beta\gamma$	$328.29\pm2.80^{ef}\gamma$
		10-20	$0.49\pm0.13^{abc}\alpha\beta$	$0.48\pm0.14^{abcde}\alpha\beta$	$0.080\pm0.013^{abcd}\alpha$	$0.70\pm0.21^{ghijklm}\beta\gamma$	$3.36\pm0.20^{efghij}\gamma\delta\varepsilon$	$679.30\pm2.43^{b}\beta$	$9.01\pm0.72^{bcd}\alpha$	$11.68\pm2.15^{cdef}\beta\gamma$	$415.62\pm3.24^{d}\beta$
		20-30	$0.53\pm0.11^{ab}\alpha$	$0.26\pm0.13^{ef}\beta$	$0.085\pm0.008^{ab}\alpha$	$0.94\pm0.06^{efgh}\alpha$	$3.65\pm0.37^{defghi}\beta\gamma\delta\varepsilon$	$728.45\pm7.39^{a}\alpha$	$8.51\pm0.99^{bcdef}\alpha\beta$	$13.42\pm1.22^{abc}\alpha\beta$	$429.10\pm2.79^{c}\alpha$
		30-40	$0.45\pm0.10^{abcdefgh}\alpha\beta$	$0.50\pm0.14^{abcd}\alpha\beta$	$0.057\pm0.006^{efghijk}\beta\gamma$	$0.77\pm0.08^{fghijkl}\alpha\beta\gamma$	$4.51\pm0.32^{abcde}\alpha\beta\gamma\delta$	$659.18\pm8.52^{c}\gamma$	$7.88\pm0.78^{cdefghij}\alpha\beta$	$15.13\pm0.98^{a}\alpha$	$328.19\pm2.68^{ef}\gamma$
		40-50	$0.39\pm0.13^{abcdefgh}\alpha\beta$	$0.43\pm0.23^{abcdef}\alpha\beta$	$0.044\pm0.004^{jk}\gamma$	$0.81\pm0.10^{fghijk}\alpha\beta\gamma$	$2.93\pm1.08^{fghijk}\varepsilon$	$534.56\pm7.12^{g}\delta$	$7.32\pm0.91^{defghijkl}\beta$	$11.48\pm1.25^{defg}\beta\gamma$	$258.77\pm2.18^{h}\delta$
		50-60	$0.42\pm0.09^{abcdefgh}\alpha\beta$	$0.43\pm0.19^{abcdef}\alpha\beta$	$0.084\pm0.013^{abc}\alpha$	$0.95\pm0.10^{efg}\alpha$	$4.60\pm0.45^{abcd}\alpha\beta\gamma$	$531.14\pm6.28^{gh}\delta$	$7.96\pm0.35^{cdefghi}\alpha\beta$	$10.31\pm1.12^{fghi}\gamma\delta$	$330.16\pm5.57^{ef}\gamma$
		60-70	$0.44\pm0.08^{abcdefg}\alpha\beta$	$0.49\pm0.15^{abcd}\alpha\beta$	$0.083\pm0.013^{abc}\alpha$	$0.74\pm0.22^{ghijkl}\alpha\beta\gamma$	$3.68\pm1.35^{defghi}\beta\gamma\delta\varepsilon$	$463.76\pm6.62^{i}\varepsilon$	$5.57\pm0.58^{klmn}\gamma$	$9.27\pm0.61^{hij}\delta\varepsilon$	$258.67\pm7.76^{h}\delta$
		70-80	$0.47\pm0.13^{abcde}\alpha\beta$	$0.43\pm0.19^{abcdef}\alpha\beta$	$0.078\pm0.015^{abcde}\alpha$	$0.90\pm0.06^{efghij}\alpha\beta$	$2.63\pm0.50^{hijk}\varepsilon$	$374.42\pm7.14^{l}\zeta$	$5.35\pm0.72^{lmno}\gamma$	$10.42\pm0.64^{fghi}\gamma\delta$	$158.10\pm5.35^{n}\zeta$
		80-90	$0.30\pm0.05^{efgh}\beta$	$0.41\pm0.24^{abcdef}\alpha\beta$	$0.079\pm0.009^{abcde}\alpha$	$0.47\pm0.10^{lmnop}\delta\varepsilon$	$4.46\pm1.21^{abcde}\alpha\beta\gamma\delta$	$352.83\pm3.57^{m}\eta$	$5.04\pm0.87^{mnop}\gamma$	$11.33\pm1.17^{efg}\gamma\delta$	$130.26\pm4.46^{o}\eta$
		90-100	$0.29\pm0.06^{fgh}\beta$	$0.63\pm0.08^{a}\alpha\alpha\beta$	$0.081\pm0.003^{abcd}\alpha$	$0.62\pm0.11^{jklmno}\gamma\delta$	$3.54\pm0.37^{defghij}\gamma\delta\varepsilon$	$344.50\pm7.24^{no}\eta$	$3.47\pm0.56^{op}\delta\varepsilon$	$8.16\pm0.52^{jklm}\varepsilon$	$328.69\pm3.12^{ef}\gamma$
		100-110	$0.31\pm0.06^{defgh}\beta$	$0.52\pm0.06^{abc}\alpha\beta$	$0.064\pm0.020^{bcdefghijk}\alpha\beta$	$0.31\pm0.06^{o}\varepsilon$	$4.91\pm0.07^{abc}\alpha\beta$	$353.53\pm6.80^{m}\eta$	$3.28\pm0.26^{o}\varepsilon$	$8.18\pm1.50^{jklm}\varepsilon$	$128.70\pm3.50^{o}\eta$
		110-120	$0.35\pm0.08^{bcdefgh}\alpha\beta$	$0.56\pm0.24^{ab}\alpha\beta$	$0.080\pm0.005^{abcd}\alpha$	$0.67\pm0.08^{ghijklmn}\gamma$	$5.42\pm0.58^{a}\alpha$	$326.93\pm5.84^{r}\theta$	$4.88\pm1.56^{mnop}\gamma\delta$	$8.21\pm0.21^{jklm}\varepsilon$	$249.33\pm1.71^{i}\varepsilon$
Oancea Wine Centre	**Chernozem**	**0-10**	$0.32\pm0.09^{cdefgh}\beta\gamma$	$0.31\pm0.05^{cdef}\alpha$	$0.071\pm0.015^{abcdefgh}\alpha\beta\gamma$	$0.90\pm0.04^{efghij}\alpha$	$3.50\pm0.20^{defghij}\beta\gamma\delta\varepsilon$	$539.23\pm6.28^{g}\alpha$	$8.17\pm0.89^{cdefgh}\alpha\beta$	$12.76\pm0.73^{cde}\alpha$	$282.76\pm7.60^{g}\gamma$
		10-20	$0.35\pm0.06^{cdefgh}\beta\gamma$	$0.34\pm0.06^{bcdef}\alpha$	$0.055\pm0.009^{fghijk}\delta\varepsilon$	$0.84\pm0.15^{efghijk}\alpha$	$3.54\pm0.50^{defghij}\beta\gamma\delta\varepsilon$	$523.60\pm3.81^{h}\beta$	$9.18\pm0.97^{bcd}\alpha$	$13.03\pm0.41^{bcde}\alpha$	$323.32\pm3.18^{f}\beta$
		20-30	$0.44\pm0.13^{abcdefgh}\alpha\beta$	$0.34\pm0.08^{bcdef}\alpha$	$0.066\pm0.011^{abcdefghij}\beta\gamma\delta$	$0.91\pm0.04^{efghi}\alpha$	$3.87\pm1.12^{cdefg}\beta\gamma$	$531.47\pm4.91^{g}\alpha$	$9.18\pm1.03^{bcd}\alpha$	$13.72\pm0.28^{abc}\alpha$	$335.80\pm6.25^{e}\alpha$
		30-40	$0.41\pm0.04^{abcdefgh}\alpha\beta\gamma$	$0.35\pm0.06^{bcdef}\alpha$	$0.060\pm0.011^{bcdefghij}\gamma\delta\varepsilon$	$0.39\pm0.07^{mno}\gamma$	$3.26\pm0.82^{fghijk}\gamma\delta\varepsilon$	$468.19\pm5.31^{j}\gamma$	$8.33\pm0.77^{cdefg}\alpha\beta$	$12.77\pm1.61^{cde}\alpha$	$214.87\pm3.39^{k}\delta$
		40-50	$0.29\pm0.06^{fgh}\beta\gamma$	$0.24\pm0.11^{f}\alpha$	$0.044\pm0.011^{jk}\delta\varepsilon$	$0.33\pm0.05^{no}\gamma$	$3.23\pm0.30^{fghijk}\gamma\delta\varepsilon$	$339.88\pm8.84^{op}\eta$	$7.87\pm0.71^{cdefghij}\alpha\beta\gamma$	$12.26\pm0.55^{cdefg}\alpha$	$175.63\pm2.20^{m}\zeta$
		50-60	$0.54\pm0.07^{a}\alpha$	$0.39\pm0.03^{bcdef}\alpha$	$0.082\pm0.004^{abcd}\alpha\beta$	$0.39\pm0.03^{mno}\gamma$	$3.08\pm0.51^{fghijk}\gamma\delta\varepsilon$	$355.79\pm3.23^{m}\varepsilon$	$6.57\pm0.80^{fghijkl}\beta\gamma\delta\varepsilon$	$9.42\pm0.49^{ghij}\beta$	$328.44\pm3.42^{e}\alpha\beta$
		60-70	$0.53\pm0.09^{a}\alpha$	$0.37\pm0.16^{bcdef}\alpha$	$0.088\pm0.003^{a}\alpha$	$0.43\pm0.04^{lmno}\gamma$	$4.58\pm1.15^{abcd}\alpha\beta$	$403.23\pm2.51^{k}\delta$	$6.90\pm0.70^{efghijklm}\gamma\delta$	$8.80\pm1.43^{hijkl}\beta\gamma$	$284.60\pm0.74^{g}\gamma$
		70-80	$0.43\pm0.08^{abcdefgh}\alpha\beta\gamma$	$0.35\pm0.07^{bcdef}\alpha$	$0.077\pm0.014^{abcde}\alpha\beta\gamma$	$0.42\pm0.06^{mno}\gamma$	$2.40\pm0.14^{jk}\varepsilon$	$328.31\pm8.80^{r}\theta$	$5.10\pm0.48^{mnop}\delta\varepsilon$	6.21 ± 1.44^{mn}	$327.54\pm5.62^{e}\alpha\beta$
		80-90	$0.44\pm0.08^{abcdefgh}\alpha\beta$	$0.30\pm0.05^{cdef}\alpha$	$0.082\pm0.003^{abcd}\alpha\beta$	$0.59\pm0.13^{klmnop}\beta$	$3.50\pm0.38^{defghij}\beta\gamma\delta\varepsilon$	$256.77\pm1.20^{s}\iota$	$4.77\pm1.34^{nop}\varepsilon$	$8.42\pm1.12^{ijkl}\beta\gamma$	$288.09\pm5.61^{g}\gamma$
		90-100	$0.29\pm0.06^{fgh}\beta\gamma$	$0.35\pm0.07^{bcdef}\alpha$	0.074 ± 0.010^{abcdef}	$0.66\pm0.11^{hijklmno}\beta$	$2.67\pm0.46^{ghijk}\delta\varepsilon$	$191.16\pm7.21^{t}\kappa$	$5.81\pm1.54^{jklmn}\delta\varepsilon$	$7.17\pm1.46^{klmn}\gamma\delta$	$329.27\pm8.54^{f}\alpha\beta$
		100-110	$0.35\pm0.09^{cdefgh}\beta\gamma$	$0.34\pm0.08^{bcdef}\alpha$	$0.078\pm0.009^{abcde}\alpha\beta\gamma$	$0.33\pm0.07^{no}\gamma$	$5.01\pm0.12^{ab}\alpha$	$260.35\pm6.97^{s}\iota$	$5.34\pm1.05^{lmno}\delta\varepsilon$	$5.88\pm0.24^{n}\delta$	$179.53\pm4.81^{l}\zeta\varepsilon$
		110-120	$0.28\pm0.07^{fgh}\gamma$	$0.30\pm0.05^{cdef}\alpha$	$0.084\pm0.003^{abc}\alpha\beta$	$0.64\pm0.08^{ijklmno}\beta$	$3.83\pm0.50^{cdefgh}\beta\gamma\delta$	$169.76\pm7.20^{u}\lambda$	$6.11\pm1.50^{hijklmn}\gamma\delta\varepsilon$	$7.04\pm0.41^{lmn}\gamma\delta$	$185.40\pm0.82^{l}\varepsilon$
Average			**0.39±0.08**	**0.37±0.09**	**0.070±0.010**	**0.84±0.11**	**3.52±0.48**	**415.40±5.70**	**7.01±0.92**	**10.38±0.95**	**302.72±4.49**

Minimum Values		0.26±0.04	0.22±0.05	0.043±0.012	0.31±0.06	2.11±0.06	114.83±3.02	3.28±0.26	5.88±0.24	128.70±3.50
Maximum Values		0.53±0.11	0.63±0.08	0.088±0.003	2.13±0.10	5.42±0.58	728.45±7.39	11.49±2.11	15.13±0.98	527.66±3.77
F		2.798	1.998	4.939	27.335	4.920	2156.599	9.406	16.893	1148.528
Sig.		***	**	**	***	***	***	***	***	***
Area	F	1.041	18.053	1.329	197.934	16.222	5329.938	12.818	8.454	3323.772
	Sig.	*in*	***	*in*	***	***	***	***	***	***
Depth	F	4.939	1.038	10.783	9.250	6.352	5553.940	24.010	46.988	1438.326
	Sig.	***	*in*	***	***	***	***	***	***	***
Area xDepth	F	1.887	1.019	1.565	20.868	3.177	169.444	1.794	2.613	805.879
	Sig.	*in*	*in*	*in*	***	***	***	*	***	***
Romić *et al.*, 2004		2.20					368.00	52.70	67.60	
Alagić *et al.*, 2014		3.14±0.03			10.70±0.01		293.00	16.67±0.09		
Bravo *et al.*, 2017							10.87±5.10			
Mihali *et al.*, 2013		26.00				3.60	165.30	9.00	27.80	
Mirlean *et al.*, 2007		0.40					211.40		54.10	

Average value ± standard deviation (n = 3). Different letters are significantly different for $p \leq 0.05$ between depths. The difference between any two values, followed by at least one common letter, is insignificant. Order of the Ministry of Waters, Forests and Environmental Protection No.756/3 November 1997, approving the regulation on the assessment of environmental pollution, Bucharest, Romania; 1997. M.L.A.* (maximum limit allowed) = Normal Values. in = insignificant.

Table 5. The $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$, $^{206}Pb/^{204}Pb$, $^{87}Sr/^{86}Sr$, isotope ratios and Pb, Sr concentration from soil samples on Bujoru, Smulti and Oancea Wine Centre

Wine Centre	Death (cm)	$^{206}Pb/^{207}Pb$	SD	RSD (%)	$^{208}Pb/^{206}Pb$	SD	RSD (%)	$^{206}Pb/^{204}Pb$	SD	RSD (%)	$^{87}Sr/^{86}Sr$	SD	RSD (%)	Pb (mg/kg) **M.A.L.* 20 mg/kg**	Sr (mg/kg) **M.A.L.** -**
Bujoru Wine Centre	0-10	1.14192$^{c}\alpha$	0.01229	1.07627	2.12959$^{bc}\beta\gamma$	0.00363	0.17050	17.36218$^{ef}\alpha\beta$	0.00492	0.02836	0.72337$^{b}\alpha$	0.00202	0.27942	7.99±0.74$^{a}\alpha$	43.06±0.86$^{ef}\alpha$
	10-20	1.13330$^{d}\beta$	0.00160	0.14131	2.12392$^{cde}\gamma$	0.00106	0.04988	17.43002$^{cdef}\alpha$	0.09840	0.56454	0.72520$^{b}\alpha$	0.00281	0.38783	5.95±1.73$^{abcdefghijk}\beta\gamma\delta$	41.55±0.62$^{efgh}\alpha\beta$
	20-30	1.13231$^{d}\beta$	0.00021	0.01887	2.13420$^{a}\alpha\beta\gamma$	0.00153	0.07163	17.37798$^{def}\alpha$	0.03421	0.19687	0.72492$^{b}\alpha$	0.00148	0.20351	5.46±0.50$^{bcdefghijkl}\gamma\delta\varepsilon$	37.53±1.04$^{hij}\gamma\delta$
	30-40	1.13340$^{d}\beta$	0.00151	0.13296	2.11065$^{fgh}\delta$	0.00437	0.20682	17.41452$^{cdef}\alpha$	0.06500	0.37327	0.72354$^{b}\alpha$	0.00296	0.40911	6.37±0.78$^{abcdefghi}\alpha\beta\gamma\delta$	41.05±0.73$^{efghi}\alpha\beta\gamma$
	40-50	1.13219$^{d}\beta$	0.00059	0.05232	2.12856$^{bc}\beta\gamma$	0.00552	0.25922	17.37402$^{def}\alpha\beta$	0.09096	0.52352	0.72283$^{b}\alpha$	0.00125	0.17308	4.80±0.40$^{fghijklm}\delta\varepsilon$	39.33±0.59$^{fghij}\beta\gamma\delta$
	50-60	1.13286$^{d}\beta$	0.00056	0.04951	2.12677$^{bcd}\gamma$	0.00765	0.35949	17.43419$^{cde}\alpha$	0.02122	0.12169	0.72464$^{b}\alpha$	0.00324	0.44777	6.62±0.39$^{abcdefgh}\alpha\beta\gamma$	41.41±2.49$^{efgh}\alpha\beta$
	60-70	1.13150$^{d}\beta$	0.00058	0.05150	2.12464$^{cde}\gamma$	0.00126	0.05911	17.26270$^{g}\beta$	0.05028	0.29128	0.72368$^{b}\alpha$	0.00096	0.13208	7.49±0.57$^{ab}\alpha\beta$	38.72±0.80$^{fghij}\beta\gamma\delta$
	70-80	1.13253$^{d}\beta$	0.00003	0.00255	2.13819$^{ab}\alpha\beta$	0.00653	0.30538	17.37004$^{def}\alpha\beta$	0.09428	0.52276	0.72308$^{b}\alpha$	0.00266	0.36729	6.86±1.13$^{abcdef}\alpha\beta\gamma$	36.53±1.42$^{ij}\delta$
	80-90	1.12992$^{d}\beta$	0.00414	0.36656	2.14084$^{a}\alpha$	0.01274	0.59503	17.39045$^{cdef}\alpha$	0.07419	0.42661	0.72477$^{b}\alpha$	0.00193	0.26678	4.75±1.49$^{ghijklm}\delta\varepsilon$	37.37±1.20h$^{ij}\delta$
	90-100	1.13339$^{d}\beta$	0.00152	0.13397	2.12487$^{cde}\gamma$	0.00203	0.09575	17.43764$^{cd}\alpha$	0.01542	0.08841	0.72477$^{b}\alpha$	0.00379	0.52298	3.84±0.66$^{lmn}\varepsilon$	37.88±2.40$^{ghij}\gamma\delta$
	100-110	1.13206$^{d}\beta$	0.00050	0.04442	2.12474$^{cde}\gamma$	0.00013	0.00595	17.35786$^{f}\alpha\beta$	0.04945	0.28488	0.72443$^{b}\alpha$	0.00215	0.29650	5.31±0.15$^{cdefghijk}\gamma\delta\varepsilon$	30.83±1.63$^{k}\varepsilon$
	110-120	1.13337$^{d}\beta$	0.00182	0.16023	2.12311$^{cde}\gamma$	0.00802	0.37778	17.45429$^{c}\alpha$	0.00267	0.01531	0.72375$^{b}\alpha$	0.00072	0.10003	6.95±0.96$^{abcde}\alpha\beta\gamma$	24.41±4.61$^{l}\zeta$
Smulti Wine Centre	0-10	1.18523$^{a}\alpha$	0.00202	0.17044	2.10286$^{hijklm}\beta$	0.00221	0.10493	17.21501$^{g}\alpha$	0.00055	0.00322	0.73086$^{a}\alpha\beta$	0.00138	0.18839	5.72±0.57$^{bcdefghijkl}\alpha\beta\gamma\delta$	55.15±6.07$^{bc}\alpha$
	10-20	1.17926$^{ab}\alpha\beta$	0.00720	0.61067	2.10393$^{hijkl}\beta$	0.00097	0.04610	17.21722$^{g}\alpha$	0.01311	0.01311	0.72987$^{a}\beta$	0.00045	0.06103	6.25±1.53$^{abcdefghij}\alpha\beta\gamma$	47.56±5.03$^{d}\beta\gamma$
	20-30	1.18079$^{a}\alpha\beta$	0.00767	0.64990	2.10402$^{hijkl}\beta$	0.00179	0.08507	17.21864$^{g}\alpha$	0.00361	0.02096	0.73072$^{a}\alpha\beta$	0.00123	0.16797	6.23±0.81$^{abcdefghij}\alpha\beta\gamma$	41.85±0.70 $^{efgh}\delta\varepsilon\zeta$
	30-40	1.18408$^{a}\alpha$	0.00154	0.13010	2.10384$^{hijkl}\beta$	0.00131	0.06231	17.22465$^{g}\alpha$	0.00165	0.00964	0.73239$^{a}\alpha\beta$	0.00043	0.05893	6.14±0.95$^{abcdefghijk}\alpha\beta\gamma\delta$	41.38±1.21 $^{efgh}\delta\varepsilon\zeta$
	40-50	1.18286$^{a}\alpha$	0.00056	0.04741	2.10880$^{gh}\alpha\beta$	0.01227	0.58163	17.21939$^{g}\alpha$	0.00692	0.04018	0.73294$^{a}\alpha\beta$	0.00346	0.47149	6.69±0.52$^{abcdefg}\alpha\beta$	38.73±0.89$^{fghij}\delta\varepsilon\zeta$
	50-60	1.18289$^{a}\alpha$	0.00054	0.04540	2.10343$^{hijklm}\beta$	0.00205	0.09744	17.21927$^{g}\alpha$	0.00666	0.03867	0.73431$^{a}\alpha$	0.00205	0.27895	6.33±0.78$^{abcdefghij}\alpha\beta\gamma$	42.93±0.3$^{ef}\gamma\delta\varepsilon$
	60-70	1.18201$^{a}\alpha$	0.00975	0.82518	2.11805$^{def}\alpha$	0.00447	0.21095	17.21582$^{g}\alpha$	0.00462	0.02681	0.73232$^{a}\alpha\beta$	0.00290	0.39589	7.20±0.95$^{abcd}\alpha$	43.99±4.07$^{de}\gamma\delta$
	70-80	1.18249$^{a}\alpha$	0.00087	0.00646	2.10417$^{hijkl}\beta$	0.00153	0.07265	17.21726$^{g}\alpha$	0.00286	0.01662	0.73339$^{a}\alpha\beta$	0.00209	0.28544	4.14±0.41$^{klmn}\delta$	36.54±1.40$^{ij}\zeta$
	80-90	1.17324$^{b}\beta$	0.00168	0.14324	2.10403$^{hijkl}\beta$	0.00148	0.07035	17.22055$^{g}\alpha$	0.00140	0.00812	0.73194$^{a}\alpha\beta$	0.00163	0.22208	4.29±0.92$^{jklmn}\gamma\delta$	39.06±0.60$^{fghij}\delta\varepsilon\zeta$
	90-100	1.17329$^{b}\beta$	0.00166	0.14128	2.10486$^{hij}\beta$	0.00201	0.09547	17.21868$^{g}\alpha$	0.00475	0.02758	0.73441$^{a}\alpha$	0.00393	0.53458	5.99±1.72$^{abcdefghijk}\alpha\beta\gamma\delta$	52.13±1.68 $^{c}\alpha\beta$
	100-110	1.18208$^{a}\alpha$	0.00052	0.04387	2.10457$^{hijk}\beta$	0.00023	0.01097	17.21577$^{g}\alpha$	0.00558	0.03240	0.73303$^{a}\alpha\beta$	0.00214	0.29212	4.65±1.39$^{ghijklm}\beta\gamma\delta$	38.39±3.30$^{ghij}\varepsilon\zeta$
	110-120	1.18349$^{a}\alpha$	0.00171	0.14416	2.11661$^{efg}\alpha$	0.01243	0.58737	17.21204$^{g}\alpha$	0.01086	0.06309	0.73184$^{a}\alpha\beta$	0.00150	0.20449	4.97±1.35$^{efghijklm}\beta\gamma\delta$	36.86±1.57$^{ij}\zeta$
Oancea Wine Centre	0-10	1.14398$^{c}\alpha\beta$	0.00145	0.12718	2.09668$^{ijklm}\beta$	0.00178	0.08496	17.63515$^{ab}\beta$	0.02839	0.16100	0.70367$^{e}\beta\gamma$	0.00162	0.23022	7.36±0.92$^{abc}\alpha$	63.65±2.24$^{a}\alpha$
	10-20	1.14254$^{c}\alpha\beta$	0.00195	0.17102	2.09858$^{ijklm}\beta$	0.00015	0.00707	17.63824$^{ab}\beta$	0.04894	0.27744	0.70414$^{e}\beta\gamma$	0.00404	0.57328	7.23±1.00$^{abcd}\alpha\beta$	53.23±1.95$^{c}\gamma$
	20-30	1.14131$^{c}\alpha\beta$	0.00174	0.15269	2.09517$^{klm}\beta$	0.00058	0.02750	17.69665$^{a}\alpha$	0.00194	0.01094	0.70344$^{e}\gamma$	0.00125	0.17810	6.68±1.34$^{abcdefg}\alpha\beta$	41.69±2.29 $^{efgh}\varepsilon\zeta$
	30-40	1.14260$^{c}\alpha\beta$	0.00263	0.23025	2.09469$^{lm}\beta$	0.00137	0.06542	17.61892$^{b}\beta$	0.01862	0.10568	0.70368$^{e}\beta\gamma$	0.00275	0.39065	5.49±1.52$^{bcdefghijkl}\alpha\beta\gamma$	57.29±0.81$^{b}\beta$
	40-50	1.14252$^{c}\alpha\beta$	0.00187	0.16378	2.09397$^{m}\beta$	0.00391	0.18677	17.64688$^{ab}\alpha\beta$	0.03115	0.17654	0.70550$^{de}\beta\gamma$	0.00344	0.48811	6.12±1.37$^{abcdefghijk}\alpha\beta\gamma$	42.35±1.22 $^{efg}\varepsilon\zeta$
	50-60	1.14436$^{c}\alpha\beta$	0.00272	0.23767	2.09587$^{jklm}\beta$	0.00280	0.13345	17.62895$^{ab}\beta$	0.02809	0.29128	0.70482$^{e}\beta\gamma$	0.00292	0.41449	4.59±0.45$^{hijklm}\gamma\delta$	47.26±1.01$^{d}\delta$
	60-70	1.14446$^{c}\alpha\beta$	0.00384	0.33587	2.09461$^{lm}\beta$	0.00123	0.05849	17.61758$^{b}\beta$	0.09428	0.54276	0.70317$^{e}\gamma$	0.00008	0.01086	4.46±0.76$^{ijklm}\gamma\delta$	39.33±2.75$^{fghij}\eta\theta$
	70-80	1.14380$^{c}\alpha\beta$	0.00072	0.06260	2.09699$^{ijklm}\beta$	0.00123	0.05849	17.63452$^{ab}\beta$	0.04478	0.25392	0.70946$^{cd}\alpha\beta$	0.00535	0.75369	6.21±2.00$^{abcdefghij}\alpha\beta\gamma$	36.54±1.40$^{ij}\theta$
	80-90	1.14428$^{c}\alpha\beta$	0.00191	0.16651	2.09477$^{lm}\beta$	0.00171	0.08148	17.60935$^{b}\beta$	0.00650	0.03690	0.71128$^{c}\alpha$	0.00532	0.74825	5.17±0.67$^{defghijkl}\beta\gamma$	39.72±1.30$^{efghij}\zeta\eta\theta$
	90-100	1.14543$^{c}\alpha$	0.00096	0.08418	2.09597$^{jklm}\beta$	0.00311	0.14821	17.64742$^{ab}\alpha\beta$	0.03223	0.18263	0.70178$^{e}\gamma$	0.00073	0.10368	3.16±1.06$^{mn}\delta\varepsilon$	32.33±2.77$^{k}\iota$
	100-110	1.14077$^{c}\beta$	0.00089	0.07823	2.09466$^{lm}\beta$	0.00013	0.00619	17.62729$^{ab}\beta$	0.02328	0.13206	0.70462$^{e}\beta\gamma$	0.00219	0.31097	2.36±0.46$^{n}\varepsilon$	43.17±1.92 $^{ef}\varepsilon$
	110-120	1.14206$^{c}\alpha\beta$	0.00296	0.25881	2.10644$^{hi}\alpha$	0.00726	0.34451	17.64762$^{ab}\alpha\beta$	0.01021	0.05786	0.70406$^{e}\beta\gamma$	0.00124	0.17674	2.98±0.08$^{mn}\delta\varepsilon$	53.20±1.97$^{c}\gamma$
Average		**1.15245**	**0.00233**	**0.45222**	**2.11041**	**0.00352**	**0.16629**	**17.41469**	**0.02637**	**0.15120**	**0.72821**	**0.00205**	**0.28116**	**5.63±0.92**	**42.05±1.86**
Minimum Values		1.12992	0.00003	0.00255	2.09397	0.00013	0.00595	17.21204	0.00055	0.00322	0.70178	0.01086	0.01086	2.36±0.46	24.41±4.61
Maximum Values		1.18523	0.01229	1.07627	2.14084	0.01274	0.59503	17.69665	0.09840	0.56454	0.73441	0.00535	0.75369	7.99±0.74	63.65±2.24
F		103.889			24.998			64.131			63.254			5.092	34.070

Wine Centre	Death (cm)	$^{206}Pb/^{207}Pb$	SD	RSD (%)	$^{208}Pb/^{206}Pb$	SD	RSD (%)	$^{206}Pb/^{204}Pb$	SD	RSD (%)	$^{87}Sr/^{86}Sr$	SD	RSD (%)	Pb (mg/kg) **M.A.L.* 20 mg/kg**	Sr (mg/kg) **M.A.L.** -
Sig.		***			***			***			***			***	***
Area	F	1785.238			367.995			1086.697			1081.963			6.754	124.283
	Sig.	***			***			***			***			**	***
Depth	F	2.555			4.468			2.472			1.437			6.918	44.507
	Sig.	**			***			*			*in*			***	***
Area xDepth	F	1.707			4.081			1.999			1.552			4.029	20.649
	Sig.	*			***			*			*in*			***	***
Rodríguez-Seijo *et al.*, 2014		1.17190	0.00004		2.09943	0.00013		18.31684	0.00160					9.00	
Tyszka *et al.*, 2012		1.17300	0.01400		2.09500	0.01700								36.00	
Ayuso *et al.*, 2016		1.19550						18.67900			0.72238			11.00	1.74
Kuang *et al.*, 2013		1.19500						18.61100							
Sherman *et al.*, 2015		0.84380			2.06200										

Average value ± standard deviation (n = 3). Romans letters represent the significance of the variety difference ($p \leq 0.05$). The difference between any two values, followed by at least one common letter, is = insignificant.***M.A.L.** for **Pb** (**Normal Values (20 mg/kg)**; **Alert threshold** (**Susceptible (50 mg/kg)** and **Less Susceptible (250 mg/kg)**); **Intervention threshold** (**Susceptible (150 mg/kg)** and **Less Susceptible (500 mg/kg)**). ****M.A.L.**for Sr = -.

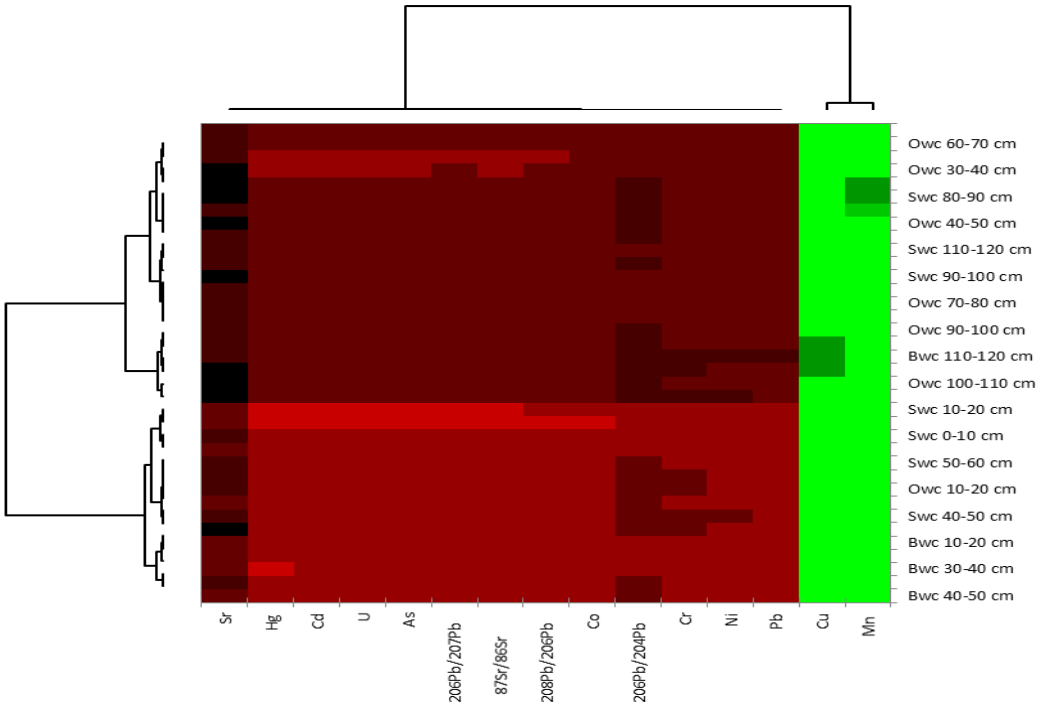

Figure 1. Heat map obtained by cluster analysis of the copper content in grape must

4. Conclusions

In this work the heavy metals concentration from vineyard soil in Bujoru, Smulti and Oancea wine-growing centers from Dealu Bujorului vineyard and to assess their ability to discriminate between geographical origins of wines. In case of Cd (1 mg/kg), Pb (20 mg/kg), Hg (0.1 mg/kg), As (5 mg/L), Co (15 mg/kg), Ni (20 mg/kg) and Cr (30 mg/kg) metals in analysed in soil samples were under Maximum Permissible Limits (MPL). Cu concentration in the soil exceeds the maximum admissible limit (20 mg/kg) having the average value of 415.40 mg/kg, this value is a common one for vineyard soils.

Regardless of Pb sources (lithogenic or anthropogenic), the average $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$ and $^{206}Pb/^{204}Pb$ ration in soil profile horizons followed the order: 1.15170 (10-20 cm $^{206}Pb/^{207}Pb$) > 1.15704 (0-10 cm $^{206}Pb/^{207}Pb$); 2.10971 (10-20 cm $^{208}Pb/^{206}Pb$) > 2.11070 (10-20 cm $^{208}Pb/^{206}Pb$) and 17. 40411 (10-20 cm $^{206}Pb/^{204}Pb$) > 17.42849 (0-10 cm $^{206}Pb/^{204}Pb$). Statistically, in the case of $^{206}Pb/^{207}Pb$ and $^{208}Pb/^{206}Pb$ there are very significant differences between the analyzed variants while $^{206}Pb/^{204}Pb$ does not show any differences with in terms of distribution on the depth of the soil profile.

Our results confirm that the $^{207}Pb/^{206}Pb$, $^{208}Pb/^{206}Pb$, $^{204}Pb/^{206}Pb$ and $^{87}Sr/^{86}Sr$ isotope ratio can be used to track the geographical origin of wine, discriminate between wine production regions, and be used to characterize wine terroirs for forensic purpose.

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CAROB SYRUP AND CAROB FLOUR (*CERATONIA SILIQUA* L.) AS FUNCTIONAL INGREDIENTS IN SPONGE CAKES

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ABSTRACT
The aim of this study was to evaluate the physicochemical and sensory characteristics of sponge cakes enriched with carob flour and carob syrup as functional ingredients and partial substitutes for wheat flour and sugar. Five formulations were prepared: a control cake, sponge cake with 25% carob flour, sponge cake with 50% carob flour, sponge cake with 25% carob syrup and sponge cake with 50% carob syrup. The replacement of wheat flour with carob flour resulted in a higher level of dietary fiber (2.45→ 18.28 g/100 g dry weight), protein (8.44 → 23.93 g/100 g dry weight) and carbohydrate content (65.40±5.20 → 86.10±2.70 g/100 g dry weight). The substitution of sugar with carob syrup increased the level of protein content (8.44 → 12.57g/100 g dry weight). Sensory evaluation of shape, color, cell size and uniformity, odor, sweetness, aftertaste, crumb tenderness was also performed.

1. Introduction

Consumer choices in the developed world are primarily dependent on socio-economic and cultural circumstances. Those consumers who have a consistently high intake of plant-derived foods have a much reduced incidence of cancer and possiblyother social and environmentally related diseases (Cencic and Chingwaru, 2010).

Functional foods are products that positively affect specific physiological functions in the body. The consumption of functional food results inimproved health, well-being, or performance beyond regular nutrition.

The beneficial effects of dietary fiber have been well-established by research data. Dietary fiber affects regulation of gastrointestinal motility, influences glucose and lipid metabolism, promotes fecal ouput, stimulates bacterial metabolic activity, detoxifies luminal contents of the colon and supports equilibrium of the colon ecosystem and integrity of the intestinal mucosa (Castillejo et al., 2006; Murakami et al., 2007; Santos et al., 2015). Dietary fiber thus qualifies as a functional food because it can affect one or more targeted functions in the body in a positive manner (Diplock et al., 1999).

Cakes are a popular dessert food for many consumers. However, most cakes have low fiber content and relatively high amounts of sucrose. Therefore, increasing the fiber content and reducing the sucrose in cakes would enhance this food's nutritional quality.

Carob pods have generally been used as animal and human food. Nowadays, the

seeds are used primarily for gum extraction. The pods are used after crushing and separation of the seeds from the pulp. The main products derived from carob pods are carob flour and syrup. Carob flour or powder could be added to cakes, bread, sweets, ice cream or beverages as a flavoring agent. Carob powder is already generally accepted as a substitute for cocoa, but it has advantages over cocoa because of lower calories and the absence of caffeine and theobromine (Hoda et al., 2006). The flavor of carob powder is not as intense as dark chocolate. In some countries such as Egypt, Turkey and Bulgaria, carob syrup is a popular drink obtained by extracting carob kibbles with water.

Carob bean seeds contain a protein called caroubin that exhibits rheological properties that are similar to those of wheat gluten (El Batal et al., 2012). This makes carob flour a promising alternative to gluten for people with celiac desease. Carob germ flour includes gallotannins, polyphenols, and proanthocyanidins which are known for their ability to inhibit reactive oxygen species and free radicals (Custódio et al., 2005).

The objective of this study was thus to evaluate the potential of carob syrup and carob flour for use in the production of sponge cakes and to determine the most appropriate level of these two foods as functional ingredients.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of sponge cakes

The standard raw materials such as Type 500 wheat flour with 0.5% an ash content (GoodMills, Bulgaria EAD), granulated sugar (Zaharni zavodi AD) and eggs (local market) used in the current study, were authorized by the Ministry of Health as manufactured in Bulgaria. The functional ingredients, syrup and carob fruit flour (*Ceratonia siliqua* L.) from Turkey (Mersin province), were produced at the University of Food Technologies – Plovdiv, as previously described (Fidan et al., 2016). The sponge cake batter formulation used as the control was prepared according to traditional technology and formulation (Angelov et al., 1974). In particular, a double mixing procedure was applied by partitioning the whipping of egg whites and egg yolks. The carob syrup was added to the sponge cake as a sugar replacement at 25% and 50% levels. The sponge cakes with carob flour were prepared by replacing wheat flour with carob flour at 25% and 50 % levels.

Each sponge cake was comprised of 95 g of batter poured into metallic forms and baked in an electric oven (Rahovetz - 02, Bulgaria) at 180°C for 30 min. The sponge cakes were stored at standard conditions (at a temperature of 18°C and 75 % relative humidity) up to the sixth day following the production date according to standard requirements (BSS, 1982). The humidity and temperature were kept constant by means of a desiccator supplied with a psychrometer and placed in a thermostat with an accuracy of ± 0.5°C.

2.2. Methods

2.2.1. Physico-chemical characteristics of the sponge batters and cakes

The specific gravity of the sponge cake batter was calculated by dividing the weight of a standard batter cup to the weight of an equal volume of distilled water at batter temperature (20.0 ± 0.5^{o}C). The physical characteristics of the sponge cakes were determined 2h after baking. Volume was measured by the small uniform seed displacement method (AACC, 1999), and porosity was assessed according to the Bulgarian State Standard method (BSS, 1992). The porosity of the sponge cake was defined as the ratio of the volume of air-

pockets in the cake crumb to the volume of the crumb. Porosity determination was made using a cylinder driller, a device of Zhuravljov. The specific volume was expressed as the ratio of the sponge cake volume to its mass. The water-absorbing capacity of the sponge cake was measured by the extent of biscuit swelling according to the Bulgarian State Standard method (BSS, 1981). Shrinkage and Springiness were determined with an automatic penetrometer (model DSD VEB Feinmess, Dresden, Germany). A hemispherical body with a diameter 12.5 mm and total weight 300 g acted on the sectional surface of a 40 mm thick sponge cake sample, detecting shrinkage levels at 5 and 10 s. Relaxation was checked by means of a hemispherical body with a diameter 25 mm and total weight of 50 g acting upon a 40 mm thick cake crumb for 5 s. This procedure was used to determine crumb springiness (Vangelov and Karadjov, 1993). Total sample moisture was determined after drying the sample at 105^{o}C up to a constant weight according to the standard method (AACC, 2000).

Nitrogen level was determined by the Kjeldahl method (ISO 20483: 2014). A multiplication factor of 6.25 was used for the calculation of protein content.

The total insoluble and soluble dietary fiber levels were determined by the enzymatic gravimetric method (AOAC 985.28, 1990), using the total dietary fiber assay kit Bioquant 1.12979.0001 (Merck, Germany) with instructions provided by the manufacturer.

The total soluble carbohydrate content was estimated according to the spectrophotometric method of Dubois et al. (1956). In brief, 0.1 ml of each extract was mixed with 1 ml of 5% phenol and 5 ml of sulphuric acid. The samples were then placed in a water bath at 30 °C for 20 minutes. The absorbance was measured at 490 nm against a blank that was prepared using the same process as that used for distilled H_2O. The reducing groups were determinated by the PAHBAH method at 410 nm (Lever, 1972). The sample preparation was performed as previously described (Petkova et al., 2014). Chromatographic determination of sucrose, glucose and fructose was performed on a HPLC Elite Chrome Hitachi instrument coupled with a refractive index detector (RID) Chromaster 5450. The separation of sugars was performed on a Shodex® Sugar SP0810 (300 mm × 8.0 mm i.d.) with $Pb^{2+}$and a Shodex SP - G (5 µm, 6 × 50 mm) guard column at 85 °C in mobile phase distilled H_2O with a flow rate of 1.0 ml/minand aninjection volume of 20 µl.

2.2.2. Sensorial evaluation

The descriptive test for a quantitative sensory profiling was used to establish the sensory characteristics (shape, color, cell size and uniformity, odor, sweetness, aftertaste, crumb tenderness) of the sponge cakes 6 h after baking following the ISO 8586:2014 and ISO 13299:2011 methods. The sponge cakes samples were ready 1 h before the evaluation. Samples of different cakes were kept in coded plates covered with aluminium foil. Twelve trained panelists were selected in order to guarantee evaluation accuracy. The intensity of each sensory characteristic was recorded on a ten-point linear scale after 1 h orientation sessions for the panelists after whom terminology and anchor points on the scale were noted. The coded samples were shown simultaneously and evaluated in random order.

2.2.3.Microbiological analyses

The microbiological analyses were carried out according to the Bulgarian State Standard (BSS, 1986). Analyses for total plate count (TPC) (ISO 4833-1: 2013), molds and yeasts (ISO 21527-2: 2011), fecal

coliforms (ISO 4831: 2006), *Salmonella* species (ISO 6579:2003) and coagulase-positive staphylococci (ISO 6888-1: 2000) were also conducted. Total plate count (TPC) in 1 g of sponge sample; total number of molds and yeasts in 1 g of sponge sample; coliforms in 1 g of sponge sample; *Salmonella* spp. in 25 g of sponge sample and coagulase-positive staphylococci in 1 g of product were determined.

2.2.4.Data analysis

Depending on the type of studied characteristic, 3 to 12 repetitions of each measurement were performed. The data were analyzed and presented as mean values ± standard deviation.

3. Results and discussions

3.1. Physical characteristics of sponge cakes

Changes in the physical characteristics of batters and sponge cakes containing carob syrup and carob flour in different amounts (25% and 50%) are summerized in Table 1.

The specific gravity of cake batter affects volume, porosity, water-absorbing capacity of the sponge cake and is important for the formation of crumb texture of the cake. The sponge batter, obtained by replacing wheat flour with carob flour, had a lower specific gravity than that of the control batter. Lower specific gravity is an indicator of more aeration, which is a desired property of cake batter. No significant differences were found in the specific gravity of the batter values between the control batter (0.71±0.01) and 25% carob syrup sponge cake batter (0.69±0.05), which could encourage the formation of larger bubbles during baking and therefore result in greater product height and volume. Another quality characteristics that is important for consumers is cake volume. The volume of cakes containing carob flour and syrup are shown in Table 1. Specific volume of cakes varied between 3.33±0.45 cm^3/g and 3.49±0.95 cm^3/g. For the control, which batter was characterized with higher value of the specific gravity, a smaller specific volume and porosity were measured. In this study, the specific volume of the control cake (3.29 ± 0.51 cm^3/g) was lower than the specific volume of sponge cake samples that contained carob flour. Berk et al. (2017) investigated the effects of partial replacement of rice flour by carob bean flour at different concentrations (10%, 20%, and 30%) on specific gravity and cake quality (specific volume, hardness). They reported that cakes prepared with 20% carob bean flour had the highest specific volume and lower hardness value and could thus be recommended for usage in gluten free cakes. According to Herranz et al. (2016) all cheackpea flour-based muffins had significantly lower specific volume than the control. The volume of the control cake (263.00±11.68 cm^3) was larger than that of sponge cakes with functional ingredients. The greatest porosity was observed in the cake with 50 % carob flour. The cake with 50 % carob syrup had the smallest volume (165.00±9.95 cm^3). In comparison with the control, a decrease in springiness was found when wheat flour was replaced by carob flour at 25% and 50% levels (25.80±4.92 PU and 21.80±2.39 PU) and by replacement of sugar with carob syrup at 25% and 50% levels (26.79±4.82 PU and 22.78±2.35 PU). Martinez-Cervera et al. (2011) reported that factors such as reological properties of the batter, the extent of air embodiment and continuance and speed of mixing and homogenisation affect the volume of bakery products. Finally, in comparison with the control, the sponge cake with 25% carob syrup had a significantly higher value of total moisture (33.65±0.98 %). It was observed that the total moisture content of the sponge cakes gradually increased with increasing levels of carob flour in the cake.

This might be due to the fact that carob flour enriches the sponge cakes with dietary fiber (see table 4). The fiber tends to hold water which could contribute to the higher total moisture content of the sponge cake.

In their study, Santos et al. (2015) noted that carob flour was a low glycemic index food and low glycemic load food. Carob flour was classified as a high fiber food, containing high levels of insoluble fiber. This high content of dietary fiber makes carob an ingredient with beneficial physiological effects. De La Hera et al. (2013) studied the batter characteristics and quality of cakes made with wheat-oat flour blends and reported that the inclusion of wheat-oat flour reduces cake volume and modifies their texture, reducing hardness, cohesiveness and springiness. Rosa et al. (2015) showed that increasing the substitution of cocoa powder by carob flour resulted in decreased cohesiveness (0.51 to 0.46), elasticity (0.95 to 0.79) and resilience in the cakes (0.26 to 0.24). Różyło et al. (2017) studied the physical and antioxidant properties of gluten-free bread enriched with carob fiber, and their results showed that increased carob fiber content induced significant favorable changes in the volume, color and texture (hardness and springiness) of the bread crumb.

Table 1. Effect of different levels of replacement of sugar and wheat flour with carob syrup and flour on the physical characteristics of the batters and sponge cakes

Physical characteristics[1]	**Sponge cake type**				
	control sample	**with 25 % carob syrup**	**with 50 % carob syrup**	**with 25 % carob flour**	**with 50 % carob flour**
Specific gravity (for batter)[2]	0.71±0.01	0.69±0.05	0.58±0.02	0.64±0.00	0.59±0.01
Specific volume, [cm^3/g]	3.29±0.51	3.33±0.45	3.39±0.34	3.38±0.28	3.49±0.95
Volume, [cm^3]	263.00±11.68	173.33±11.55	165.00±9.95	236.67±15.28	226.67±13.95
Porosity, [%]	66.68±1.54	68.37±3.74	69.25±2.51	67.90±2.14	74.08±3.71
Water-absorbing capacity, [%], [PU] [3]	329.00±18.05	303.19±9.02	336.02±14.05	344.00±11.01	329.00±14.91
Spinginess, [PU]	45.00±1.40	26.79±4.82	22.78±2.35	25.80±4.92	21.80±2.39
Shrinkage, PU	63.80±2.87	68.37±8.29	62.02±4.74	67.60±8.29	62.00±4.74
Total moisture, [%]	23.42±0.44	33.65±0.98	27.62±1.14	27.81±1.13	30.86±0.97

[1] The values are mean ± SD ($p \leq 0.05$).

[2] The temperature of the batter is on the average 20.0 ± 0.5 °C.

[3] PU - Penetrometer Units.

3.2. Carbohydrate content of sponge cakes, supplemented with different concentration of carob syrup and carob flour

Detailed information about the carbohydrate profiles of sponge cakes - carob syrup and flour was presented. The carbohydrate content of cakes containing different levels of carob syrup, flour and the control cake are summarized in Table 2. The total carbohydrate content of different cakes varied from 65.4±5.2g/100 g dry weight to 86.1±2.7 g/100 g dry weight, being lowest for the control cake (65.4±5.2 g/100 g dry weight) and highest for the sponge cake sample with 50% carob flour (86.1±2.7 g/100 g dry weight). It was observed that total carbohydrate content increased in sponge cakes with increased levels of carob flour and carob syrup in the batter.

In all analyzed samples with carob ingredients the presence of monosaccharide glucose and disaccharide sucrose was detected. The content of reducing sugars varied between 1.3 and 5.3 g/100 g dry weight. The sponge cake, obtained by replacing 50% wheat flour with carob powder, was estimated to be a rich source of carbohydrates (86.1 g/100 g dry weight).

Rosa et al. (2015) studied the effect substituting of cocoa powder for carob flour in cakes made with soy and banana flour and found that the obtained cakes had a higher level of dietary fiber (16.42 the 25.89 %), a lower carbohydrate content (20.65 the 8.42 %) and lower calorie content (208.89 the 148.07 kcal).

For more detailed characterization of the carbohydrate profile, sponge cake samples were analyzed by the HPLC-RID method (Figure 1).

The protein and fiber content of four different cake samples processed with different levels of carob syrup and flour are shown in Table 3.

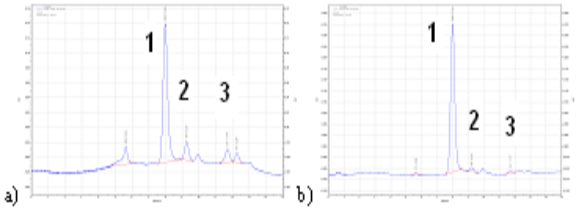

Figure 1. HPL-RID chromatograms of carbohydrates composition in sponge cakes, supplemented with different concentration of carob (*Ceratonia siliqua* L.) syrup and carob flour, showing peaks for sucrose (1), glucose (2), and fructose (3).

a) sponge cake with 50% carob syrup

b) sponge cake with 50% carob flour

Table 2. Means ± standard deviations of carbohydrate content in sponge cakes, supplemented with different concentration of carob syrup and flour (g/100 g dry weight)

Type of sponge cake	Total carbohydrates	Reducing sugars	Glucose	Fructose	Sucrose
control	65.4±5.2	0.7±0.2	0.4±0.1	ND[1]	26.8±0.6
with 25 % carob syrup	67.1±3.5	2.8±0.2	1.4±0.1	0.4±0.1	27.7±0.2
with 50 % carob syrup	74.3±4.1	5.3±0.4	1.3±0.2	2.7±0.2	28.5±0.2
with 25 % carob flour	83.5±2.4	1.3±0.2	0.7±0.1	0.5±0.1	35.4±0.2
with 50 % carob flour	86.1±2.7	4.9±0.7	2.4±0.2	2.1±0.1	43.90±0.2

[1] ND = Not detected

Table 3. Mean values of the dietary fibers and of the protein of the sponge cake samples without and with functional ingredient (per g/100g dry weight)

Type of sponge cake	Dietary fibers [g/100 g dry weight]			Total protein*, [g/100 g dry weight]
	insoluble	soluble	total	
control	2.00±0.10	0.63±0.09	2.63±0.11	8.44
with 25 % carob syrup	1.89±0.15	0.56±0.09	2.45±0.15	10.63
with 50 % carob syrup	1.95±0.14	0.63±0.10	2.58±0.17	12.57
with 25 % carob flour	10.68±0.15	1.74±0.10	12.42±0.21	15.81
with 50 % carob flour	16.22±0.19	2.06±0.10	18.28±0.22	23.93

*Protein = % Nitrogen × 6.25

In the present study it was observed that the protein content of all samples (10.63-23.93 g/100g dry weight) was higher than that of the control sample (8.44 g/100g dry weight). It was also noticed that the protein content in sponge cake samples increased with increased substitution levels of carob syrup and flour. The results for total dietary fiber content revealed that increasing the replacement levels of wheat flour with carob flour from 25% to 50%, respectively, led to a concomitant increase in values for the fiber content in sponge cake samples from 2.63 g/100g dry weight (control) to 12.42 g/100g dry weight (sponge cake with 25% carob flour) and 18.28 g/100g dry weight (sponge cake with 50% carob flour). This could be due to the fact that carob flour contains a higher percentage of fiber (28.17 g/100g dry weight) than wheat flour (0.45 g/100g dry weight) (Fidan, 2017). A similar observation of dietary fiber content increase in sponge cakes enriched with powder from the outer leaves of white cabbage was made by Prokopov et al. (2015).

Table 4. Sensory characteristics of the sponge cake samples without and with functional ingredient

Sensory characteristics[1]	Sponge cake type				
	control sample	with 25 % carob syrup	with 50 % carob syrup	with 25 % carob flour	with 50 % carob flour
Shape	7.93±1.03	7.13±1.41	7.47±1.11	7.47±1.19	7.73±1.33
Colour	8.07±1.10	7.53±1.46	7.60±1.19	7.53±1.51	7.73±1.79
Cells size and uniformity[2]	6.93±1.03	7.40±1.18	7.07±1.24	7.47±1.25	6.87±1.30
Odour	7.20±1.37	7.53±1.13	7.33±1.32	6.67±1.68	6.47±1.64
Sweetness	6.67±2.06	7.00±2.04	6.67±1.12	6.87±1.55	7.33±1.76
Aftertaste	4.07±1.14	2.87±0.70	3.67±1.02	3.93±1.08	4.13±1.07
Crumb tenderness	6.67±1.63	7.53±1.19	6.93±1.16	7.40±1.12	7.80±1.37

1 The values are mean ± SD ($p \leq 0.05$).

2 A scale from 0 to 9 was used to evaluate sensory characteristics. Nine is ideal for the third sensory characteristic when the cells are small and equal in size

3.3. Sensorial evaluation of sponge cakes, supplemented with different concentrations of carob flour and carob syrup

Sensory analysis evaluations were performed in order to determine the optimum sensory characteristics of sponge cakes with regard to the panelists' preferences. The results of the sensory evaluation are given in Table 4.

Ratings test revealed that sensory characteristics including shape, color, cell size and uniformity, odor, sweetness, aftertaste and crumb tenderness were perceived without significant differences between the control cake and those supplemented with different concentrations of carob flour and carob syrup. Our investigation showed that the control sample and the cake with 50% carob flour both had approximately similar shape and color. In terms of their shape, the cakes with the addition of carob flour were perceived very well. The data showed that there was not a great difference in the values of size and uniformity of cells and crumb tenderness for the investigated cakes with 25% carob flour and 25% carob syrup. The cells of the new sponge cakes with carob ingredients were small and equal, uniformly distributed in the crumb, and were thin-walled. The control sample had a lightly yellow color, while the other cakes had a medium to dark surface and a brown crumb due to the presence of carob flour and syrup. Despite its color, the sponge cake made with the addition of 50% carob flour ws perceived very well by the panelists. Rosa et al. (2015) reported that in terms of color, the control and those made with 25% and 50% carob flour had the highest average scores with no significant difference between them. The researchers concluded that with regard to color, the cake with 100% carob flour had the lowest acceptability, a factor that may have been associated with the dark color caused by the carob flour. With regard to aftertaste, there were differences in the control and sponge cake with 25% carob syrup which could be explained by the fact that carob is rich in condensed tannins, which, when used in excess, tend to give an astringent aftertaste to products (Silanikove et a., 2006). The sample with 25% carob syrup had the least aftertaste (2.87±0.70). These results were in agreement with Hafez (2012) who reported that there was no remarkable difference among samples with and without marjoram powder substitution for the scores of crumb color, texture and overall acceptability, while in taste there were differences in the control and samples with other concentrations. As a result of sweetness, samples supplemented with 50% carob syrup (6.67±1.12) had the same score as the control (6.67±2.06). The mean score of sponge cakes with functional ingredients evaluated in terms of crumb tenderness was higher than that of the control sample.

3.4. Microbiological analysis

In this study, the storage (within six days from production at a temperature of up to 18 °C and relative humidity $\varphi < 75\%$) of sponge cake samples supplemented with different concentrations of carob flour and carob syrup was considered for microbial analyses.

The microbiological characteristics of the sponge cakes during storage are presented in Table 5.

No pathogenic bacteria such as coagulase-positive staphylococci in 1 g of *Salmonella* spp. in 25 g and fecal coliforms in 1 g, respectively, were detected. Up to the 6th day of storage at room temperature, no evidence of mold was detected on the samples. At the end of the storage period, the microbial loads in the control and the variants were similar, and this was likely due to secondary air contamination on the surface of the samples.

Table 5. Microbiological characteristics of the sponge cake samples without and with functional ingredient during sixth-day storage

Storage time, [day]	**Type of sponge cake**	**Total plate count, [CfU/g][1]**	**Coliforms, [CfU/g][1]**	**Coagulase-positive staphylococci, [CfU/g][1]**	***Salmonella* spp. in 25 g**	**Molds and yeasts, [CfU/g][1]**
0	control sample	0	ND[2]	ND	ND	0
	with 25 % carob syrup	0	ND	ND	ND	0
	with 50 % carob syrup	0	ND	ND	ND	0
	with 25 % carob flour	0	ND	ND	ND	0
	with 50 % carob flour	0	ND	ND	ND	0
3	control sample	2.2×10^4	ND	ND	ND	0
	with 25 % carob syrup	0	ND	ND	ND	0
	with 50 % carob syrup	0	ND	ND	ND	0
	with 25 % carob flour	0	ND	ND	ND	0
	with 50 % carob flour	0	ND	ND	ND	0
6	control sample	2.1×10^4	ND	ND	ND	1.3×10^4
	with 25 % carob syrup	5.6×10^2	ND	ND	ND	0
	with 50 % carob syrup	0	ND	ND	ND	4×10^1
	with 25 % carob flour	0	ND	ND	ND	0
	with 50 % carob flour	1.9×10^3	ND	ND	ND	0

[1] CfU/g - Colony forming Units per gram

2 ND = Not detected

4.Conclusions

The results of this study showed that it is possible to design and prepare sponge cakes that contain high protein and dietary fiber. The successful replacement of wheat flour with carob flour and replacement of sugar with carob syrup, was also demonstrated. The physical analyses revealed that partial replacement of wheat flour by carob flour increased the total moisture content of the sponge cakes. The chemical composition of the cakes with carob flour showed higher dietary fiber and protein content compared to the control sponge cake. The cakes with carob flour and syrup as functional ingredients showed differences in cell size and uniformity as well as sweetness and crumb tenderness, confirming that the replacement ingredients positively influenced sensory characteristics.

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EFFECT OF AUTOCLAVING-COOLING ON THE PHYSICAL PROPERTIES, MICROSTRUCTURE AND STARCH HYDROLYSIS OF MILLED RICE

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ABSTRACT

The research aimed to determine the effect of autoclaving and cooling on physical properties, particularly texture and color of cooked rice grains, the microscopic images and starch hydrolysis. Autoclaving and cooling were applied on rice grains to slow down rice starch hydrolysis. Three rice varieties were selected based on amylose level (high, medium and low amylose content of rice varieties). Autoclaving-cooling was conducted by autoclaving rice grains in excess water at 120^{o}C for 15 minutes, followed by cooling the rice grains at temperature of 4^{o}C for 24 hours. The process of autoclaving and cooling were repeated for the treatments of two and three autoclaving-cooling cycles. Results showed that rice variety (based on amylose content) and number of autoclaving-cooling cycles had significant effect on the rate of starch hydrolysis, on the other hand, they had no significant effect on the texture and color of cooked rice. Microstructure images showed that the autoclaved-cooled rice grains displayed more condensed structure and a continuous network with irregular shape formed. The higher amylose rice variety and more autoclaving-cooling cycles applied on rice grains resulted in a lower rate of constant (k) of total dissolved solids content during starch hydrolysis. The condensed microstructure in a rice grain and a lower rate of starch hydrolysis might indicate the content of resistant starch in rice grains increased; therefore it would result in a slower glucose release during digestion.

1. Introduction

Rice contributes the largest amount of energy in daily energy requirement for most people in Indonesia. Rice is usually consumed as steamed rice grains. Starch is the major constituent in rice, and it can be easily broken down into glucose during digestion, thereby increasing blood sugar. There are some products that have been developed to overcome the difficulties for people with diabetics, such low glycemic indexed milk, and foods that are made of resistant starch, however there were not so many against the whole rice grains. Some manufacturers have developed analog rice by using ingredients with low glycemic indexed starch. However, it is difficult for the Indonesian people to change the main diet of steamed rice grains into analog rice. Accordingly, this study was designed to address the problem by changing the components of the starch in rice grains into starch retrogradation or resistant starch. Resistant starch is starch that is not digested by the digestive enzymes so there is no nutrition absorption by the intestine (Hassan et al., 2010; Yun et al., 2010; Ranawana et al., 2009). Re-arrangement of the structure in the starch fraction during processing which includes the process of gelatinization and crystallization can cause starch to retrograde (Hug-Iten et al., 2003). It is possible to produce high resistant

starch in rice grains due to substantial amount of starch in rice grains. A few experimental works has been reported in increasing resistant starch in rice grains (Kim et al., 2006; Milasinovic et al., 2009; Widowati et al., 2010). Those papers mostly focused on the amount of the resistant starch produced through various methods such as parboiling, autoclaving–cooling, or the combination of autoclaving and hydrolysis by enzyme. It has no doubt that those methods successfully increased the amount of resistant starch in rice grains, however, none of those works report on the physical properties of autoclaved and cooled rice grains particularly the microscopic images, texture and color of the rice. In this study, the rice grains were processed by autoclaving-cooling method, and the product was called as "autoclaved-cooled" rice grains.

Rice consumers are increasingly selective in determining the type of rice for consumption. They not only concern about the nutritive aspect of cooked rice but also the physical properties such as texture and color of rice. Texture is one of physical properties that are important in judging the eating quality of cooked rice. The texture which could indicate hardness of rice could be measured by texture analyzer that was expressed as the energy needed to disintegrate the cooked rice and texture is affected by cohesiveness of rice grains. The harder of the texture of cooked rice was indicated by higher energy (gram force) to penetrate into rice grains.

One of the components that determine the eating quality is amylose content. Rice grains can be classified into three groups based on amylose level, namely rice with high amylose content (25-33%), medium (20-25%), and low (2-9%). Different rice varieties will result in different texture and color of cooked rice. Therefore this experimental work determined the physical characteristics (texture and color) of cooked autoclaved-cooled rice from some selected rice varieties differing in amylose content. Autoclaving-cooling was chosen as method to reduce the starch digestibility of rice grains due to its environmental friendly method. The repeating process of autoclaving-cooling which was so called as "cycle" could increase the amount of retrograded starch, and therefore it could slow down the rice starch digestibility. Starch digestibility could be indicated by starch hydrolysis during which starch was broken down into simpler carbohydrate.

Most previous studies investigated the effect of autoclaving-cooling on some properties of starches rather than on grains (Babu and Parimalavalli, 2013; Zhao and Lin, 2009; Kim et al., 2009). Therefore, this study was undertaken to investigate the effects of autoclaving-cooling cycles of rice grains differing in amylose content on the physical properties, microscopic images and rate of starch hydrolysis.

2. Materials and methods

2.1. Materials

The selected rice varieties were high amylose content of rice variety (IR 42 with the amylose content of 25.5%), medium amylose content of rice variety (Karawang with the amylose content of 11.3%), and low amylose content of rice variety (waxy rice with the amylose content of 1.4%). IR 42 and Karawang rice varieties were obtained from the Rice Central Production in South Sumatera, Indonesia. Waxy rice was purchased from local traditional market in Palembang city, South Sumatera. Enzyme used in the analysis of rate of starch hydrolysis was α-amylase solution (Sigma-Aldrich, A8220).

2.2.Autoclaving and cooling on rice

Each milled rice grain variety (IR 42, Karawang, waxy rice) was weighed as amount of 250 g and 750 mL of aquadest was added. It was cooked in a cooking pot until all of the aquadest was absorbed by the rice grains. The partial gelatinized rice grains were transferred into a one Liter-beaker glass, covered by aluminium foil and placed in a plastic bag and it was heated in an autoclave at the sterilizing temperature (121^{o}C) for 15 minutes. The completely gelatinized rice grains were taken out from autoclave and cooled for one hour at room temperature. The gelatinized grains were placed in a refrigerator at 4^{o}C for 24 hours. The rice grain that was processed up to this stage was

called as one cycle of autoclaving-cooling. For two cycles of autoclaving-cooling, the gelatinized rice grains that had been refrigerated were then re-heated in the autoclave for 15 minutes (121^{o}C), followed by cooling at a temperature of 4^{o}C for 24 hours. The similar process was conducted for three cycles of autoclaving-cooling. The last step was drying the rice grains at 50 °C in a hot air oven until the moisture content of the rice grains reached approximately 8%. Each treatment was performed in triplicates.

2.3. Microscopic images, texture and color analysis

The microscopic images of internal structure of autoclaved-cooled rice grains were determined by using Scanning Electron Microscopy (SEM 5200 (JEOL) at 1000 magnifications.

Texture and color analysis were conducted on autoclaved-cooled rice grains that had been cooked in a rice cooker (ratio of rice and water = 1:3). The texture measurement was performed in individual cooked rice grain by applying trigger 0.9 g, distance 1 mm and speed 1mm/s.

Analysis of the texture of cooked rice was conducted by using texture analyzer (LFRA 1500 Texture Analyzer, Brookfield, USA) with flat end (cylinder type) stainless steel probe (probe specification was TA 39 in 2 mm of diameter, 20 mm in length). The color measurement of cooked rice was carried out by using a color reader (Konica Minolta CR-10) for lightness (*L*), chroma (*C*) and hue (*h*).

2.4.Starch hydrolysis by α-amylase (*in vitro*)

Starch hydrolysis was measured by measuring the total dissolved solids of rice grains during hydrolysis. Dried rice grains were ground and weighed for 2 g, and placed in a 25 mL of Beaker glass. An amount of 10 mL aquadest was added into sample and heated on a hot plate to gelatinize the sample. The gel was cooled at room temperature and measured for its initial total dissolved solids by using a refractometer (Atago, 0-85 Brix%). Enzyme solution of α-amylase (0.1 mL) was added into the gel solution and gently stirred with a magnetic stirrer. The total dissolved solids were periodically measured every 30 minutes to 120 minutes. The rate of starch hydrolysis by enzymatic process was analyzed by the Arrhenius equation.

2.5. Statistical Analysis

An analysis of variance (ANOVA) was performed on physical analysis of cooked rice grains, and the significant difference treatments were further analyzed by Tukey's test using the Statistical Analysis System. P values < 0.05 were considered significant. Rice variety was as factor A, and number of cycles of autoclaving and cooling was as factor B.

2.6. Rate of Starch Hydrolysis

The rate of starch hydrolysis during enzymatic process was modeled as:

$$-\frac{dC}{dt} = kC^{n} \quad \text{..... (1)}$$

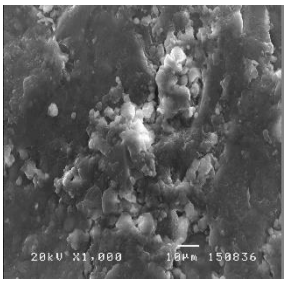
Where *C* is total dissolved solids (Brix%), *t* is hydrolysis period (minute), *k* is a pseudo-rate of constant, *n* is reaction order. The reaction order (*n*) in the starch hydrolysis was assumed to follow the first reaction order. If *n* is equal to one, equation (1) collapses to equation (2) (Labuza and Riboh, 1982).

$$-\frac{dC}{dt} = kC \quad \text{..... (2)}$$

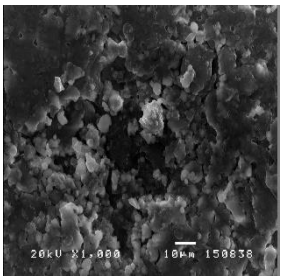
3. Results and discussions

3.1. Microscopic Images

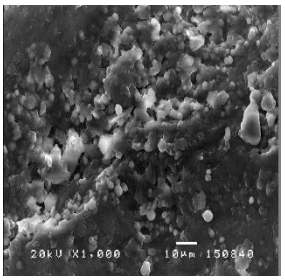
Microscopic images of rice were presented in Figures 1 and 2. Figure 1 exhibited granular appearances in the image, while Figure 2 clearly illustrated that the granular structure disappeared and a continuous network with irregular shape formed. The microstructure images displayed more condensed microstructure in the rice grain along with more cycles of autoclaving-cooling.



IR 42 rice



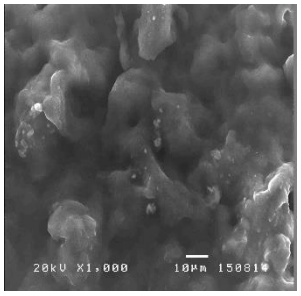
Karawang rice



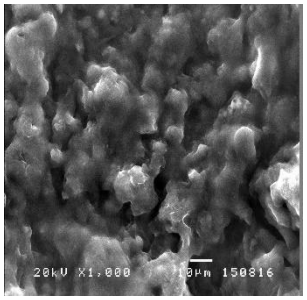
Waxy rice

Figure 1. Microstructures of untreated IR 42, Karawang and waxy rice at magnification of 1000x under SEM.

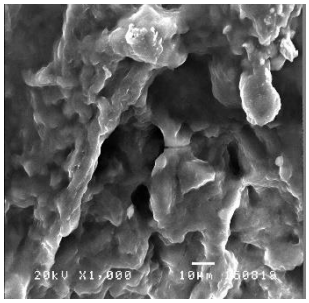
Figure 1 shows that the untreated rice grain of IR 42 variety had a denser region compared to Karawang variety and waxy rice. It might be due to higher amount of amylose (linier fraction of starch) in IR 42 variety. During autoclaving and cooling process in a rice grain, amylose and amylopectin intensively interacted each other (Hoover, 2010). The re-arrangement of amylose and amylose fractions, amylose and amylopectin fraction during autoclaving-cooling resulted in a denser gelatinized starch region than the untreated rice grains. As shown in Figure 2, more cycles of autoclaving and cooling applied on a rice grain, a denser gelatinized starch region in rice grains. The re-arrangement of linier chains in amylose fraction was more readily compared to amylopectin; therefore the IR 42 rice variety that contained higher amylose content showed a dense gelatinized starch than others.



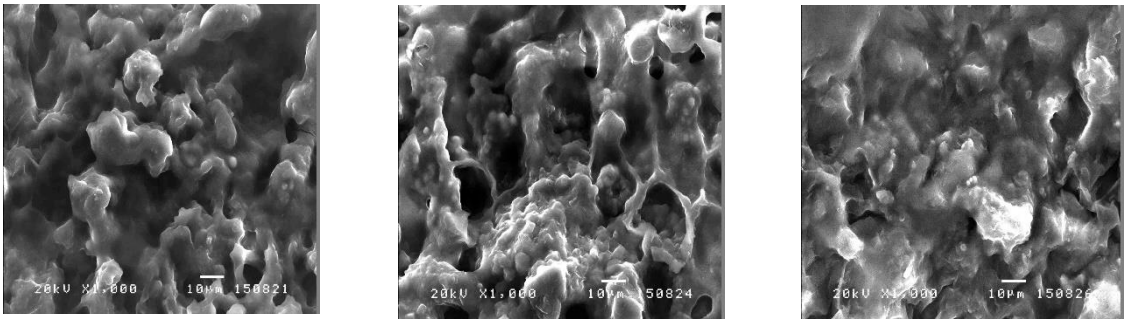
IR 42 – 1 cycle



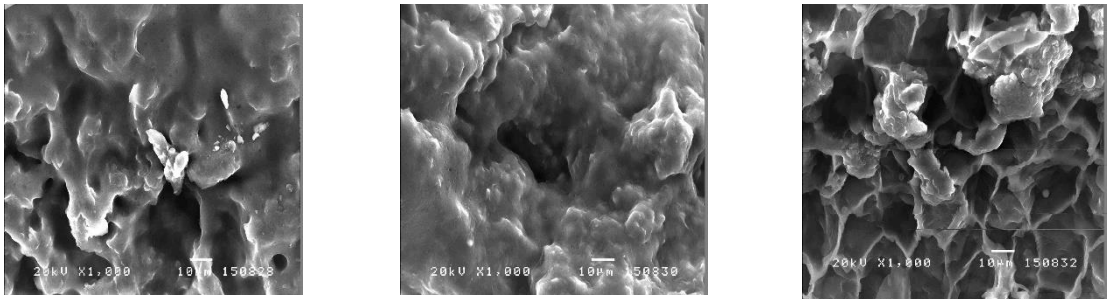
IR 42- 2 cycles



IR 42- 3 cycles



Karawang – 1 cycle | Karawang – 2 cycles | Karawang – 3 cycles



Waxy Rice – 1 cycle | Waxy Rice – 2 cycles | Waxy Rice – 3 cycles

Figure 2. Microstructures of autoclaved and cooled rice at magnification of 1000x under scanning electron microscope.

3.2. Hardness

Analysis of variance showed that the rice variety (based on amylose content), number of autoclaving-cooling cycles and their interactions had significant effect ($P<0.05$) on the texture (hardness) of the cooked rice samples. Post Hoc comparison using Tukey's test were presented in Table 1 to 3.

Table 1. Pos Hoc comparison using Tukey's test on rice variety

Rice variety	Hardness (gf)
Waxy rice	$(12.41\pm0.36)^{a}$
Karawang	$(17.05\pm0.41)^{b}$
IR 42	$(22.16\pm0.39)^{c}$

Means with different superscript letters are significantly different ($P<0.05$) (Tukey's$_{0.05}$ = 0.15)

Table 2. Pos Hoc comparison using Tukey's test on number of cycles

Number of cycles	Hardness (gf)
1 cycle	$(16.00\pm0.15)^{a}$
2 cycles	$(17.33\pm0.52)^{b}$
3 cycles	$(18.30\pm0.49)^{c}$

Means with different superscript letters are significantly different ($P<0.05$) (Tukey's$_{0.05}$ = 0.15)

Table 3. Pos Hoc comparison using Tukey's test on interaction of rice variety and number of cycles

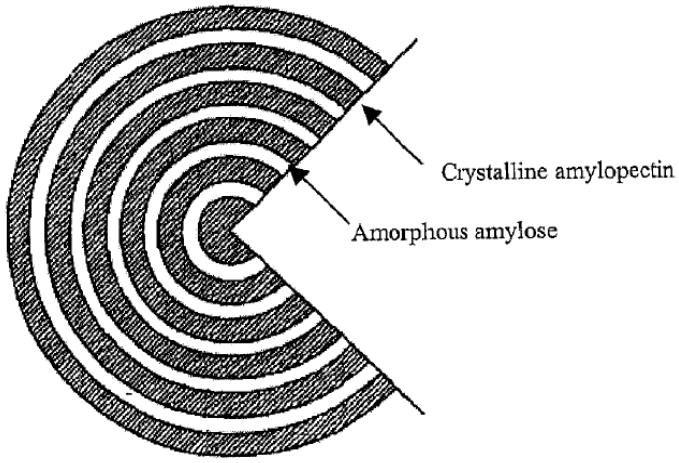
Rice variety	Number of autoclaving-cooling cycle		
	one cycle	two cycles	three cycles
IR42	$(20.36\pm0.12)^{f}$	$(22.09\pm0.61)^{g}$	$(24.04\pm0.45)^{h}$
Karawang	$(16.36\pm0.12)^{d}$	$(17.04\pm0.45)^{e}$	$(17.15\pm0.66)^{e}$

Waxy rice	$(11.27\pm0.23)^{a}$	$(12.87\pm0.49)^{b}$	$(13.10+0.36)^{c}$

Means with different superscript letters are significantly different ($P<0.05$) (Tukey's $s_{0.05} = 0.79$)

Factors affecting the cooked rice have long been recognized, owing to extensive research in this area (Cameron and Wang, 2005; Asghar et al., 2012; Widjajaseputra, 2012; Han and Lim, 2009; Narkrugsa and Saeleaw, 2009). However, texture and color are quality attributes that mostly affect the consumers' preference of cooked rice. The texture of cooked rice in this research was measured as hardness. The hardness of the rice samples are recorded as peak load and final load in grams force per millimeter (gf/mm). Peak load value indicates the amount of grams force needed to disintegrate the rice sample, while the final load indicates the magnitude of gram force after the probe entered into the sample. Final value of peak load and load in all the samples were relatively equal, and therefore the values of the final load were reported in this experiment.

IR42 rice varieties contained the highest amylose content (25.5%) among the selected rice varieties; therefore, as expected, more retrograded starch occurred during cooling due to easier re-arrangement of amylose. Amylose exists within a starch granule as an entity that is largely separated from the amylopectin fraction. Amylose is located in bundles between amylopectin clusters, and randomly interdispersed among the clusters in both amorphous and crystalline regions. A schematic diagram of the structure of a starch granule, showing the amorphous amylose and crystalline layers of amylopectin is reproduced in Figure 3.



Source: Morrison et al. (1994)

Figure 3. A schematic model of a starch granule that shows amorphous amylose and crystalline amylopectin domains

The retrograded starch was clearly shown in the microstructure images by the dense gelatinized starch region. Unlike IR 42 and Karawang rice varieties, waxy rice only contained 1% of amylose content, therefore only slight retrograded starch occurred during cooling of gelatinized rice grains. As a consequence, the texture of cooked autoclaved-cooled rice grains of waxy rice was lower than the other two rice varieties. Amylose fractions are more readily retrograded than amylopectin fraction.

3.3. Color analysis

Color attributes included lightness, chroma and hue. The lowest lightness value

(69.37%) and chroma value (2.20%) were found in cooked rice of IR 42 variety (high amylose variety) that was processed by one cycle of autoclaving-cooling, on the other hand, the highest lightness value (77.80%) was found in medium amylose rice variety (Karawang). The highest chroma value (7.20%) was obtained in cooked waxy rice that was processed by three cycles of autoclaving-cooling. Hue showed the dominant color appeared on the surface of the cooked rice. The hue values ranged from 87.67 ^{o} to 129.47^{o} that has the spectrum of yellowish white. Lightness, chroma and hue values of all treatments were shown in Figures 4, 5 and 6, respectively.

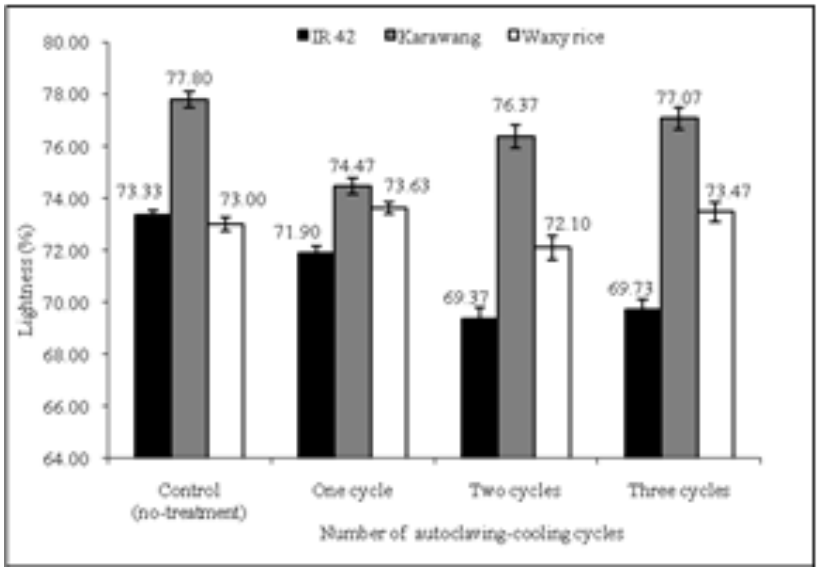


Figure 4. Lightness value (%) of cooked autoclaved– cooled rice grains

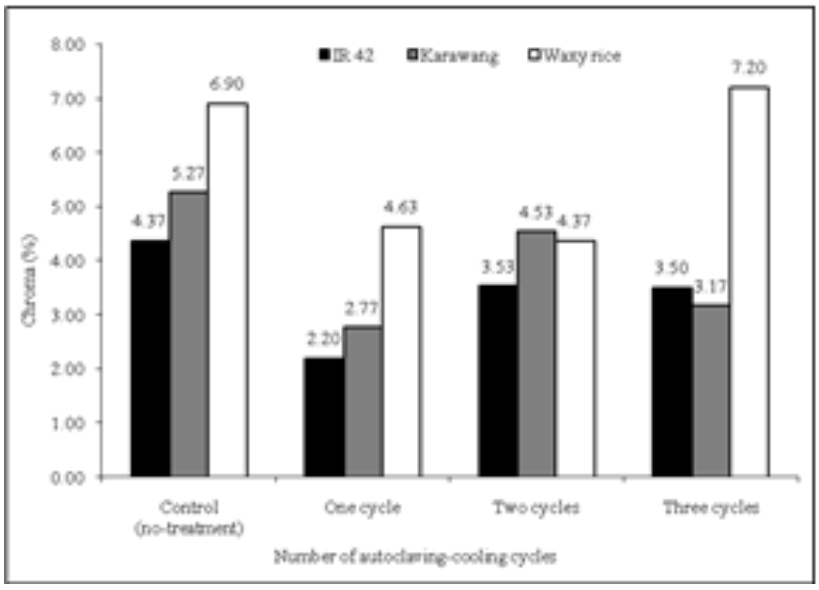


Figure 5. Chroma values (%) of cooked autoclaved–cooled

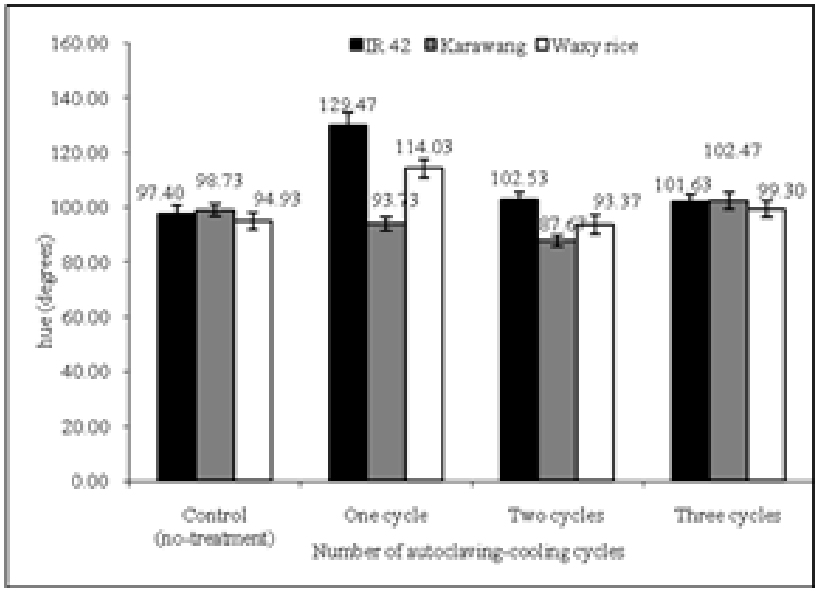


Figure 6. Hue value (^{o}) of cooked autoclaved–cooled

No significant difference was observed on lightness, chroma and hue of cooked rice samples (P>0.05) for any of the variables. These insignificant differences (P>0.05) in the cooked rice sample gave possibilities of this product to be accepted by most rice consumers in respect of the similar appearance of color as the untreated cooked rice.

The rice grains used in the research were white milled rice in which all of the bran layers and germs were brushed off during milling and polishing. With all of the bran layers removed resulted in white endosperm of rice, therefore, there was no significant effect of rice amylose content and number of autoclaving-cooling cycles on the color of treated rice grains.

3.3. Rate of starch hydrolysis

Hydrolysis of starch would increase the amount of solids dissolve in the liquid, therefore the rate of starch hydrolysis in this experiment was based on the total dissolved solids. The rates of starch hydrolysis (*k*) of all samples are summarized in Table 4. The *k* values decreased along with the increase number of autoclaving-cooling cycles. It indicated that starch in rice had been depreciated by retrogradation.

Table 4. Rate of constant (*k*) of total dissolved solids during starch hydrolysis (according to first order reaction)

Treatments	Rate of starch hydrolysis (*k*) based on total dissolved solids content (Brix% minute^{-1})
Rice processed by one cycle of autoclaving-cooling	
IR 42	1.4×10^{-3} **c**
Karawang	2×10^{-3} **e**
Waxy Rice	2.94×10^{-2} **h**
Rice processed by two cycles of autoclaving-cooling	

IR 42	10^{-3} **b**
Karawang	1.8×10^{-3} **d**
Waxy Rice	2.87×10^{-2} **g**
Rice processed by three cycles of autoclaving-cooling	
IR 42	8×10^{-4} **a**
Karawang	8×10^{-4} **a**
Waxy Rice	2.28×10^{-2} **f**

Means with different superscript letters are significantly different (P<0.05)

The main component in the rice is starch that can exceed 85%. Yin and Cheng (1980) explained that starch dissolves in water when heated and can form crystalline structure of amylopectin, whilst amorphous amylose is damaged and form a gel. When the gel is cooled, the linear part of amylose and amylopectin linear part will condense and form a crystalline structure by way of mutual binding through hydrogen bonding. Temperatures between -8^{o}C to 8^{o}C can lead to the formation of the crystalline structure. As further stated by Kim et al. (2009) that starch was completely gelatinized and amylose could be leached out during autoclaving; whereas the crystalline regions (amylopectin) of clusters of branched amylopectin chains were broken down. The leached out amylose would re-arrange and lead to formation of retrograded starch or resistant starch. In other words, the retrograded starch formed a tightly packed starch and hardened the texture of rice grains. It resulted in a slower rate of starch hydrolysis. This finding is in agreement with Vatanasuchart et al. (2009) who stated that rice snacks made of low amylose rice had low resistant starch or retrograded starch.

Autoclaving and cooling of rice grains would be able to increase the amount of resistant starch; therefore autoclaving and cooling of rice grains could retard the rice starch to be hydrolyzed. The slow rate of starch hydrolysis was indicated by lower *k* values (Table 2). Higher value of *k* indicates that starch in rice sample could be rapidly hydrolyzed by amylase. Our findings are in agreement with Ozturk et al. (2009) and Pongjanta et al. (2009) who stated starch with higher content of amylose could be more readily formed resistant starch that that of lower content of amylose. As stated earlier that waxy rice contained the lowest amount of amylose content, and therefore, the *k* value of starch hydrolysis in waxy rice was the highest among the rice samples. The *k* value of starch hydrolysis decreased with increasing number of autoclaving-cooling cycles applied on rice samples which was due to the formation of more resistant starch with more cycles of autoclaving-cooling.

4. Conclusions

The rice variety and number of autoclaving-cooling cycles significantly affected the rate of starch hydrolysis but not on texture and color of cooked autoclave-cooled rice grains. The internal structure of autoclaved-cooled rice grains were more condensed rather than that of without treatment. The texture of cooked rice tend to be harder after experiencing more cycles of autoclaving-cooling particularly in rice grains with higher amylose content. The rate of starch hydrolysis (*k*) decreased with more autoclaving-cooling cycles. The lowest *k* value (rate of starch hydrolysis) was found in cooked rice with three autoclaving-cooling cycles of both high and medium amylose rice varieties.

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USE OF ACID WHEY IN TECHNOLOGY OF ENRICHED JELLY DESSERT

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ABSTRACT

Jelly based on milk whey is recommended for all population groups as a functional product for increasing immunity, especially for people with connective tissue diseases, cardiovascular diseases, high blood pressure, metabolic disorders, and diabetis. To improve the technological properties of the product, the dietary fibre was swollen in the acid whey mixed with water. The solubility of pectin improved with an increase in temperature. According to the results of the study, the nutritional value of the test sample exceeded the one of the control sample in the content of dietary fibre (which was achieved by adding citric fibre to the jelly) and the content of essential macro- and microelements - Ca, Mg, Zn and P (by replacing water with the acid whey). Thus, the developed product along with the functional properties had an increased biological and nutritional value and possessed high organoleptic characteristics.

1.Introduction

One of the most promising areas for food industry is currently the development of healthy food products, including those enriched with functional ingredients (Tihomirova, 2001).

The production of desserts on the basis of secondary dairy raw material allows to make the diet more various, and improve the taste of the product, reducing its cost (Yadav, et al., 2016).

Milk whey is a by-product in the production of protein-fat products, such as cheese, cottage cheese, casein (Boland, 2011). The yield of milk whey from 1 ton of milk in the production of high-protein products ranges from 65 to 82%: natural cheeses – 80%; defatted cheeses– 65%; low-fat cheeses – 65%; brynza – 65%; cottage cheese – 80%; technical casein – 75%; food casein – 82% (Jeewanthi, et al., 2015; Smithers, 2008).

It's clinically proved that the use of whey products can be effective in the treatment and prevention of diabetes, bowel diseases, hypertension, infections, diseases of integumentary and bone tissues, immunodeficiency, complications after surgery (Jayaprakasha, et al., 1999; Jelen, 2009).

Moreover, it is established that whey proteins influence the feeling of satiety (so that a person can eat less), and the speed of digestion, which allows to control one's weight (Yetmin, et al., 2001).

Dietary fibre is one of the most popular and most widely used food ingredients due to its multifunctionality. On the one hand, dietary fibre is used as technological additives that changes the structure and chemical properties of food products, on the other hand, dietary fibre is an excellent functional ingredient that can have a beneficial effect both on individual systems of the human body and on the whole organism (Petrov, et al., 2001).

Citrus dietary fibre is extracted from the cellular material of dried citrus pulp mechanically, without any chemical reagents, by opening and expanding the structural cell of citrus fibre. It possesses a high fat-binding capacity, emulsifying, stabilizing, structure-forming properties, antioxidant effect, reduces

contamination by microorganisms, thereby increasing the shelf life and improving the freshness of food products, it is resistant to cycles of frosting and defrosting. It improves nutritional value, being a product of functional purpose, thanks to the content of dietary fibre, beneficial for human health (Kosikowski, 1979; Alamri, et., 2014).

A rational way of processing acid whey is the production of various structured desserts on its basis (Pescuma, et al., 2015).

During the study we faced the task of developing a new kind of enriched product, jelly dessert by way of example. Acid whey rich in minerals and essential nutrients was chosen as an enriching component, and citrus pectin «Citrus Fibre 7000» as a stabilizing component and a source of dietary fibre (Kozlov, 2004).

The nutritional trend for foods enrichment has been and is very important. However, when new components are incorporated into an existing dairy formulation, the effect of such modification on food properties should be researched.

Jha et al. developed a process to extend the shelf life of a dairy dessert enriched with dalia (cooked and shredded wheat) and determined its physicochemical properties (Jha, et al., 2012).

Qasem et al. carried out a study of high soluble-fiber pudding by incorporating okra (2–8%) in a dessert formulation, trying to improve both the rheological (flow and texture) and nutritional (soluble fiber) properties of desserts and refer good results for the 2% incorporation level (Qasem, et al., 2016).

In the light of the above, the development of technology and formula for food products with dietary fibre is a relevant task. In addition, the use of secondary dairy raw materials (milk whey) as a basis for such products simultaneously solves the problems of ensuring the adequate nutrition of the population, and using all milk components to the full, which in turn affects the cost of finished products, minimizing waste disposal costs (Tutelian, et al., 2000).

2. Materials and methods

2.1. Materials

As the object of the study, the orange jelly recipe, listed in the collection of recipes for catering, was used. The recipe included orange juice without pulp, gelatin, sugar, water and citric acid.

In our technology, water was replaced with acid whey, and Citrus Fibre 7000 (manufactured by Bernello Ingredients GmbH) was used as the stabilizing and functional-technological component.

2.2. Methods

The content of dry substances was determined by the hydrometric method. The arithmetic mean of the results of two indications ρ_1 and ρ_2 obtained under repeated conditions was taken for the average value of the hydrometer readings at the temperature of the sample (20.0±2.0) °C.

The mass fraction of lactose was determined using Shimadzu LC-20 Prominence liquid chromatograph equipped with spectrophotometric and refraction index detectors; an aminopropyl stationary phase column for the separation of carbohydrates (Zorbax Carbohydrate 250x4.6 mm, 5 μm, manufactured by Agilent) and the corresponding pre-column). The method was based on the determination of lactose in a filtrate obtained after removal of fat and protein from the sample by high performance liquid chromatography when separated on an anion exchange column. The obtained measurement results were compared with the values of the mass fraction of lactose in a standard sample using a calibration curve.

The mass fraction of protein was determined by a Kjeldahl-based method of the sample's mineralization and photometric measurement of the indophenol blue colour intensity, proportional to the amount of ammonia in the mineralase.

The content of milk fat was determined by the acid method. The method was based on the release of fat from the whey under the action of concentrated sulfuric acid and isoamyl alcohol,

followed by centrifugation and measurement of the volume of released fat in the graduated part of the oleometer.

The acidity of the whey was determined by titrating it with an alkali solution in the presence of phenolphthalein.

The density was determined using a lactodensimeter.

The temperature and time of swelling and dissolution of dietary fibre were determined by the test tube method. The method was based on the evaporation of water from the product during heat treatment and determining the change in its mass by weighing.

The content of vitamin C was determined by the fluorimetric method on Fluorat-02 fluid analyzer. The method was based on extracting vitamin C from a food product, treating the extract with activated carbon in order to purify it and simultaneously oxidize ascorbic acid to dehydroascorbic acid, which interacts with o-phenylenediamine in a weak acid medium to form a fluorescent product, and registering the fluorescence on a Fluorat-02 analyzer.

Dietary fibre was determined by enzymatic-gravimetric method. The method was based on enzymatic hydrolysis of starch and non-starch compounds. Dietary fibre was precipitated with ethyl alcohol, dried and its content was determined gravimetrically (Pascual, et al., 2000).

The content of calcium, magnesium and zinc in products was determined with MGA 915/1000 atomic absorption spectrometer with electrothermal atomization. The method was based on measuring the resonant absorption of light by free metal atoms, which occurs when it passes through a layer of atomic vapor in an electrothermal atomizer of an atomic absorption spectrometer.

The mass concentration of elements is determined by the value of the integral signal of absorption and is calculated automatically according to a pre-set calibration curve.

The phosphorus content was determined using its ability combining with ammonium molybdenum to form phosphomolybdic acid, which was reduced by the amidol reagent and gave a blue color (Skurikhin, et al., 1998).

To conduct an organoleptic evaluation of Jelly Dessert a 9-point scale was used, with weight coefficients taken into the account.

All analyses were carried out in triplicate unless otherwise stated and the average values were calculated. The results were expressed as mean value ± standard deviation. Significant differences between mean values at significance level $p<0.05$ were established using the One way analysis of variance and Student`s test. Microsoft Excel version 2010 was used as the statistical analysis software.

3.Results and discussions

Milk processing enterprises in Chelyabinsk region produce a lot of cottage cheese, and therefore the search for optimal ways of processing acid whey – a valuable secondary resource – is a relevant task.

The physical, chemical and organoleptic parameters of the acid whey are given in Table 1.

Table 1. Physical, Chemical and Organoleptic Parameters of Acid Whey

Parameter	**Characteristics**
Dry substances, %	6.8±0.09
including:	
lactose	3.1±0.05
protein	1.1±0.08
milk fat	0.2±0.08
Acidity, °T	73
Density, kg/m^3	1026
Appearance and consistency	Homogeneous liquid. Possible protein precipitate

Colour	Pale-yellow
Taste and smell	Characteristic of whey, sour

As can be seen from Table 1, acid whey contains a significant amount of mineral substances, which explains its biological value. Globular proteins in the whey – 65% β-lactalbumin, 25% α-lactalbumin and 8% serum albumin – are well absorbed by the body (Kosaric, et al., 1982).

Citrus Fibre 7000 is a cream powder made from dried orange pulp. The taste and smell are neutral.

Citrus pectin gives the product the desired structure without adding calcium cations and does not react to the pH value.

We determined the parameters of swelling and dissolution of pectins used in the study (see Table 2).

Table 2. Parameters of Swelling and Dissolution of Dietary Fibre

Concentration, %	**Swelling Parameters**		**Dissolution Parameters**	
	time, minutes	*temperature, °C*	*time, minutes*	*temperature, °C*
0.5	10	20	20	60
1	20	20	30	60
1.5	30	20	40	60
2	45	20	50	60
2.5	50	20	60	60
3	60	20	65	60

Taking into account the organoleptic characteristics of the secondary dairy raw material under study, and the difficulty in extracting valuable food components from it, it is advisable to preserve the biopotential of the acid whey by changing its aggregate state and enhancing the flavor and aromatic characteristics. In other words, the best solution is to develop a jelly dessert based on whey with the addition of orange juice, a consistency regulator and a flavor component (Yadav, et al., 2015).

Table 3. Formula of Jelly Dessert Based on Acid Whey

Ingredient	**Quantity, g**
Orange juice without pulp	200
Sugar	140
Citric acid	1
Gelatin	30
Acid whey	620
Citrus Fibre 7000	9

A series of preliminary model experiments were carried out to select the optimal ratio of the formula components of the target product (see Table 3) and justify the technology of its production.

A classic recipe of jelly dessert based on orange juice (without pulp), gelatin, water and sugar was used for the control sample.

The production of the enriched jelly dessert was based on the abovesaid recipe, with water replaced by acid whey and citrus fibre added. Prepared gelatin was added to the whey heated with sugar to 60 °C. After the gelatin dissolved, juice extracted from oranges and the citrus fibre powder were added. Then citric acid was added and the mixture was stirred constantly for 2 minutes. The mixture was allowed to cool to the room temperature, then poured into molds and cooled off.

To improve the technological properties of the product, the dietary fibre was swollen in the acid whey mixed with water. The solubility of pectin improved with an increase in temperature.

As can be seen from the formula in Table 3, the development of an enriched product does not require expensive raw materials and additional equipment, which positively influences the cost of the dessert.

The citric dietary fibre contained in the developed product is an essential component of human nutrition (the daily requirement is 25 g), as it promotes the normalization of the gastrointestinal tract. It also stabilizes the consistency of the finished product by pre-dissolution in the mixture before homogenization and subsequent swelling.

At the next stage of the study, we tested the physical and chemical parameters of the control sample (the jelly was prepared according to the traditional formula) and the test sample (the jelly based on acid whey). The physical and chemical parameters and nutritional value of the jelly are given in Table 4.

Table 4. Physical and Chemical Parameters and Nutritional Value of Jelly

Parameter	**Control Sample**	**Test Sample**
Acidity, °T	15 ± 0.04*	32 ± 0.02*
Mass fraction of soluble solids, %	17.4 ± 0.04*	41.5 ± 0.05*
Mass fraction of vitamin C, mg%	53.3 ± 0.07*	55.5 ± 0.06*
Mass fraction of dietary fibre, %	not detected	1.9 ± 0.09*
Calcium content, mg kg^{-1}	103.83 ± 14.21*	212.83 ± 29.47*
Magnesium content, mg kg^{-1}	87.32 ± 31.11*	241.49 ± 40.51*
Zinc content, mg kg^{-1}	6.13 ± 0.87*	17.05 ± 1.01*
Phosphorus mass fraction, %	0.110 ± 0.010*	0.189 ± 0.013*

Note: * denotes statistically significant difference at p <0.05 level

According to the results of the study, the nutritional value of the test sample exceeded the one of the control sample in the content of dietary fibre (achieved by adding citric fibre to the jelly) and the content of essential macro- and microelements - Ca, Mg, Zn and P (by replacing water with the acid whey).

At the final stage, an organoleptic evaluation of the jelly dessert was carried out.

The organoleptic evaluation of the samples is shown in Fig. 1 and 2.

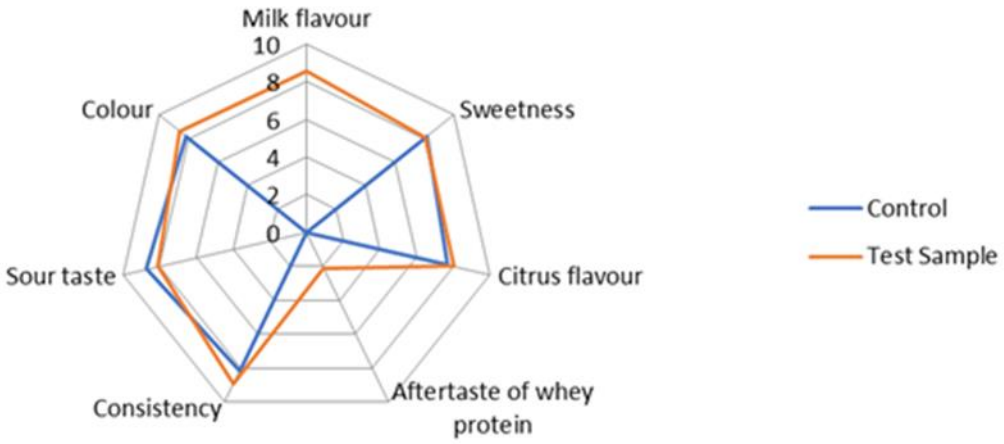
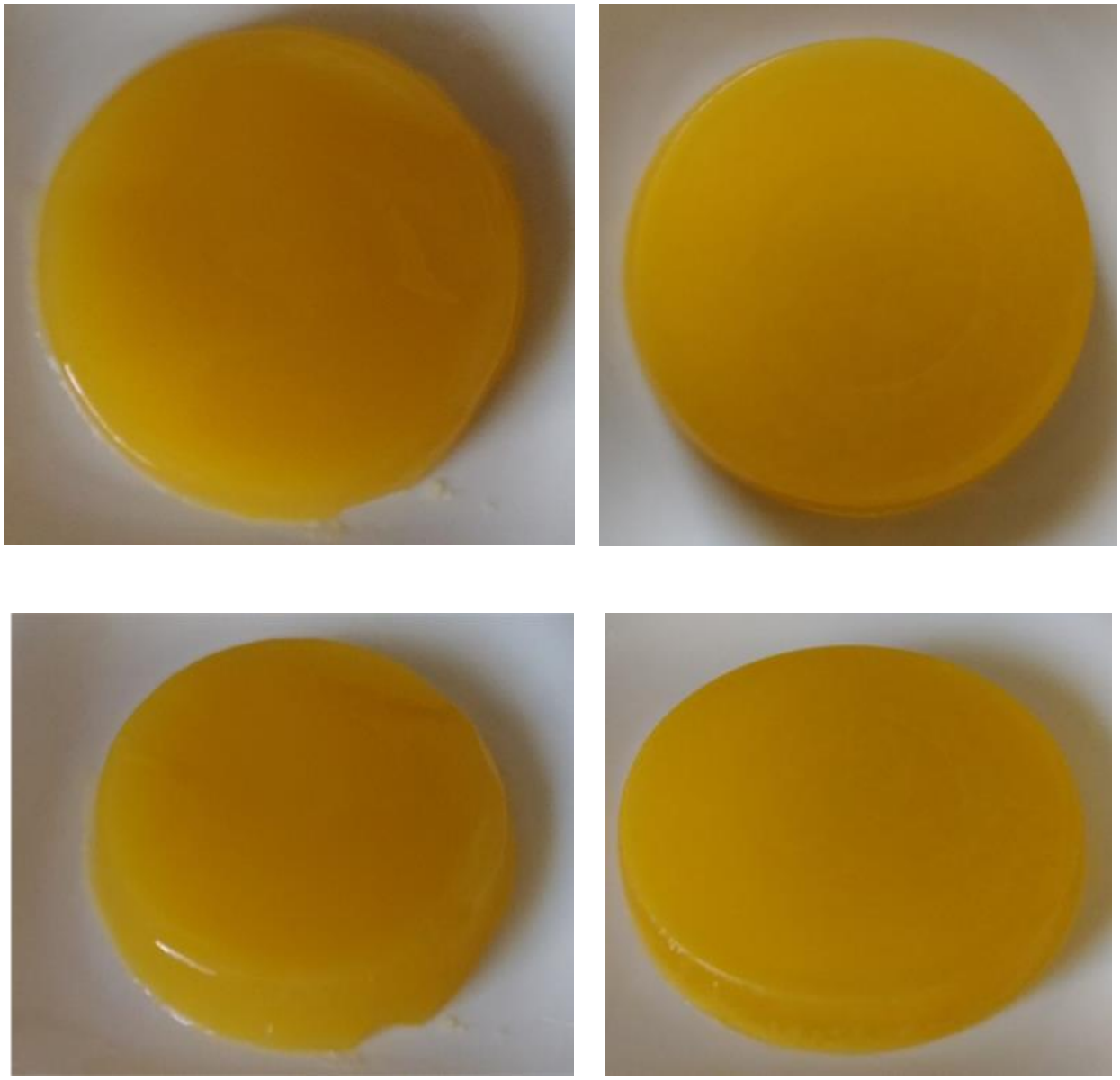


Figure 1. Profilogram of Jelly Desserts

The obtained product possessed pleasant gastronomic characteristics. The taste profilogram (see Figure 1) demonstrates the unusual combination of taste profiles of the main components of the product formula – the sour-milk taste of acid whey was perfectly combined with the taste of orange (Surmacka Szczesniak, 2002).

The test sample was considered the best one judging by its organoleptic properties – it had a distinct citrus flavour, natural color and taste, which is characteristic of the raw materials used.



Control Sample Test Sample

Figure 2. Photos of the control and test samples

Adding citrus fibre to the formula increased the density of the dessert, without separation of the liquid. Adding the acid whey gave the dessert a pleasant milk taste, increased sweetness and reduced the sour taste of the jelly.

According to the results, we can conclude that in the course of the study we obtained an enriched jelly dessert, containing acid whey and citrus fibre. Acid whey increases the nutritional and biological value of the product, while citrus

fibre not only improves the functional and technological characteristics of the dessert, but also enriches it with valuable dietary fibre. The organoleptic evaluation indicates that such dessert can be used in the everyday diet of a wide range of consumers.

4. Conclusions

We have for the first time developed and tested the technology of obtaining the enriched jelly dessert based on the acid whey and citrus fibre.

Prepared gelatin was added to the whey heated with sugar to 60 °C. After the gelatin dissolved, juice extracted from oranges and the citrus fibre powder was added. Then citric acid was added and the mixture was stirred constantly for 2 minutes. The mixture was allowed to cool to room temperature, then poured into molds and cooled off.

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Adding citrus fibre to the formula increased the density of the dessert, without separation of the liquid. Adding the acid whey gave the dessert a pleasant milk taste, increased sweetness and reduced the sour taste of the jelly.

Jelly based on milk whey is recommended for all population groups as a functional product for increasing immunity, especially for people with connective tissue diseases, cardiovascular diseases, high blood pressure, metabolic disorders, and diabetis.

Thus, the developed product along with the functional properties had an increased biological and nutritional value and possessed high organoleptic characteristics.

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OPTIMISATION OFA READY TO USE "NUTRITIOUS MIX" INCORPORATING INDIAN HERBS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT
Indian medicinal herbs are the natural and healthy alternative source of medications possessing side effects for various ailments. Their incorporation in food products can make it both nutritious and healthful. Nutritious mix was formulated as an instant food that can be reconstituted for consumption effortlessly. The purpose of the study was to optimise the amounts of Indian herbs as functional foods for incorporation in the powder to enhance its nutritional and functional properties.RSM (response surface methodology) and CCRD (central composite rotable design) were utilised for optimisation with three process variables (namely, amounts of apple powder, *Rauvolfiaserpentina* and black cumin seeds) and potassium, sodium, fibre and overall acceptability as response variables. The response surface plots along with regression models were produced and regression coefficients and lack of fit tests were used to test the adequacy. The optimum levels that were attained for in range potassium (477.71 mg), minimum sodium (39.85 mg), maximum fibre (4.09 g) and maximum overall acceptability (87.61) were: 5.00 g apple powder, 0.70 g *Rauvolfia serpentina* and 10.00 g black cumin seeds powder. Optimum recipe was nutritionally adequate and highly acceptable. Nutritious mix can provide beneficial roles to the people in maintaining their health without changing their regular diet patterns.

1. Introduction

Medicinal plants are regaining importance as a result of side effects caused by modern synthetic drugs. However, herbal medicines have sustained to be in demand among the developing countries as a result of being easily accessible, cost effective and culturally acceptable (Sewell and Rafieian-Kopaei, 2014). Herbal medicine remains to be the core of approximately 75 to 80 percent world's population, mostly among the developing world, for primary health care as a result of having enhanced cultural acceptance, being more compatible with the human's body, and possessing fewer side effects (Vidyarthi*et al.*, 2013). India is an immense repository of medicinal plants being traditionally utilised in the treatment of various ailments (Agrawal *et al.*, 2010). Hypertension is a leading public health problem worldwide. Chemical medicines for hypertension generally cause side effects making the usage of medicinal herbs necessary (Pourjabali *et al.*, 2017). There are many scientifically studied and frequently used

naturally occurring medicinal plants for the management of hypertension including Black cumin seeds, Rauwolfia and others (Agrawal *et al.,* 2010).

The roots, leaves, seeds, fruits and juice of *Rauvolfia serpentina* having medicinal benefits have drawn the attention of those practicing indigenous therapies. It has been used as a therapy for combating anxiety, epilepsy, excitement, gastrointestinal disorders, hypertension, insanity, mental agitation, traumas, schizophrenia, sedative insomnia(Malviya and Sason, 2016), body aches, burns and skin diseases(Poonam *et al.,* 2013).Current scientific researches on black cumin seeds ((*Nigella sativa* L.) and its oil have shown numerous bioactivities for the plant including anticarcinogenic, antihyperlipidemic, anti-inflammatory, antipyretic and analgesic, antiulcer, antibacterial and antifungal, antihypertensive, hepatoprotective and antioxidant activities as well that includes scavenging the reactive species of oxygen, preventing rheumatoid arthritis in rat models(Toma *et al.,* 2015).

In recent times, the rising health issues have resulted in the transference towards the optimal nutrition diet. Thus, food manufacturers are tended to produce such food products that can satisfy both consumer's appetite and desires for health promotion(Olaiya*et al.,* 2016). Nutraceuticals have come out to be an alternative source of the modern medicines and have shown positive results in decreasing the conventional medicines requirement along with reducing the possibilities of adverse effects (Sharma *et al.,* 2017). Currently, nutraceuticals and functional foods have gained the attention as potential alternative therapies in the hypertension treatment (Chen *et al.,* 2009). Thus, incorporation of medicinal herbs like *Rauvolfia serpentina* and black cumin seeds as nutraceuticals can come out to be a potential alternative source of medicines for hypertension management. Additionally, incorporation of heart healthy food like apple can help in enriching the food product's health benefits.

In spite of having highly beneficial roles in several diseases, incorporation of above mentioned herbs and apples in higher quantities can compromise the overall acceptance of various sensory attributes of developed food product. Therefore, there is a need for such techniques that can help in getting optimum solutions to produce a recipe which is adequate from nutritional point of view and is accepted organoleptically as well. Process optimisation is the one of current techniques being utilised in the formulation of optimum food products with increased nutritional properties. "Response surface methodology (RSM) is a powerful mathematical model with a collection of statistical techniques where in, interactions between numerous process variables can be recognized with fewer experimental trials. It is extensively used to study and optimize the operational variables for experiment designing, model developing and factors and conditions optimization(Karuppaiya *et al.,* 2010)".

Therefore, in the present study, a ready to use nutritious mix incorporating Indian herbs, appropriate for hypertensive people was formulated as instant food with the objective to get the statistically valid optimum combination of amount of apple powder, *Rauvolfia serpentina* and black cumin seeds powder as process variables for their incorporation and in range potassium, minimum sodium, maximum fibre and overall acceptability as response variables through CCRD of response surface methodology.

2. Materials and methods

The present work was done in Banasthali Vidyapith, Rajasthan, India, during the time span of July, 2014 to April, 2015. The raw ingredients and apple as functional food were purchased from Banasthali Vidyapith's local

market. Black cumin seeds of brand with a good reputewere acquired from general store of Ghaziabad whereas *Rauvolfiaserpentina* was obtained from a reputed ayurvedic pharmacy.

2.1. Formulation of nutritious mix

Rauvolfia serpentina powder (0.5 g), black cumin seeds powder (5 g), apple powder (10 g), tomato powder (5 g), whole wheat flour (15 g) and roasted bengal gram (15 g) were incorporated for the preparation of nutritious mix. Apples were cut into thin slices, blanched, oven dried at 60°C for 48 hours and then powdered. Black cumin seeds were cleaned and powdered. No specific treatment was given to root powder of *Rauvolfia serpentina* for the purpose of product development. Drying of tomatoes and tamarind was done at 60°C in an oven for 2 days and then were grinded. Roasted bengal gram was grinded to make powder. Black cumin seeds powder and wheat flour were roasted followed by addition of apple powder, *Rauvolfia serpentina* powder, tomato powder and tamarind powder proportionally to make nutritious mix. Auto seal sachets were used for storage of the prepared mix and these were kept in container being air tight. Sensory analysis of reconstituted thick drink was conducted. Reconstitution was done by addition of 100 ml butter milk (*Saras*, plain buttermilk) to the weighed quantity of 25 g nutritious mix and a pinch of powder of cuminseeds after roasting was mixed to it. The formulation of total product of 50g was done which was sufficient for a couple of servings.

2.2. Design of experiments

Developed food product was process optimised through RSM. RSM consists of statistical and mathematical techniques that are beneficial in development, improvement and optimisation procedure(Carley *et al.*, 2004). CCRD comprising 3 independent variables (process variables) at 5 levels was utilised to define the optimum conditions in formulating nutritious mix as presented in table 1. Twenty experimental runs were generated as a result when replication was carried at the center point (0) combination for six times. CCRD comprises of 3 points that are factorial points, centre points and star points and these let to estimate the curvature. The distance in-between the centre of design space and star point is ±α(Singh *et al.*, 2007). Depending upon the one-at-a-time preliminary experiments, the critical factors (process variables), amounts each of apple powder, *Rauvolfia serpentina* and black cumin seeds powder were selected for process optimisation. As per the central composite rotable design, the experiments number in totality is $(2)^n$ + 2n + central points, where n stands for sum total of variables. In present study, there are 3 variables in total for which the experiments' total number for every critical factor will be 20. The codes, -α, -1, 0, 1 and α were given for different 5 levels in every experiment; where $\alpha=2^{n/4}=2^{3/4}$ = 1.682. Thus, the codes were -1.682 (lowest), 0 (middle) and 1.682 (highest) for process variables. Each ofthe critical factors was analysed for its effect on the dependent variables (response variables)– calculated potassium, calculated sodium, fibre and overall acceptability. 'Design-Expert software (9.0)' (Statease Inc., Minneapolis, MN, USA) came out to generate 20 sample combinations (table 2) by the use of

Table 1. Levels of process factors to optimisenutritious mix

	Name	Units	-1 level	+1 level	-alpha	+alpha
A	Apple powder	G	5.0	15.0	1.591040	18.4090000
B	*Rauvolfiaserpentina*	G	0.3	0.7	0.163641	0.836359
C	Black cumin seeds powder	G	3.0	10.0	0.613725	12.386300

design matrix and combinations of variables in experimental runs. Each one of the sample combinations was produced in food preparation laboratory of Banasthali Vidyapith. The values for dependent variable, fibre were estimated through laboratory analysis whereas calculation of potassium and sodium content was done by the use of values provided in Nutritive Value of Indian Food (Gopalan *et al.,* 2007). The semi trained panelists were asked to score in between 1 to 100 depending upon liking of each combination for overall acceptability. The data sheet of the software was entered with all of these values. Order for carrying out the experiments was random.

Table 2. Experimental designs generated and observed responses of nutritious mix

	Generated			**Estimated**			
S. No.	**Apple powder (g)**	***Rauvolfia serpentina* (g)**	**Black cumin seeds powder (g)**	**Potassium (mg)**	**Sodium (mg)**	**Fibre (g)**	**Overall Acceptability**
1	10.00	0.50	12.39	535.35	51.17	3.97	86.53
2	10.00	0.50	6.50	431.35	44.32	2.80	88.73
3	5.00	0.30	10.00	492.52	39.90	3.74	85.40
4	10.00	0.84	6.50	431.35	44.32	3.42	86.60
5	10.00	0.50	6.50	431.35	44.32	3.01	85.80
6	10.00	0.50	0.61	327.33	37.80	2.01	86.80
7	15.00	0.30	10.00	493.78	57.02	3.92	88.00
8	18.41	0.50	6.50	432.39	58.70	2.94	88.60
9	10.00	0.50	6.50	431.35	44.32	3.52	87.53
10	15.00	0.30	3.00	370.18	49.08	2.04	89.53
11	10.00	0.50	6.50	431.35	44.32	2.98	88.66
12	10.00	0.50	6.50	431.35	44.32	3.28	86.06
13	5.00	0.70	10.00	492.52	39.90	4.32	87.33
14	5.00	0.70	3.00	368.91	31.96	2.04	88.06
15	10.00	0.16	6.50	431.35	44.32	2.81	86.60
16	1.59	0.50	6.50	430.28	29.92	3.02	88.13
17	15.00	0.70	3.00	470.18	49.08	3.16	85.80
18	15.00	0.70	10.00	493.78	57.02	4.97	87.60
19	10.00	0.50	6.50	431.35	44.32	2.96	87.13
20	5.00	0.30	3.00	368.91	31.96	2.08	88.46

2.3. Data analysis and optimisation

The data obtained by performing experiments on different combinations were then dispensed for a second order polynomial regression analysis by the use ofleast square regression method and the analysis of the significant ($p<0.05$) effect of allthe process variables on the responses was conducted. The second order polynomial equation given below can define the system behaviour:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x^2 + \sum\sum \beta_{ij} x_i x_j \quad (1)$$

Where Y stands for predicted response, β_0 for the interception coefficients, β_i for the linear term, β_{ii} for the quadratic term, β_{ij} for the interaction term and x_i and x_j are representatives of the levels coded for process variables.

Goodness of fit and the significance of linear, quadratic and interaction effects were calculated through the ANOVA of the regression equation. The independent variables for ANOVA were amounts of apple powder, *Rauvolfia serpentina* and black cumin seeds powder whereas potassium, sodium, fibre and overall acceptability were the dependent ones. Estimation of the validity attained of the models was the function of their coefficients of determination (R^2) values and the lack of fit analysis. A good model should be significant and lack of fit should be insignificant. The value of predicted R^2 should be in reasonable agreement with adjusted R^2. It can be described as the ratio of explained variation which was a degree of fit measure (Chan *et al.,* 2009). The coefficient of variation (CV) can be defined as the dimensionless numeral that measures the degree of variability relative to the mean. Various interactions of any two independent variables along with hold of the third variable's value at the midpoint are depicted through generation of response surfaces and contour plots. Accuracy in geometrical representation as well as useful information accuracy is provided about the system behaviour within the experimental design by the generated contour plots. The aim of optimisation process was to find the levels of process variables that would give potassium, sodium, fibre and overall acceptability as per the set goals. Design-Expert Software's (9.0) numerical optimisation technique was utilised for the concurrent optimisation of these responses. As evident from table 3, desired goals and responses were chosen for each factor in accordance to which the software generated certain optimum solutions. An optimum solution with the highest desirability was chosen as the optimised recipe. This optimised recipe was formulated in food preparation laboratory and further analysis of its nutritional properties was carried out.

Table 3. Optimisation criteria for different process variables and response variables of nutritious mix

Factors/responses	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Apple powder (g)	In range	5.00	15.00	1.00	1.00	3.00
Rauvolfiaserpentina (g)	In range	0.30	0.70	1.00	1.00	3.00
Black cumin seeds powder (g)	In range	3.00	10.00	1.00	1.00	3.00
Potassium (mg)	In range	327.33	535.35	1.00	1.00	3.00
Sodium (mg)	Minimize	29.92	58.70	1.00	1.00	3.00
Fibre (g)	Maximize	2.01	4.97	1.00	1.00	3.00
Overall acceptability	Maximize	85.40	89.53	1.00	1.00	3.00

2.4. Sensory analysis

A selection of semi trained panel of 15 members was done using triangle test to conduct the sensory evaluation (Jellinek, 1985). The overall acceptability (dependent variable of process optimisation) of produced combinations of nutritious mix was evaluated through 100 pointscale. This scale was utilised to acquire fitness of the model for overall acceptability in process optimisation.

2.5. Nutritional analysis

Nutritional evaluation was conducted of the optimised recipe only. Estimations of moisture and ash were done by standard AOAC(2002) procedures. Semiautomatic instrumentation technique was utilised for protein and fat

analysis where, assessment of protein was done through microkjeldahl method using Kel Plus (model no. KES06L, manufactured by Pelican, India) and fat was analysed through soxhlet method by the use of Socs Plus (model no

SCS6, manufactured by Pelican, India). Carbohydrate was calculated using substraction method and estimation of crude fibre was done through acid alkali digestion method(AOAC, 2002). Iron through Wong's method and vitamin C and calcium using titrametric methods were estimated(NIN, 2003).

3. Results and discussion

3.1. Results

3.1.1. Optimisation of parameters for ANOVA

Selection of a suitable model for a response to compare the models on the basis of p-values was done by fit summary statistics. The model is said to be "significant if the p value comes to be <0.05". ANOVA is importantly used to evaluate whether the regression model and individual model coefficients are significant and the goodness of fit of regression model(Fentie *et al.,* 2014). The results of ANOVA for the independent variables' effect on potassium specified that, the two factor interaction design model (2FI) had a significant ($p<0.05$) effect on potassium (dependent variable). The effect of independent variables on sodium indicated that the quadratic model had significant ($p<0.05$) effect on sodium as a dependent variable. Effect of independent variables on fibre depicted that, the linear model had a significant ($p<0.05$) effect on fibre as a dependent variable. Lack of fit had non-significant($p>0.05$) effect on the model, suggesting that model fits the data well. The model (2FI) had a non-significant effect on overall acceptability, which was a response variable, when observed with respect to the process variables. Lack of fit had non-significant ($p>0.05$) effect on the model for this response, depicting that the model fit the data well.

3.1.2. Optimisation of parameters for regression coefficients (R^2)

Table 4 represents the parameters acquired by fitting of potassium, sodium, fibre and overall acceptability data. It also presents regression coefficients of model's intercept, linear, quadratic and cross product terms. The coefficient of determination was utilised to evaluate if the model is fit and adeuate. The model with the higher order polynomial where the model is significant is said to be a suitable model. The nearer is the R^2 value towards the unity, the better is the empirical model said to fit the actual data(Zaibunnisa *et al.,* 2009). R^2 value for sodium was 1.00 which suggested that the model completely fits the actual data. Gan *et al.* (2007) recommended that to obtain good fit model, value of R^2 should be at least 80% (R^2 = 0.80). R^2 value for fibre was 0.84 suggesting a good fit of model. Evidence indicated that generated models were highly adequate if the value of R^2 was > 90% (R^2 >0.90)(Das *et al.,* 2012; Demirel and Kayan, 2012; Seth and Rajamanickam, 2012). R^2 value for potassium was 0.92 suggesting the model to be highly adequate. The model's R^2 value denotes the "proportion of variation in the model rather than random error". The regression model could explain 92% of variations in potassium content, 84% of variations in fibre content, 48% variations in overall acceptability and no variation in sodium content of nutritious mix (table 4). The results of being precise and reliable were depicted by lesser CV values of potassium, sodium and overall acceptability. The greater CV values of fibre revealed the results to be comparatively less precise and reliable.

3.1.3. Effect of process conditions for calculated potassium

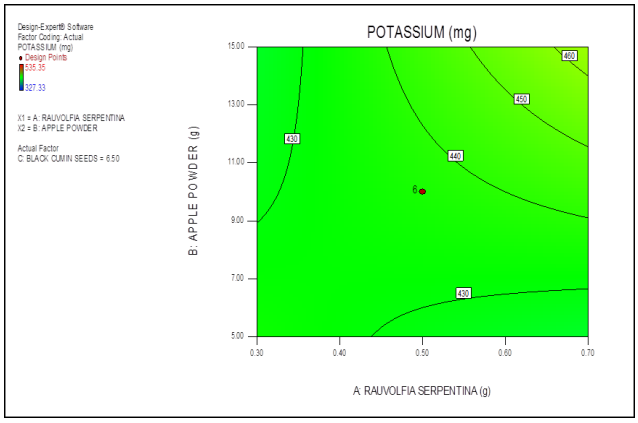
Table 2 depicts the observations for potassium along with the different combination of independent variables. The process variables' effect on potassium as a response of nutritious mix is described by the regression equation given as:

Potassium= 258.95181 + 27.68308 * *Rauvolfia serpentina* - 0.015678 * Apple powder + 31.64362 * Black cumin seeds powder + 12.50000 * *Rauvolfia serpentina* * Apple powder -17.85714 * *Rauvolfia serpentina* * Black cumin seeds powder - 0.71443 * Apple powder * Black cumin seeds powder

Linear curves with *Rauvolfia serpentina* and apple powder are evident from developed response surface (figure 1(a)). The observation was that linear term of *Rauvolfia serpentina* (p=0.125) and cross product of *Rauvolfia serpentina* with black cumin seeds powder (p=0.052) had non-significant effect on the potassium content of nutritious mix. Centre points (6) are depicted through red colour in middle of each graph. Curvilinear plots were observed with *Rauvolfia serpentina* and black cumin seeds powder (figure 1(b)). It was depicted that linear term of black cumin seeds powder (p<0.000) had significant effect and cross product of apple powder with black cumin seeds powder (p=0.052) had non-significant effect on the potassium. The linear curves with apple powder and black cumin seeds powder (figure 1(c)) were developed. The linear term of apple powder (p=0.099) and cross product of *Rauvolfia serpentina* with apple powder (p=0.052) had non-significant effect on the potassium of nutritious mix.

Table 4. Regression coefficients of predicted quadratic polynomial models of nutritious mix (generated by design expert)

Coefficient	Potassium	Sodium	Fibre	Overall acceptability
Intercept	436.350	44.320	3.150	87.370
Linear				
A	7.320	-4.625E-015	0.270	-0.190
B	7.950	8.560	0.130	0.180
C	54.500	3.970	0.800	-0.290
Quadratic				
A^2		0.024		
B^2		0.021		
C^2		0.083		
Cross product				
AB	12.500	5.321E-015		-0.710
AC	-12.500	5.782E-015		0.710
BC	-12.500	6.647E-015		0.510
R^2	0.928	1.000	0.846	0.482
Adjusted R^2	0.894	0.999	0.817	0.243
CV%	3.790	0.150	10.71	1.150



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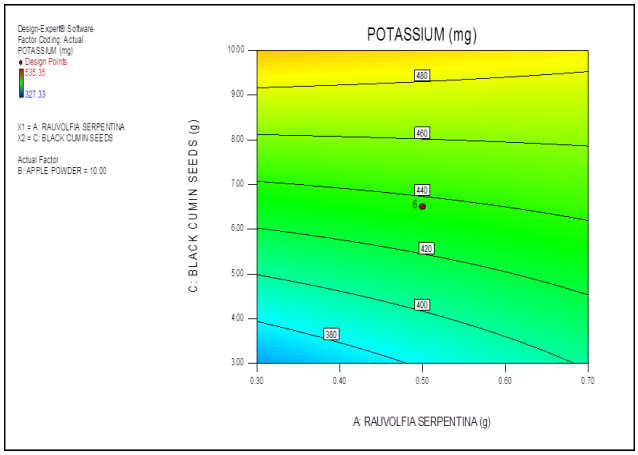

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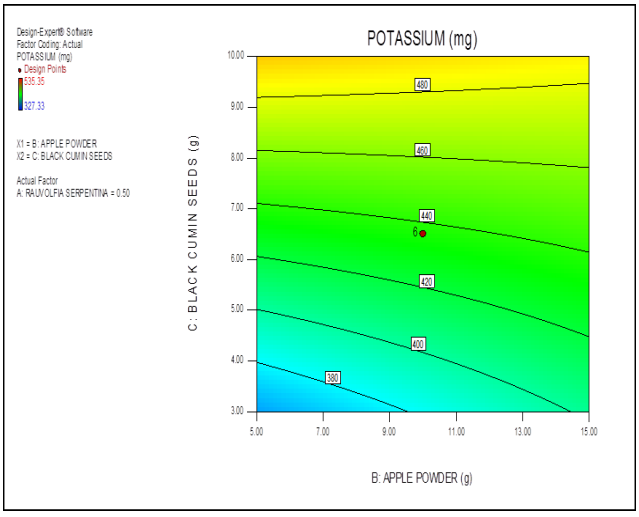


(c)

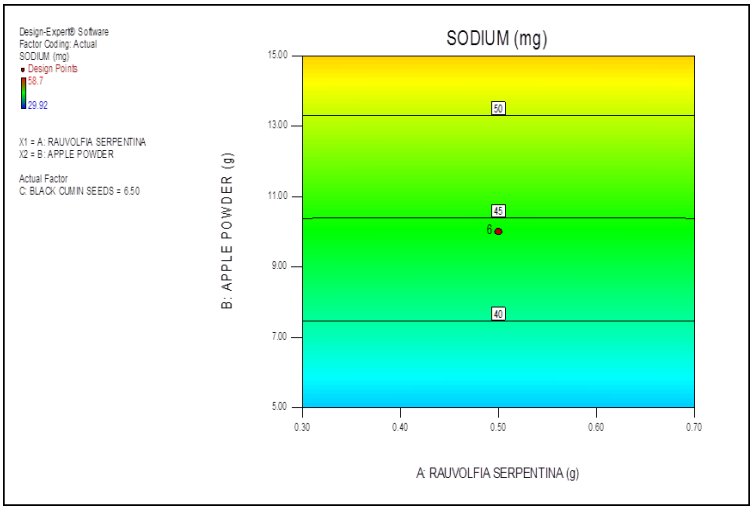
Figure 1. Interactive effect of *Rauvolfia serpentina* and apple powder (a), *Rauvolfia serpentina* and black cumin seeds powder (b) and apple powder and black cumin seeds powder (c) on potassium content of nutritious mix

3.1.4. Effect of process condition for calculated sodium

Table 2 represents the observations for sodium as a response variable with different combination of independent variables. The independent variables' effect on response, sodium of nutritious mix in terms of actual level of variables is described by the regression equation given as:

Sodium= 20.34274 - 0.60775 * *Rauvolfia serpentina* + 1.69508 * Apple powder + 1.04716 * Black cumin seeds powder - 1.50553E-0.15 * *Rauvolfia serpentina* * Apple powder - 1.67980E-015 * *Rauvolfia serpentina* * Black cumin seeds powder + 4.09840E-017 * Apple powder * Black cumin seeds powder + 0.60775 * *Rauvolfia serpentina*^{2} + 8.30971E-004 * Apple powder^2 + 6.74662E-003 * Black cumin seeds powder^2

The response surface developed in figure 2(a) shows linear curves with *Rauvolfia serpentina* and apple powder. The observation was that the linear term of *Rauvolfia serpentina*(p=1.000), cross product of *Rauvolfia serpentina* and black cumin seeds powder (p=1.000) and quadratic term of *Rauvolfia serpentina* (p=0.18) had non-significant effect on sodium (dependent variable). Curvilinear plots were observed with *Rauvolfia serpentina* and black cumin seeds powder (figure 2(b)). Black cumin seeds powder shows significant influence (p<0.000) in terms of linear model, whereas it shows non-significant effect in terms of cross product with apple powder (p=1.000) and quadratic term of black cumin seeds (0.000) shows significant effect on sodium. Linear curves were developed with apple powder and black cumin seeds powder (figure 2(c)). As observed, the linear term of apple powder (p=<0.000) had significant influence whereas cross product of apple powder and *Rauvolfia serpentina* (p=1.000) had non-significant influence and quadratic term of apple powder (p=0.254) had non-significant influence on the response, sodium.



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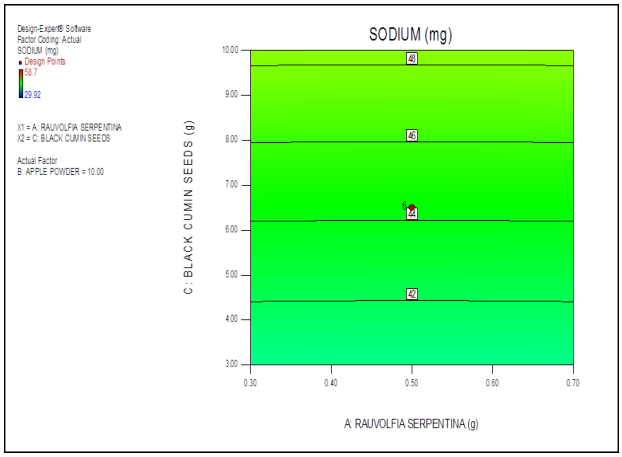

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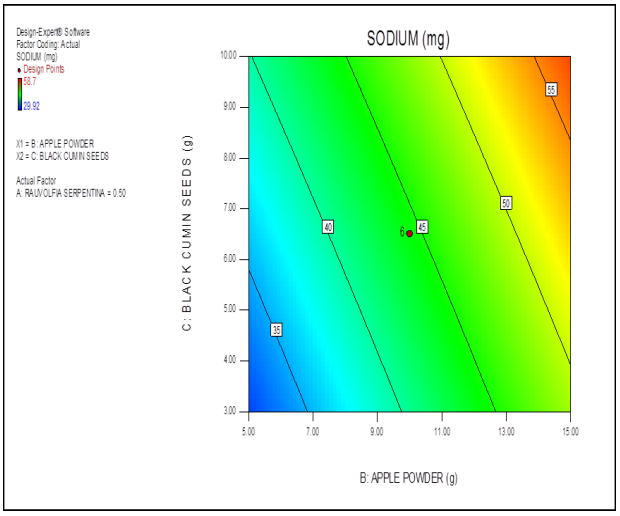


(c)

Figure 2. Interactive effect of*Rauvolfia serpentina* and apple powder (a), *Rauvolfia serpentina* and black cumin seeds powder (b) and apple powder and black cumin seeds powder (c) on sodium content of nutritious mix

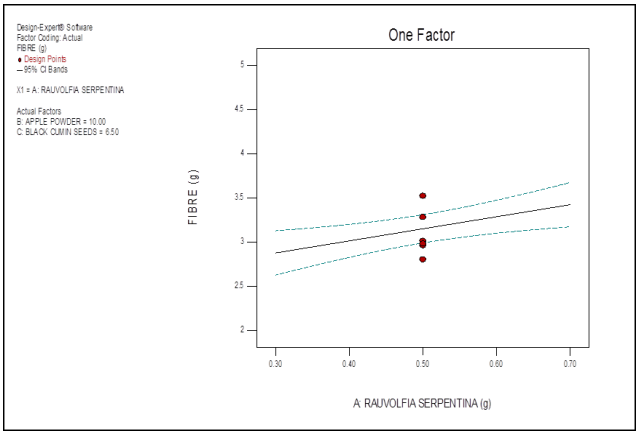
3.1.5. Effect of process condition for fibre

Table 2 depicts the observations for fibre as a response variable with different combination of independent variables. The effect of the independent variables on response, fibre of nutritious mix in actual level terms of variable is described by regression equation given as:

Fibre= 0.86560 + 1.12247 * *Rauvolfia serpentina* + 0.012182 * Apple powder + 0.23267 * Black cumin seeds powder

Linear plots with *Rauvolfia serpentina* (figure 3(a)), apple powder (figure 3(b)) and black cumin seeds powder (figure 3(c)) are shown on the response plots. The interactive effect of amount of *Rauvolfia serpentina* on independent variable, fibre in figure 3(a) indicates maximum fibre content (3.3 g) obtained at 0.70 g of *Rauvolfia serpentina* and minimum fibre content (2.8 g) attained at 0.3 g of *Rauvolfia serpentina*. The interactive effect of apple powder with fibre in figure 3(b)indicates maximum fibre content (3.2 g) observed at 15.0 g of apple powder and minimum fibre content (3.0 g) obtained at 5.0 g of apple powder. The interactive effect of black

cumin seeds powder on fibre (figure 3(c)) specifies the maximum fibre content (3.8 g) observed at 10.0 g black cumin seeds powder and minimum fibre content (2.4 g) was observed at 3.0 g black cumin seeds powder.



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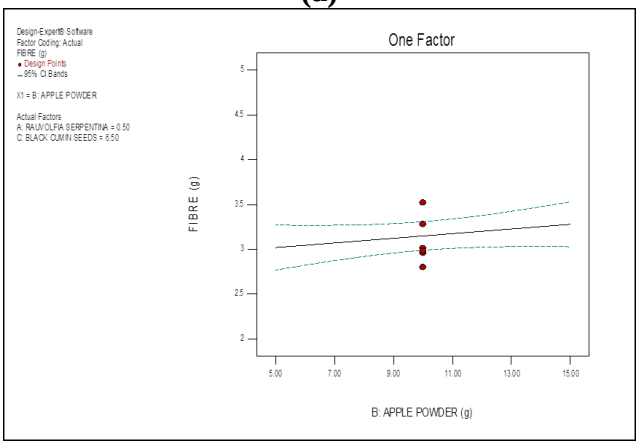

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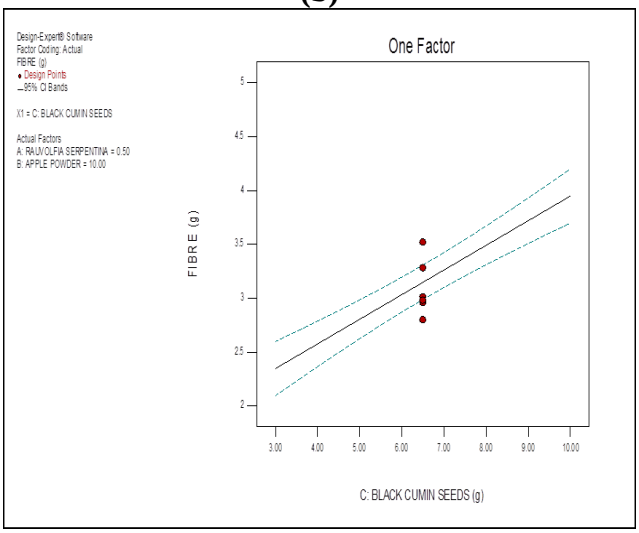


(c)

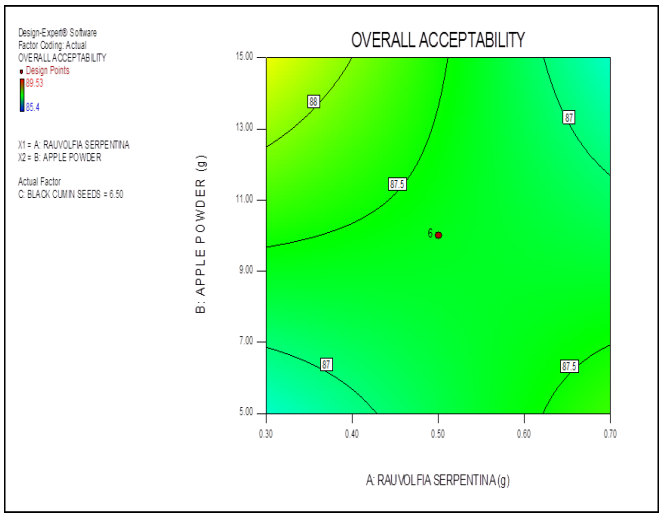
Figure 3. Interactive effect of *Rauvolfia serpentina*(a), apple powder (b) and black cumin seeds powder (c) on fibre content of nutritious mix

3.1.6. Effect of process condition for overall acceptability

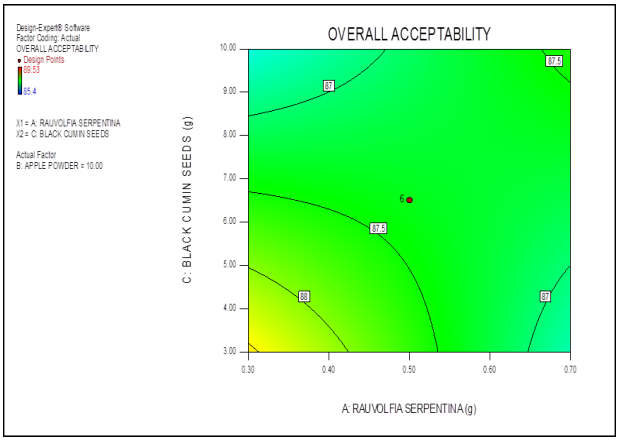
Organoleptic characteristics have a significant importance in modifying, improving, developing and accepting the innovative food products (Yadav *et al.,* 2007). Overall acceptability is a significant factor having direct relation to the likeability of any developed novel food product. Table 2 represents the results observed for overall acceptability with different combination of independent variables. The effect of the independent variables on overall acceptability of nutritious mix in terms of actual level of variables is described by regression equation given as:

Overall acceptability= 89.65441 - 0.44655 * *Rauvolfia serpentina* + 0.20143 * Apple powder -0.87850 * Black cumin seeds powder - 0.70750 * *Rauvolfia serpentina* * Apple powder + 1.01071 * *Rauvolfia serpentina* * Black cumin seeds powder + 0.029000 * Apple powder * Black cumin seeds powder

Curvilinear plots were observed with *Rauvolfia serpentina* and apple powder (figure 4(a)). *Rauvolfia serpentina* in its linear term (p=0.494) and in its cross product term with black cumin seeds powder (p=0.067) had non-significant effect on the response, overall acceptability. The response surface developed in figure 4(b) shows linear curves with *Rauvolfia serpentina* and black cumin seeds powder. Linear term of black cumin seeds powder (p=0.302) and cross product term of *Rauvolfia serpentina* and apple powder (p=0.671) had non-significant on the overall acceptability. Linear curves were developed with apple powder and black cumin seeds powder (figure 4(c)). Linear term of apple powder (p=0.516) and its cross product with black cumin seeds powder (p=0.175) as observed had non-significant influence on the response, overall acceptability.



(a)



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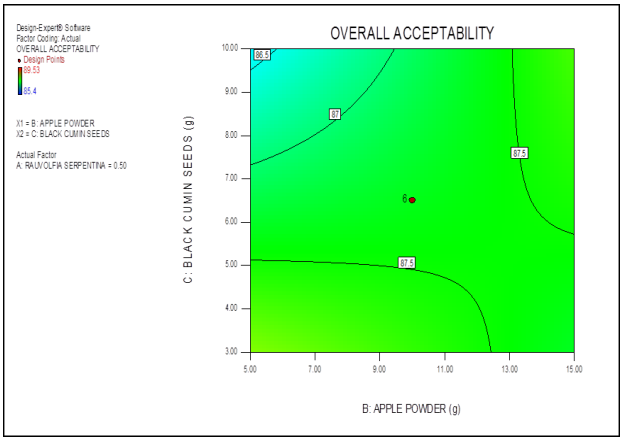

(c)

Figure 4. Interactive effect of *Rauvolfia serpentina* and apple powder (a), *Rauvolfia serpentina* and black cumin seeds powder (b) and apple powder and black cumin seeds powder (c) on overall acceptability of nutritious mix

3.1.7. Optimisation of process parameters

The above mentioned results signify the fact that the quality of nutritious mix does not depend on the particular key factor. The properties of the nutritious mix were determined by significant role of all process variables leading to the next step that was to get the best combination of process variables having the ability of producing the expected properties of end product. Thus, numerical optimisation of the process parameters was done to obtain best combination of nutritious mix. Simultaneous optimisation of the multiple response variables took place through the Design Expert (9.0). Table 3 depicts the chosen desired goals for each factor and response. Thirty solutions of independent variables with the predicted responses were generated through the software. The range between 0.569-0.627 was obtained for desirability of optimum solutions. Four optimum solutions were attained depending upon the highest desirability. The optimum recipes consisted (i) 5.00 g apple powder, 0.70 g *Rauvolfia serpentina* and 10.00 g black cumin seeds powder with 477.71 mg potassium, 39.85 mg sodium, 4.09 g fibre and 87.61 overall acceptability score; (ii) 5.00 g apple powder, 0.70 g *Rauvolfia serpentina* and 9.96 g black cumin seeds powder with 477.15 mg

potassium, 39.81 mg sodium, 4.08 g fibre and 87.61 score of overall acceptability; (iii) 5.00 g apple powder, 0.70 g *Rauvolfia serpentina* and 9.92 black cumin seeds powder with 476.49 mg potassium, 39.76 mg sodium, 4.07 g fibre and 87.61 score of overall acceptability, (iv) 5.04g apple powder, 0.70 g *Rauvolfia serpentina* and 10.00 g black cumin seeds powder with 477.78 mg potassium, 39.92 mg sodium, 4.09 g fibre and 87.61 overall acceptability score in about 51 g of products. The range of processes which might possibly be contemplated as the optimum range for best quality food product in terms of potassium, sodium, fibre and overall acceptability was provided through these optimum solutions. These were suitable conditions to formulate nutritious mix providing nutritional adequacy without compromising the organoleptic characteristics. Formulation of optimised and enhanced nutritious mix was done using the best solution chosen. Solution 1 with the maximum desirability value of 0.627 in a range of 0.569-0.627, along with in range potassium, minimum sodium, maximum fibre and maximum overall acceptability was chosen for subsequent laboratory estimation.

3.1.8. Nutritional analysis

The nutrients estimation was done as per 100 g quantity. The optimum recipe was adequate in terms of nutrition having 72.46 g carbohydrate, 5.99 g moisture, 0.94 g ash, 15.63 g protein, 3.29 g fat, 1.69 g crude fibre, 9.03 mg iron, 236.29 mg calcium and 1.09 mg vitamin C.

3.2. Discussion

Rapid urbanisation, industrial development and consequential variations in lifestyles of individuals have resulted in progressive formulations of instant dry mixes and ready-to-eat convenient food products(Balasubramanian *et al.,* 2014). These products are gaining popularity as a result of ease of consumption and increased shelf life (Bunkar*et al.,* 2014) along with reducing the time for preparation by eradicating numerous steps of cooking (Balasuramanian *et al.,* 2014). Several researches have been carried out for the development of instant foods including soy-fortified instant *upma* mix (Yadav and Sharma, 2008), *halwa* dry mix (Yadav *et al.,* 2007), pearl millet based *upma* dry mix (Balasubramanian *et al.,* 2014) and instant wheat porridge (*dalia*) mix (Khan *et al.,* 2014).

With convenience, there also comes an increased demand of consumers for value added products with health advantages (Gadhiya *et al.,* 2015). The improvements in the understanding of association between nutrition and health lead to the functional foods development which is a practical and new approach for the achievement of optimum status by promoting the state of being healthy and thus probably decreasing the diseases' risk (Siró *et al.,* 2008). Such products that are claimed to be healthy and have functional and/or heath properties are gaining priority in researches in production of novel foods(de Sousa *et al.*, 2011). Nutraceutical potential of medicinal plants makes them beneficial to be used in medicine and for therapeutic purposes (Harsha and Aarti, 2015). Various herbs possess many therapeutic properties including antioxidative, antihypertensive, anti-inflammatory, antidiabetic, antimicrobial, etc (Oraon *et al.*,2017). Thus incorporation of these herbs asfunctional foods can provide several health benefits to the consumers.Several researches related to development of food products incorporating herbs and other functional foods have been conducted including herbal juice development from traditional Indian plants using *Citrus limetta* as base (Harsha and Aarti, 2015) powdered food developed with addition of *Spirulina* (Santos *et al.,* 2016), development of an apple snack rich in flavonoid (Betoret *et al.,* 2012), development of blended papaya- Aloe verayready to serve

beverage(Boghani *et al.,* 2012)to enhance the nutritional properties of the food products. In the present study *Rauvolfia serpentina*, black cumin seeds and apples were incorporated as functional ingredients to enhance the nutritional properties of the food product.

Rauvolfia serpentina and black cumin seeds were found to significantly increase the amount of fibre, whereas apple powder and black cumin seeds significantly decreased the amount of sodium in nutritious mix. This recipe has low sodium content that can be an additional benefit as a result of direct relation of sodium consumption with hypertension in humans (Malviya and Sason, 2016). Apples provide a good source of carbohydrates and vitamins and have less contribution in calories along with no contribution in fat, sodium or cholesterol (Harris *et al.,* 2007). Black cumin seeds constitutes of proteins, minerals, vitamins, enzymes, carbohydrates and fats having about overall fat contained in the form of omega-3 and omega-6 fatty acids in rich amount. They also contain vitamins A, B_1, B_2, B_3 and C as well as calcium, iron, magnesium, zinc and selenium(Hussain and Hussain, 2016). Thus, the incorporation of apples, *Rauvolfia serpentina* and black cumin seeds in nutritious mix have together contributed in obtaining the goal of low sodium and high fibre content.

Optimisation of ingredients in the food formulation is essential for the development of a product. There are number of techniques that are available to draw the best levels of input variables that in turn optimise their responses (Nadeem *et al.,* 2012). RSM is the one which is stated to be an effective measure for optimising a process when the independent variables are hypothesised to possess a dominant or accumulative effect on the desired responses(Martínez *et al.,* 2004). The observations of effect of independent variables on fibre in this study represented the significant effect of linear model on the response. The results for effect of independent variables on sodium showed that the quadratic model had significant effect on response. The 2FI model indicates the non-significant process variables' effecton the response, overall acceptability. Similar studies were conducted, (i) process optimisation for formulating cowpea incorporated instant *kheer* mix by the use ofRSM was conducted in which amounts of cowpea and malted wheat flour and cowpea soaking time and were the process variables and protein, crude fiber and overall acceptability were the responses. Results revealed that the models had non-significant effect on the response, crude fibre and overall acceptability (Gupta *et al.,* 2014); (ii) optimisation of multigrain premix (MGP) to develop high protein and dietary fibre biscuits through RSM was conducted in which levels of MGP and wheat flour concentration were the process variables and protein, soluble, insoluble fibres, biscuit dough hardness, breaking strength and overall acceptability were the response variables. Results revealed that the incorporation of MGP significantly increases the soluble and insoluble fibres content of biscuits(Kumar *et al.,* 2015a); (iii) process optimisation of vegetable cereal mix using RSM was conducted in which amounts of *Trigonella foenum-graecum* and *Gymnema sylvestre*and soaking time of *Trigonella foenum-graecum* were the independent variables and fat, fibre, carbohydrate and overall acceptability were the responses. Results for effect of process variables on the response showed that 2FI model had a significant effect on fibre(dependent variable) whereas 2FI model had non-significant effect of process variables on the response, overall acceptability (Gupta *et al.,* 2016). There are some other similar studies in which food product development of various premixes was conducted using RSM to enhance their nutritional characteristics like optimisation of instant dalia dessert pre-mix formulation by the use of RSM (Jha *et al.,* 2015) and production of

multigrain premixes-its effect on rheological, textural, and micro- structural characteristics of dough and quality of biscuits(Kumar *et al.*, 2015b).

The nutritional analysis of optimum recipe resulted in recipe being nutritionally adequate and was rich in iron and good source of calcium. Bhadana *et al.* (2016) carried out a study on product development and nutrients evaluation of value added product incorporated with spirulina powder, soya flour and rice flour. for nutritional analysis revealed that the sample The results containing spirulina powder 20 g, soya flour and rice flour had moisture content of 2.48 percent per 50 g and 1.10 percent per 50 g fat content that were similar to the present study. The instant foods are beneficial in saving very important resources such as time and energy(Lohekar and Arya, 2014) and the value addition of functional ingredients in optimum levels can enhance the nutritional properties of these foods without compromising their acceptability. The nutritious mix owing to low sodium can be of benefit to hypertensive patients as rise in blood pressure is a common disorder in India(Raghupathy *et al.*, 2014) affecting all age groups.

4. Conclusions

RSM came out to be a successful tool to derive the best combination of different processes (amount of apple powder, *Rauvolfiaserpentina* and black cumin seeds) for formulation of nutritious mix. Out of 30 suggested combinations, 4 combinations had highest desirability value (0.627) in comparison to others. Recipe having 5.00 g apple powder, 0.70 g *Rauvolfia serpentina* and 10.00 g black cumin seeds powder with 477.71 mg potassium, 39.85 mg sodium, 4.09 g fibre and 87.61 overall acceptability score was selected optimum recipe and subjected for further nutritional analysis. Optimum recipe had adequate potassium, low sodium, high fibre and high overall acceptability. It was rich in iron and good source of calcium. Nutritious mix is the instant food which is convenient to be used, affordable and of nutritional importance as well. Incorporation of Indian medicinal herbs into it makes it highly beneficial for various ailments like hypertension.Thus, it can be effortlesslyutilised by the consumers as food add-on devoid of any variation in their regular diets.

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IMPACT OF ALGAE ADDITION ON BREAD PROPERTIES AND CONSUMERS BEHAVIOR-PRELIMINARY RESEARCH

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ABSTRACT

The paper presents some preliminary results related to the effects of *Spirulina* (*S. platensis*) powder addition in ratios of 1%, 3% and 5%, respectively on some properties of white bread. Experimental data indicate that the fortification of bread results in significantly higher values of polyphenols and proteins contents, antioxidant activity and minerals level, the higher *Spirulina* ratio the higher values were obtained. On the other side, the consumers studies indicated that spirulina-bread is not accepted, the highest ratio of addedd *Spirulina* the lowest scores for sensorial attributes being obtained. The main reasons indicated by the consumers for the spirulina-breads rejection were the grey-green color of breads often associated with mold presence, the caverns in the bread crumb and the "herbal" taste. Further investigations are required to find spirulina-bread formulations able to meet both the high functionality and consumers sesnsorial expectations.

1. Introduction

Bread is the staple food in many countries as an important source of complex carbohydrates and proteins. Its main disadvantage is related to the low nutritional content because the vitamins and minerals are in the bran and germ which are discarded during milling. Bread fortification (EU regulation 1925/2006) corrects the nutrients deficiencies, balances the total nutrient profile and restores nutrients lost during baking. Although fortified flours with iron exist on the market of 68 countries (around 2 billions beneficiaries) the resulted products are usually rejected by the consumers due to poor organoleptic and sensory quality and side effects (digestive distress) caused by the minerals addition in inorganic state (sulphate, oxide). Multiples studies demonstrated that organic bond minerals are absorbed more efficiently than inorganic compounds (Rider et al., 2003). Spirulina (*Spirulina platensis)*, a blue-green microalgae has arisen as a promising sustainable, balanced and complete source of organic bioactive compounds which help nurture and sustain physical and mental health (Ama Moor et al., 2016).

The paper presents some preliminary research related to the impact of *Spirulina* (*Spirulina platensis*) powder addition on some nutritional values of bread as well on the consumer acceptance of spirulina-bread.

2. Materials and methods

Spirulina (*Spirulina platensis*) powder was purchased from Hofigal Company whils the other ingredients (wheat flour, salt, yeast) from the local market.

2.1. Bread preparation

Four bread formulations in which the amount of *Spirulina* powder was 1%, 3% and

5% were prepared in respect of the same amounts of flour, white yeasts and water (100g, 5 g, 50 mL). The ingredients were mixed for 30 minutes and the dough was allowed to rest for about 30 minutes at room temperature. After moulding in round shape, dough pieces were proofed at 30ºC for 45 minutes, remixed 2 minutes, moulded in rectangular shape put into baking tins at 30ºC for another 25 minutes. The tins were kept for 30 minutes at 220ºC in a MATINA Oven prior pre-heated at 150ºC. After baking, the bread loaves were allowed to cool at ambient temperature for 3 h and then slices and send to analysis.

2.2. Polyphenols content

Bread extract was prepared accordint to the method proposed by Michalska et al. (2007). According to it, 1g of bread samples was mixed with 10 mL methanol for 2h at 37ºC and the centrifuged at 12,000 x g rpm for 15 minutes. The supernatant was used for both polyphenol and DPPH analyzes.

For polyphenol content 0.25 mL of bread extract was mixed with 0.25 mL of diluted Folin-Ciocalteu's reagent (water 1:1 v/v), 0.5 mL of sodium carbonate (Na_2CO_3) solution (0.2 g/ml) and 4 mL of water and allowed to rest for 25 minutes at room temperature. After centrifugation for 10 minutes at 2,000 x g the absorbance of supernatant was red at 725 (UV-VIS spectrometer Lambda 35) against gallic acid standards Michalska et al. (2007). The results were expressed as mg gallic acid/g of bread.

2.3. Antioxidant activity (DPPH method)

The method proposed by López-de-Dicastillo et al. (2010) adapted to our study was used to measure the antioxidant activity of bread varieties. 5 mL of bread extract was put in contact with 5 mL DPPH for 30 minutes in the darkness at room temperature. The absorbance of sample was read at 515 nm using as compared to blank made of 5 mL ethanol and 5 mL DPPH. The absorbance was calculated as:

$$\text{Antioxidant activity (\%)} = (1- Abs_s /Abs_b)x100 \quad (1)$$

where: Abs_s, Abs_b are the absorbance of sample and blank, respectively;

2.4. Mineral content (Ca, Mg, Fe, Zn)

Bread digestion occurred according to the method described by Berghof (MWS–2/ Food, Pharma, Cosmetics which provides the contact of 300 mg of bread with 6 mL of HNO_3 and 1 mL HCl followed by heating in the microwave oven (MWS-2) for 5, 10 and 15 minutes at 145ºC, 170ºC and 200ºC. The content of Ca, Mg, Zn and Fe was read by using atomic absorbtion spectrometer Perkin Elmer AAnalyst 800.

2.5. Proteins content

Kjeldahl method was used to determine the protein in bread samples (AOAC 945.18-B method). Around 2 g of ground bread, sieved at 2 mm and dried at 105ºC to constant weight was digested (7 g K_2SO_4, 5 mg powder of Se, 12 mL H_2SO_4, 5 mL H_2O_2 35%) for 20 minutes at 420ºC. After cooling and dilution with 50 ml of deionised water the solution was distilled and the distilled was collected in 25 mL boric acid solution of 4%. After titration with HCl 0.2 N the protein content was calculated according to the Eq (2):

$$\text{Protein content (mg N-NH}_3\text{)} = V \cdot 2.803 \quad (2)$$

where V is the volume of HCl used for titration, mL; 2.803 is the amount of $N\text{-}NH_3$ corresponding to 1 mL of HCl 0.2 N

2.6. Consumer acceptance study

Fifty assessors of which 25 were females and 25 male were included in the sensorial analysis of the spirulina-bread varieties. Prior to analysis they were instructed in order to develop a consensual vocabulary for the

interested issues: color, taste, texture, smell and overall acceptability. They were asked to rank each descriptor according to their intensity in a range of 1 (weak) to 5 (strong).

2.7. Statistically processing of experimental data

All analyzes were performed in triplicate and results are expressed as mean ±standard deviation. The statistical significance of the differences obtained between bread varieties was evaluated using the student *t*-test. Probability value $p < 0.05$ was considered statistically significant.

3.Results and discussions

3.1.Pholyphenols content and antioxidant activity

As in Figure 1 is displayed, the highest level of polyphenols can be noticed in the bread enriched with *Spirulina* powder. By increasing the amount of incorporated *Spirulina* with 1%, 3% and 5% the polyphenols content rises 1.19-fold, 1.41-fold and 2.-73-fold as compared with un-supplemented bread.

Higher antioxidant activity was also obtained after the addition of *Spirulina* in the bread (Figure 1). It increased to 11.06% in bread with 1% *Spirulina*, 13.21% in bread with 3% *Spirulina* and 15.46% in bread with 5% *Spirulina* from 10.24% in plain bread. Several bioactive compounds founded at high level in Spirulina (phycocyanin, total carotenoids and β-carotenoids, phenolic compounds) can be considered responsible for the high polyphenols content and antioxidants activity of fortified bread (Saharan and Joode, 2017; Marco et al., 2014). Good correlation was observed between polyphenols content and antioxidant activity of spirulina-bread (R= 0.9369). Our values for polyphenols content and antioxidant activity are close to those obtained by Saharan and Joode (2017) of which bread formula included *Spirulina* powder at 98:2, 96:4, 94:6 and 92:8 (yeast 3g, sugar 10g, salt 1.75g, water ± 60 ml).

3.2. Minerals content

Significant improvement in the content of Ca, Mg, Fe and Zn of fortified bread as compared to plain bread was noticed (Figure 1). Thus, Ca increased 1.21-fold in 1% spirulina-bread, 1.88-fold in 3% spirulina-bread and 2.06-fold in 5% spirulina-bread whilst Mg displayed higher growth increments of 1.39-fold in spirulina-bread, 2.42-fold in 3% spirulina-bread and 2.60-fold in 5% spirulina-bread. The highest amount of Fe content was obtained in 5% spirulina-bread of 5.01 mg/100 as compared to 3.87 mg/100 g in 3% spirulina-bread, 2.01 mg/100 g in 1% spirulina-bread and 0.96 mg/100 g in un-fortified bread. The increase in Zn content was 1.61-fold, 1.99-fold and 2.31-fold, respectively much higher than the results of Saharan and Joode (2017) who reported that the Zinc content was differed non-significantly. It is obviously that the addition of *Spirulina* powder resulted in the increase of minerals content in bread.

3.3. Proteins content

The fortification of bread with *Spirulina* powder also resulted in the increase of the proteins level up to 1.88-fold in the 5% spirulina-bread explained by the high level of proteins in the *Spirulina* powder (Simo et al., 2005). Ak et al. (2016) reported close values of proteins content in white bread enriched with 10% *Spirulina*.

3.4. Consumer acceptance study

The results of sensory evaluation study of spirulina-bread varieties are displayed in the Figure 2. According to it, the addition of *Spirulina* decreases all the sensory descriptors, the highest decrease being noticed in the case of bread enriched with 5% *Spirulina* and the lowest for the bread with 1% *Spirulina*.

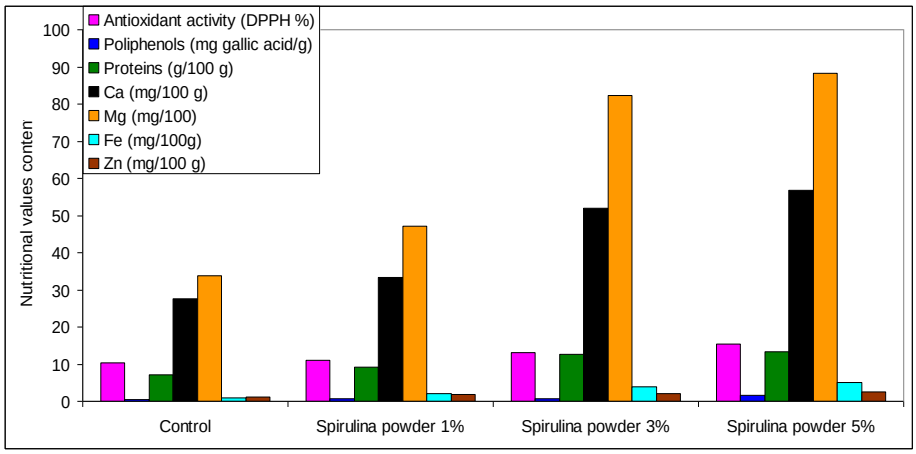

Figure 1. The characteristics of spirulina-bread

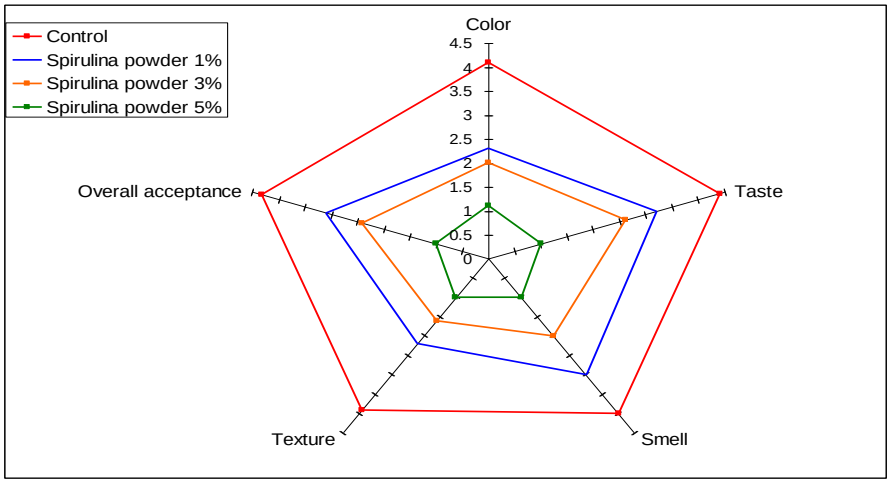

Figure 2. The sensorial chart related to spirulina-bread

Figure 3. Appearance of spirulina-breads

Thus, in the 1% spirulina-bread the color descriptor decrease 1.78-fold, taste 1.37-fold, smell 1.32-fold, texture 1.77-fold and overall acceptability 1.38-fold. In the 5% spirulina-bread, the decreases were 3.72-fold for color and 4-fold for the other descriptors. The main reasons indicated by the consumers for the spirulina-breads rejection were the grey-green color of breads often associated with the mold presence, the caverns in the bread crumb and the "herbal" taste (Figure 3).

4. Conclusions

Preliminary research indicates that by addition of Spirulina the nutritional value of bread in terms of proteins, polyphenols and minerals levels is significantly enhanced. The antioxidant activity of bread also increases. These confer functional property to spirulina-

bread and recommend it not only for the vulnerable consumers (osteoporosis, calcium and magnesium defficiencies, anemia) but also to the healthy consumers interested in the pro-health-food.

On the other side, the consumers studies indicated that spirulina-bread is not accepted, the highest ratio of addedd *Spirulina* the lowest scores for sensorial attributes being obtained. Further investigations are required to find spirulina-bread formulations able to meet both the high functionality and consumers sesnsorial expectations.

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PERSPECTIVES OF RASPBERRY USE IN OENOLOGY

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ABSTRACT

The main physical-and-chemical parameters of raspberry of Polka and Zieva varieties were given. Reliable difference between berries of raspberry depending on the variety and the harvest year was determined. Very high correlation dependencies between physical-and-chemical parameters of raspberry berries were established. Organoleptic parameters of raspberry berries were studied and the best variety was defined.

Output of juice from raspberry berries of two pomological varieties was shown, and essential influence of the variety factor and method of squash processing was ascertained. Qualitative parameters of fresh juice from raspberry berries including phenolic substances were under research and the best variety by this parameter was found. Calculations of raspberry juice strength with alcohol to volume fraction of alcohol by 16% were made. The way of using raspberry alcohol juice was proposed.

1.Introduction

Market of alcohol production is quite saturated with quality alcoholic beverages, the volume of production and consumption of beverages is increasing. There is a large number of new varieties of multicomponent alcoholic beverages on the consumer market, which include compositions that can change biological activity of ethyl alcohol.

A wide range of liqueur-and-alcoholic products is based on the use of various vegetative raw materials in the production of semi-finished products - aromatic alcohols, liqueurs, alcoholic juices, fruit drinks, etc. On the one hand, it opens practically unlimited possibilities for the formation of taste, aroma, colour of liqueur-and-alcoholic products, on the other hand, difficulties in determining and rationing of its chemical composition, ensuring of constant and predictable consumer characteristics arise (Kuzmin and others, 2012).

Liqueur-and-alcoholic industry uses a large amount of raw materials which assortment is constantly replenished by new types. Specification and deepening of knowledge regarding biochemical principles of blending of receipt ingredients during production of alcoholic beverages promotes the improvement of the quality of semi-finished products and finished products. The analysis of accumulated domestic and foreign data makes it possible to make corrections in the technological process and expand the raw material assortment for alcohol industry (Holovko and others, 2017, Altinier et al., 2007, Ashurst, 2005), in particular viniculture industry.

Variety of beverages is achieved by using semi-finished products of liqueur-and-alcoholic production which include alcoholic juices, fruit drinks, liqueurs and aromatic alcohols (Domaretsky, 2005; Litovcenko and Tiurin, 2002).

Alcoholic juice is a semi-finished product of liqueur-and-alcoholic or wine production with strength of 16 ... 25%, made by pressing of fruit-berry squash and by bleaching of received juice with rectified ethyl alcohol from food raw materials (Kovalevskaya, 1997).

The main source of many vitamins is natural raw materials, including berries. Berries as a source of vitamins, medicinal remedy and a human digestive regulator have an advantage over other foods. Raspberry is among these berries. This culture is characterized by high nutritional value, a pleasantly delicate aroma, a sweet taste.

Raspberry is a valuable berry culture, spread in all natural-and-climatic zones of Ukraine. It takes the second place after strawberries by taste and dessert qualities. Raspberry berries are eaten fresh, dried, frozen, canned and processed (drinks, juices, syrups, extracts, wines, preserves, jams, etc.). They are delicate, sweet, with a specific aroma. Contain 5-10% of sugars (mainly fructose and glucose), 0.7-2.5% of organic acids (apple, lemon, wine, salicylic), fiber - 5,1%, pectin - 0,6%, vitamin C - up to 50-70 mg/100 g, catechins up to 80 mg/100 g and anthocyanins up to 250 mg/100 g, potassium - 225 mg/100 g, as well as iron, manganese, copper, boron, iodine and vitamins B_2, E , antibiotics, essential oils, sterols. Raspberries prevail over black currant and strawberries by the number of carotene, vitamins B_2 and E. Harmonious ratio of vitamins makes it possible to use raspberry berries for the treatment of ulcerous diseases and various vascular permeability disorders (Georgievsky and others, 1990; Tomenko, 2013; Domaretskyi and Prybylskyi, 2005). Phytotherapists used leaves and branches of raspberry for the preparation of tinctures for treating cough and angina. Ointments for treatment of skin diseases can be made from fresh raspberry leaves. It belongs to the category of the most valuable berries (Yanovsky and others, 2009).

Raspberry berries, in which raw materials of Ukraine are rich, are the real treasury of biologically active substances. They have a clear expressed physiological effect on the human body. Natural resources allow not only to prepare them for local needs, but also to use in a wine-making sector.

The purpose of our work is to study the quality of the use of raspberry berries in viniculture.

2. Materials and methods

The research was conducted in 2015-2016 in conditions of the laboratory of the Department of technology of storage and processing of fruits and vegetables of Uman National University of Horticulture with raspberry of pomological varieties Pegas and Zieva. Raspberry was gathered at the stage of technical ripeness. Preparation of raspberry berries included sorting, washing, removing from the fruit-stalks, inspecting and crushing. It was received juice from raspberry berries by pressing in a laboratory. Enzyme preparation: Fructosim Gx20 in the amount of 0.03% of the weight of the squash in order to obtain a larger output of juice before pressing.

The content of the components of the chemical composition in berries and juice was determined from at least two kilograms of raw materials.

Physical-and-chemical parameters were determined according to generally accepted methods, sampling and preparation for analysis - according to GSTU 46.067-2003; mass concentration of sugars (in terms of invert) by ferricyanide method according to DSTU 4954:2008; mass concentration of titrated acids by titration of 0,1 n alkaline solution (NaOH) according to DSTU 4957:2008; vitamin C by iodometric method

according to GOST 24556-89; mass fraction of dry soluble substances in raw materials and juices by refractometric method according to DSTU ISO 2173: 2007, mass concentration of phenolic substances by using Folin–Ciocalteu reagent. Rectified alcohol with strength of 96% was used for bleaching. Calculations of fortifying by alcohol to volume fraction of alcohol of 16% were carried out according to the general rules KDU 00011050-15.94.10-1: 2008 for processing fruits and berries for wine materials (Lytovcheko and others, 2002).

Researches were conducted in four replications randomized in time. Data processing was performed by using Microsoft Office and Statistica 10 programs. Replicates of each experiment were processed by descriptive statistics to determine the coefficient of variation. In the case of a weak variation of the samples data of each experiment, received values were statistically processed. Connections between the factors were determined by using dispersion and correlation-and-regression analysis (Table 1).

Experiment characteristics:

- number of factors – 2;
- number of blocks – 2;
- number of runs – 10 (nc=4, ns=4, nc_0=1, ns_0=1);
- alpha for rotatability – 1,4142, alpha for orthogonality – 1,4142.

The number of analytical replications – 3. The coefficients of variation by analytical replications were less than 10 which corresponded to a non-significant variation (Moiseichenko, 1992, Maltsev and others, 1973).

Table 1. Steps and levels of experiment variation

Factor	Low Value	Low Label	Center Value	Center Label	High Value	High Label	StarLow Label	StarHigh Label
Temperature, C	30	Low	45	Center Pt	60	High	23,7	66,2
Duration of infusion, minutes	8	Low	16,5	Center Pt	25	High	4,4	28,5

3.Results and discussions

The leading factor determining the activity of plant development was heat and moisture supply. However, these factors were extremely unstable, so two climatic years and two yields can never be the same (Haidai, 2011). The influence of weather conditions affected the formation of raspberry quality. The main parameters characterizing the quality of raw materials for fruit-and-berry winemaking were the mass concentration of dry soluble substances and sugars. For two years of research, berries of Zieva variety in 2016 (Figure 1, a, b) accumulated the mass concentration of dry soluble substances and sugars better – 13.4% and 8.8% respectively.

The important feature of the fruits suitable for processing in fruit-and-berry winemaking is titrated acidity. There was no significant difference between the varieties by accumulation of titrating acids. The mass concentration of titrating acids varied within 1.60-1.92% over two years of research, that is, was quite high (Figure 1, c). Therefore, you need to add water in order to normalize the mass concentration of titrating acids if you have the purpose to make unstrong wine materials from raspberry.

One of the main parameters of consumer quality of fruits in fresh state is the content of vitamin C (Hernández et al., 2006). There was no significant difference between the varieties by accumulation of vitamin C (Figure 1, d).

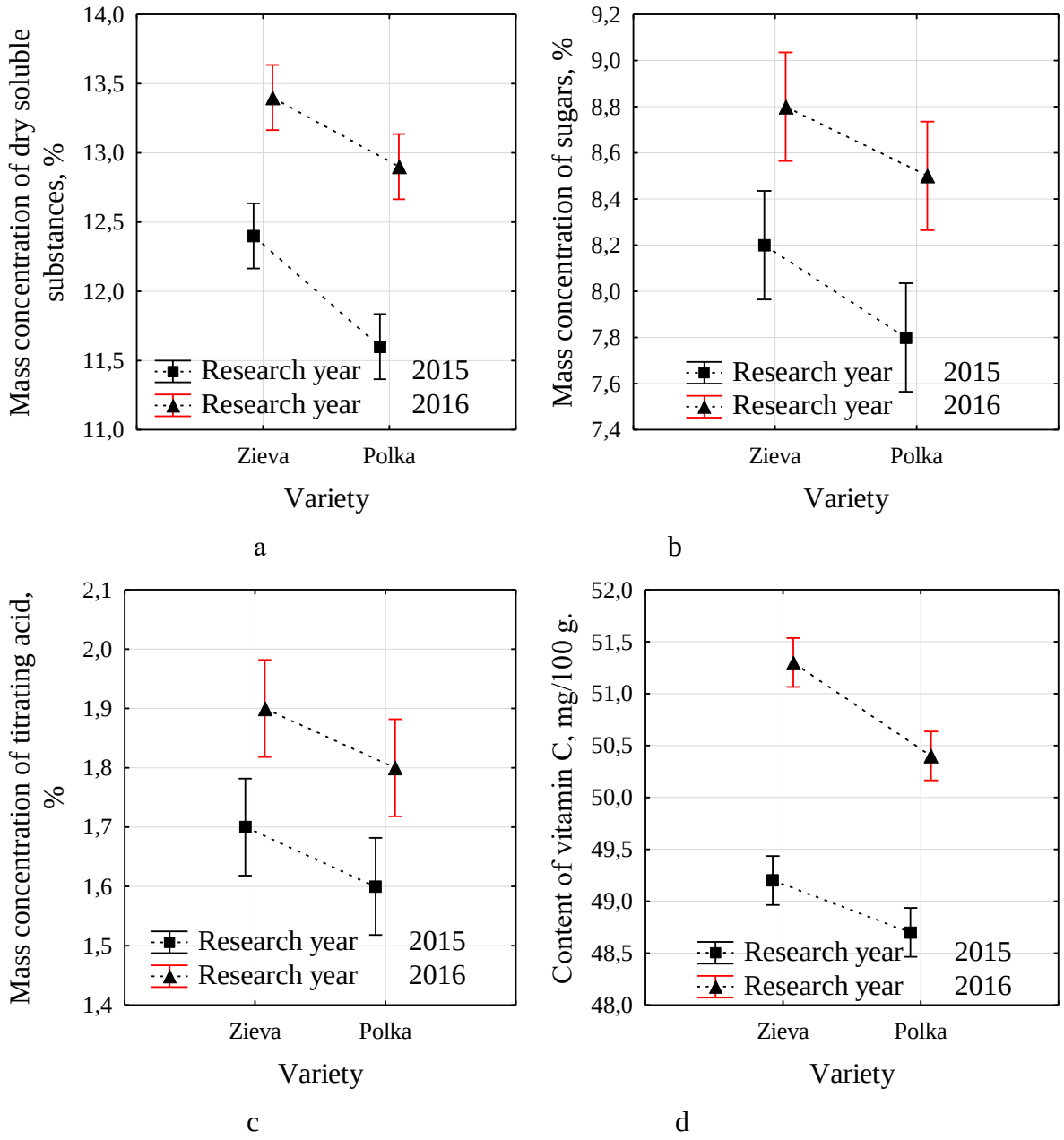

Figure 1. Amount of the components of chemical content of raspberry berries a – Mass concentration of dry soluble substances, %; b – Mass concentration of sugars, %; c – Mass concentration of titrating acid, %; d – Content of vitamin C, mg/100 g.

The content of vitamin C in raspberry berries of Zieva variety in 2015 was 51.3 mg/100g, and the amount of vitamin C in Polka variety in the same year was 50.4 mg/100g.

It was found that the quality of raw materials significantly differed depending on the year of yield and variety.

It was determined very high correlation dependence (r=0.98) between accumulation of dry soluble substances and mass concentration of sugars (Figure 2); (r=0.98) between titrated acidity and sugars content (Figure 3); (r=0,96) between the content of vitamin C and mass concentration of dry soluble substances (Figure 4); (r=0,95) between titrated acidity and dry substances (Figure 5); (r=0.93) between the content of vitamin C and content of sugars (Figure 6); (r=0.92) between the content of vitamin C and titrated acidity (Figure 7).

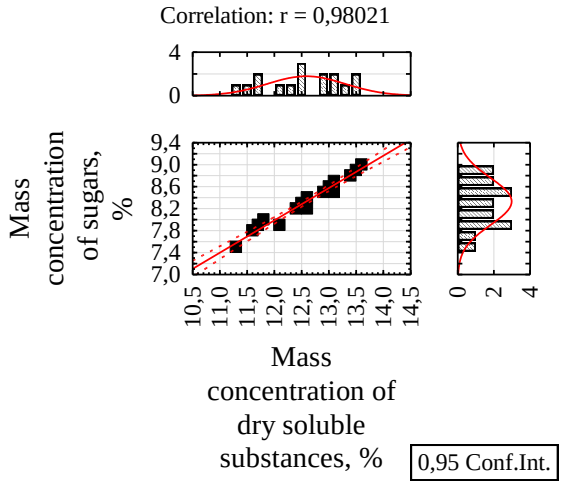

Figure 2. Accumulation of dry soluble substances and mass concentration of sugars

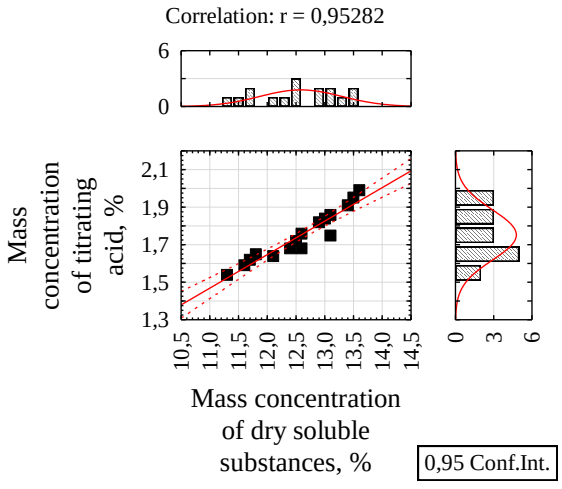

Figure 3. Correlation connection between titrated acidity and sugars content

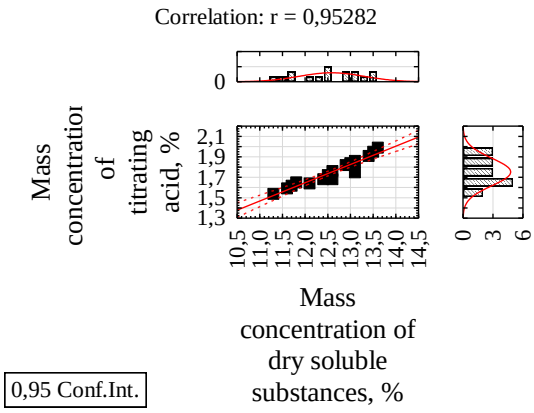

Figure 4. Correlation connection between the content of vitamin C and mass concentration of dry soluble substances

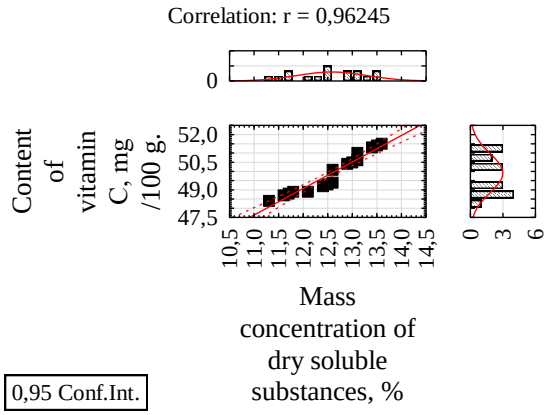

Figure 5. Correlation connection between titrated acidity and dry substances

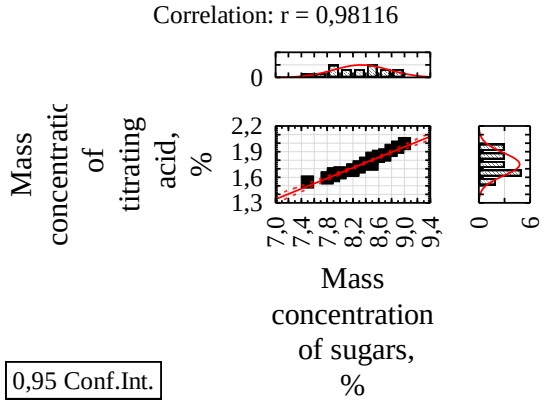

Figure 6. Correlation connection between

the content of vitamin C and content of sugars

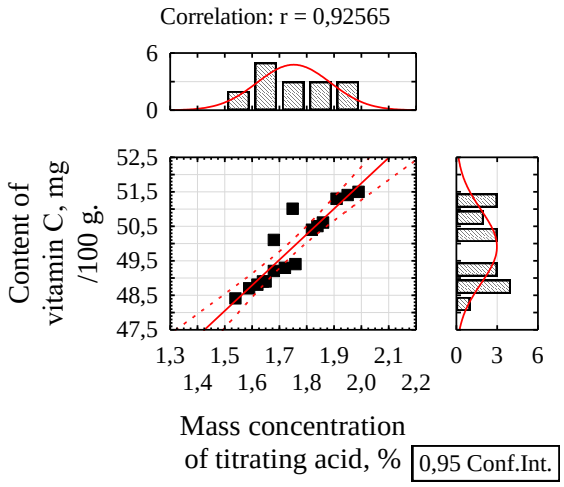

Figure 7. Correlation connection between the content of vitamin C and titrated acidity

The quality evaluated organoleptically is an important market factor. Tasting estimation of raspberry varieties was carried out according to five main features: appearance, colour, aroma, taste and consistency. The analysis of the results showed (Figure 8) that organoleptic characteristics depended to a large extent on the pomological variety of raspberry berries.

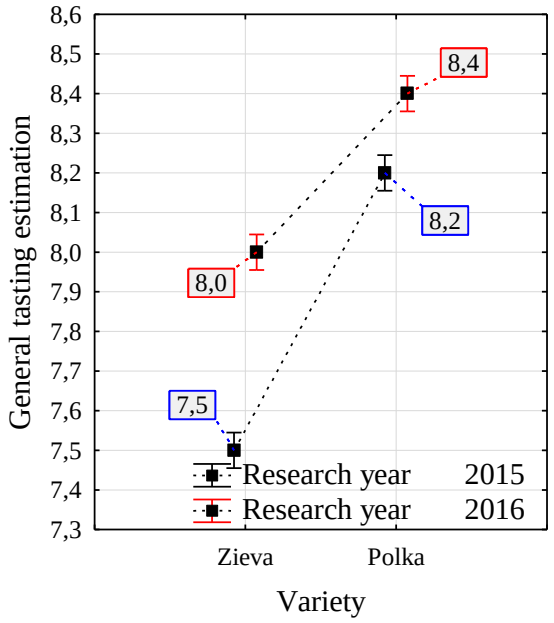

Figure 8. General tasting estimation of raspberries berries, grades

The best berries by general tasting estimation were Zieva variety.

Heat treatment and squash treatment with a pectolytic enzyme preparation was used to increase juice output. The influence of the methods of raspberry pretreatment of Polka and Zieva varieties on juice output is shown on Figure 9.

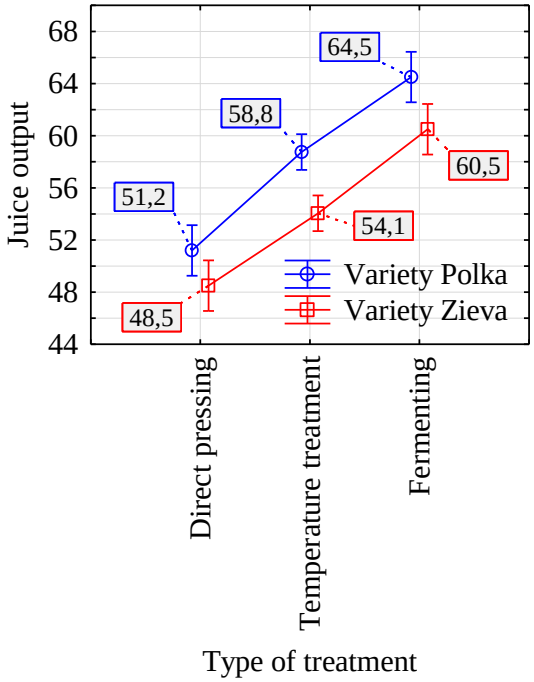

Figure 9. Influence of the methods of raspberry pretreatment of Polka and Zieva varieties on juice output, %

Infusing under temperature regime and extraction increased juice output for both varieties of raspberry. Juice output increases, for Polka variety by 6.5% compared to the control, and for Zieva veriety by 8.3% respectively, comparing the effect of temperature treatment in variants without fermentation.

Fermentation of squash increased juice output for Polka variety by 10.5%, and for Zieva variety by 12% compared with the control variant, that described these variants

as optimal. Factor of the variety and method of squash processing greatly influenced the juice output.

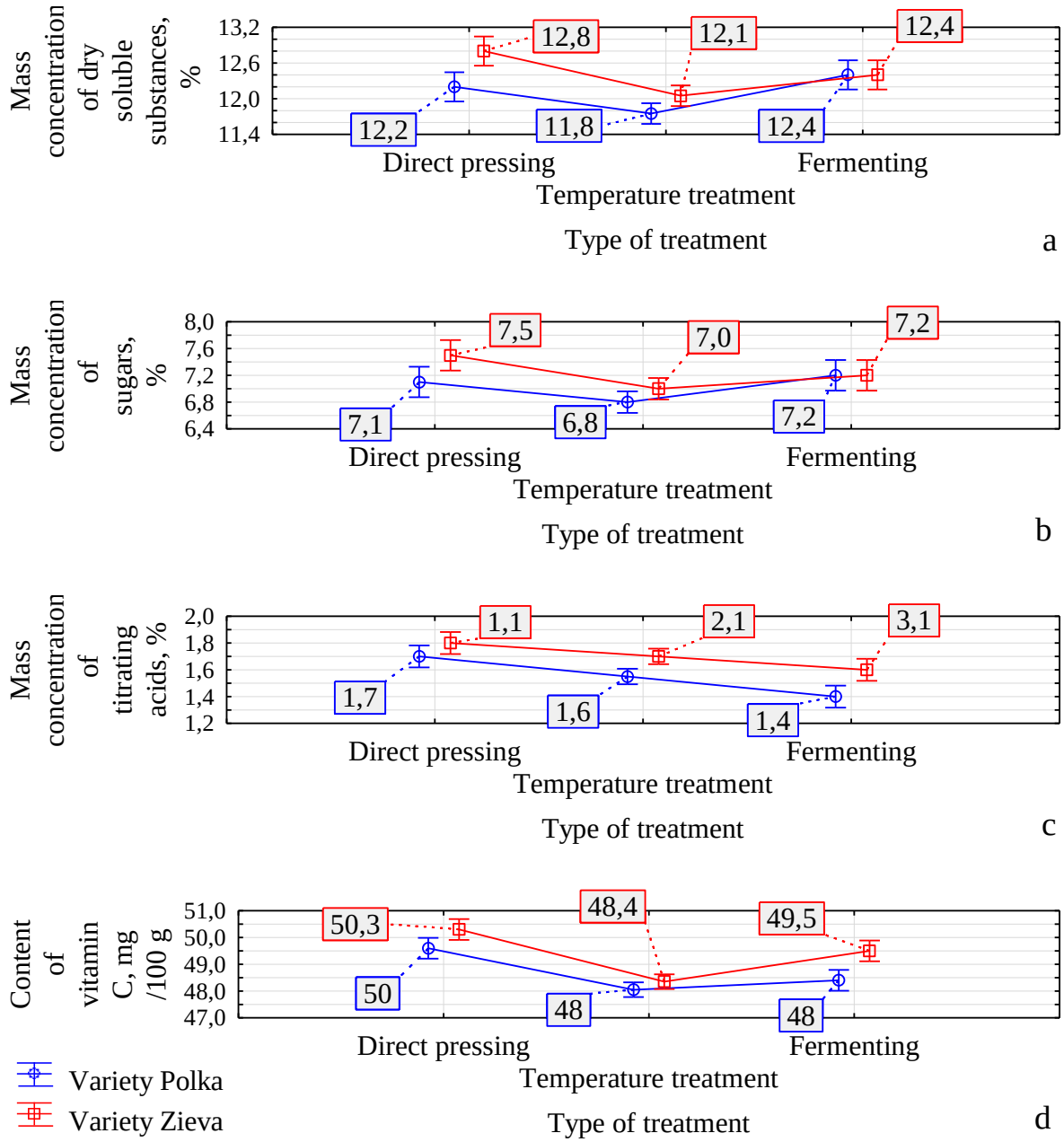

Figure 10. Physical-and-chemical parameters of fresh juice from raspberry berries: a – Mass concentration of dry soluble substances, %; b – Mass concentration of sugars, %; c – Mass concentration of titrating acids, %; d – Content of vitamin C, mg/100 g.

The quality of fruit-and-berry juices and wines depended on the quality and characteristics of raw materials. It was established that mass concentration of dry soluble substances (Figure 10, a), sugars (Figure 10, b), titrating acids (Figure 10, c)

and vitamin C content (Figure 10, d) in raspberry juices of both varieties after temperature treatment and fermentation of squash unsignificantly decreased in relation to the control variant, which meant that physical-and-chemical parameters of raspberry juice of these varieties did not greatly affect the main technological parameters of juices.

Content of phenolic substances became less depending on the method of squash treatment of both varieties (Figure 11).

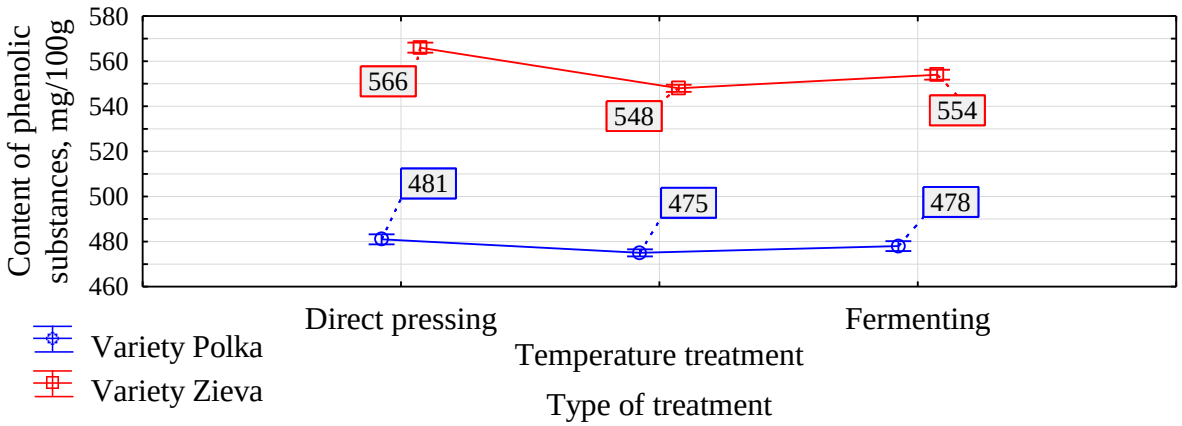

Figure 11. Influence of the methods of raspberry pretreatment of Polka and Zieva varieties on the content of phenolic substances in juices, mg/100g

The difference in mass concentration of phenolic substances between varieties was 85 mg/100 g, and in the variant with heat treatment was 74 mg/100 g in the control variant. Variants with squash fermentation were characterized by reasonably large difference between the content of phenolic substances of 76 mg/100 g comparing the change in the content by mass concentration of phenolic substances; in the result raspberry of Zieva variety turned out to be the best one.

Having studies peculiarities of raspberry berries of Polka and Zieva varieties for the content of the components of chemical composition and the best juice output, we decided to choose Zieva raspberry variety for the further use, which is characterized by a high content of phenolic substances, for enlarging alcoholic juices with biologically active substances in fruit-and-berry winemaking. Rectified alcohol with strength of 96% was used for vinage. Calculations of fortifying raspberry juice of Zieva variety berries with alcohol to the volume fraction of alcohol 16% are given in Table 2.

Table 2. Use of rectified alcohol and output of alcoholic juice on 100 decalitre

Experiment variant	Juice output, %	Amount of fresh juice, decalitre	Use of rectified alcohol 96 %, decalitre	Concentration, decalitre	Output of alcoholic juice, decalitre /100 kg
1. Direct pressing	48,5	46,2	9,102	0,70	54,6
2. Temperature treatment	56,8	54,3	10,698	0,89	64,2
3. Fermentation	60,5	57,7	11,368	0,95	68,2

4. Conclusions

Experiments present practicability of using raspberry of Zieva variety in winemaking which is characterized by a high content of phenolic substances (566-547 mg/100 g), for fortification of alcoholic juices with biologically active substances.

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CHEMICAL, RHEOLOGICAL AND SENSORY CHARACTERISTICS OF PROCESSED CHEESE SPREAD ANALOGUES

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ABSTRACT

The present work as undertaken to assess information on the physico-chemical composition, fatty, texture profile, microstructure and sensory evaluation of processed cheese analogue made by substituting 50% and 100% of butter fat with vegetable fat (peanut cream fat). Both the control processed cheese and the processed cheese spread analogue containing peanut cream were found to be within the composition range of processed cheese. Meanwhile replacing butter fat with vegetable fat at level of 50 and 100% did not considerably affect the chemical composition of cheese analogues. Concentration of total and individual volatile fatty acids of control processed cheese spread were higher than that of processed cheese spread analogue in which milk fat was substituted with vegetable fat at 50 or 100% level. The highest concentration of major short chain volatile fatty acids namely butyric, caproic, caprylic and capric acids were found in control processed cheese spread. Partial substitution of butter fat with peanut cream fat has led to promoted significant modification in the texture, microstructure and sensory properties of processed cheese spread analogue. Control processed cheese spread was firmer than the experimental processed cheese analogue which is related to the presence of high percentage of peanut cream fat. In addition, this treatment decreased the cohesiveness and hardness of the resultant analogue. The size of fat globules increased and the uniformity of their distribution in protein matrix decreased with increased the percentage of vegetable fat used. Control processed cheese spread showed a uniform protein network in which numerous small fat particles were dispersed whilst in processed cheese analogue the fat globules were present in smaller numbers and with greater diameter, a behavior intensified by increasing the proportion of vegetable fat. Control processed cheese spread and processed cheese spread analogue in which butter fat was replaced with peanut cream fat at 50% level had the better sensory characteristics compared with that of analogue made with 100% vegetable fat.

1.Introduction

Developed products, known as ‘imitation’ processed cheese spread analogue, are widely produced, and are made from mixtures of dairy and/or non-dairy proteins and dairy fat or vegetable oil (Jihan *et al.*, 2017) These products are variously labelled as ‘analogues’, ‘analogs’ imitation’, ‘substitute’, ‘artificial’, ‘extruded’, ‘synthetic’, and/or ‘filled’ (Shaw, 1984; McCarthy, 1990 and Federation, 1995). Imitation cheese spread analogue or cheese spread analogue products are cheese spread analogue like products manufactured by blending water, fat, proteins and other ingredients into a homogeneous mass in the presence of the necessary heat, mechanical shear

and emulsifying salts (Guinee, 2003 and Salek *et al*., 2015). The main advantages of this type of cheese spread analogue include its simplicity in production, low manufacturing costs and its amenability to be easily formulated with customized physicochemical and nutritional attributes. Cheese spread analogues have gained importance in different areas. Firstly, largely because of a tremendous increase in the consumption of pizza pie and the fact that cheese spread analogue is among the costliest components of a pizza pie, attention has focused on the development of cheese spread analogue substitutes. In addition, the manufacture of an imitation cheese spread analogue allows manufacturers greater scope in manipulating constituents toward nutritional, textural, and economic ends (Solowiej *et al*., 2014 and Mohamed and Shalaby, 2016). A wide variety of formulated imitation cheese spread analogues in which non-fat milk solids and milk fat are replaced by caseinates and vegetable oils are available in the US (Kiely, McConnell *et al*., 1991). Secondly, due to rapidly increasing prices cheese spread analogue is being gradually priced out of lower income groups. Making cheese spread analogue -like products by substituting the higher priced milk-derived ingredients with lower priced ingredients from vegetable sources may be a possible solution for this economic problem (Guirguis, Abdel Baky *et al*., 1985 and Awad, Salama *et al*., 2014). The cost of producing cheese spread analogues can be considerably less than that of their natural counterparts. As well as savings in the manufacturing process, raw materials are considerably cheaper than milk (Shaw 1984). Thirdly, the short supply of milk production in some parts of the world has led to increased interest in the utilization of substitute ingredients from vegetable sources in producing some dairy analogues (Ahmed and Hassan 1995; McNutt 1989; Bachmann, 2001 and Tamime, 2011). In developing countries where dairy products are expensive and insufficient in quantity, dairy substitutes prepared from legumes provide a nutritious alternative (Santos, Resurreccion *et al*., 1989). Fourthly, there is an ever-increasing interest among consumers in food products which contain less total fat, saturated fat, cholesterol, and calories. Such products are useful in controlling body weight and reducing the risk of heart and artery disease (Kong-Chan, Hellyer *et al*., 1991 and Mortensen, 1991). Thus, cheese spread analogue may offer an excellent opportunity for substituting a traditional product with a new one which offers the same or better nutritional and texture characteristics, by using caseinate as protein sources and the use of polyunsaturated vegetable fats and oils, which produce a cholesterol free product (Giese, 1992 and Kneifel and Seiler, 1993).

It is better to formulate foods with ingredients that help to lower health risks, as in the case of substituting animal fats by vegetable fats and oils, to give foodstuffs low in cholesterol and saturated fat contents (Lobato-Calleros, Vernon-Carter *et al*., 1997). An important area of research for cheese spread analogue is to build up the polyunsaturated fat level, thus improving the health benefits of cheese spread analogue (McNutt, 1989; Bachmann, 2001 and Tamime, 2011). The present work has been carried out to evaluate the effect of replacing butter fat with 50 and 100% of peanut cream on chemical composition, fatty acids, texture properties and sensory evaluation of processed cheese spread analogue.

2. Materials and methods

2.1.Materials

2.1.1. Cow's milk

Cow's milk was obtained from dairy technology unit, Faculty of Agriculture, Zagazig University.

2.1.2. Emulsifying Salts and Nisin

Commercial emulsifying salts (sodium salts of phosphates and polyphosphates) and nisin were obtained from Greenland factory, 10th of Ramadan City, Egypt.

2.1.3. Peanut seeds

Red-skinned peanut (*Arachis hypogaea*) seeds were purchased from a local market in Zagazig Egypt. Good quality and mold-free seeds were selected.

2.1.4. Skim milk powder

The skim milk powder was purchased from private dairy plant in Zagazig Egypt. Chemical characteristics **(**Butterfat 1.25% max – moisture 4.00% max– protein (n x 6.38) 35.5% approx. – lactose 51.0% approx. – minerals/ashes 8.50% approx. – titratable acidity 0.15% max – solubility index 1.25 ml max– sediment / scorched particles disc b max– wpn index not more than 1.5 mg/g).

2.1.5. Sucrose

Good quality sucrose was obtained from a local market in Zagazig, Egypt.

2.1.6. Ras cheese

Ras cheese which was made by the method of (Abdel-Hamid, El-Shabrawy *et al*., 2000) at the Department of Food Science, Faculty of Agriculture, Zagazig University, Zagazig, Egypt was used in the preparation of cheese spread analogue.

2.1.7. Butter

Butter was obtained from Dairy Technology Unit, Faculty of Agriculture, Zagazig University, Zagazig, Egypt and used in the preparation of cheese spread analogues.

2.1.8. Starch

Maize starch was obtained from the local market in Zagazig, Egypt.

2.1.9 Chemicals

All chemical used in this analysis was of analytical grade quality.

2.2.Methods

2.2.1. Preparation of peanut milk and peanut cream

Peanut-milk was prepared by the method described by (Isanga and Zhang, 2009). Briefly, sorted peanut seeds were roasted (130°C for 20 min), de-skinned and soaked in 0.5 g/100 ml $NaHCO_3$ at 5°C for 12 h. After washing with water, the kernels were then mixed with water at a ratio of 1:5 W/V (peanuts (g): water (ml), transferred to a blender, and blended for at least 5 min. Resultant formed slurry was filtered using three-layered cheese cloth to obtain peanut milk. Then it was pasteurized at 71˚C/15 seconds. cooled and stored at freezer (- 10°C) till uses. Peanut cream was prepared at Dairy Technology Unit, Faculty of Agriculture, Zagazig University. Peanut milk was prepared as described before then cream was separated using milk separator. The separator was flush with warm water to be clean and warm. Peanut milk was heated to 45°C, then poured into the bowl and permit to pass it through the central tubular shaft with turning on the motor and crank for 10 minutes, the force of spin causes the peanut cream to separate from the peanut milk. The resultant peanut cream was stored in the refrigerator at 5°C after pasteurization until usage.

2.2.2. Manufacture of processed cheese spread analogue

Control processed cheese spread and other cheese analogue containing 50 and 100% peanut cream were manufactured with the following formulation as shown in Table 1.

Table 1.Formulation of different ingredients set in processed cheese analogue spread preparation (Total batch 1500 g).

Ingredients	**Substitution levels of milk fat by peanut cream**		
	0%	**50%**	**100%**
Mature Ras cheese	127.50	120	112.50
Fresh Ras cheese	510	480	468.75
Butter (84% fat)	107.25	59.25	-
Peanut cream (45% fat)	-	108.30	224.70
Starch	30	30	30
Skim milk powder	60	58.50	52.50
Emulsifying Salts	37.50	37.50	37.50
Water	627.75	606.45	574.05
Total	**1500**	**1500**	**1500**

Manufacture of processed cheese spread of all treatments as done in the Dairy Technology Unit, Food Science Department, Faculty of Agriculture, Ain Shams University. Mature (2 months) and fresh Ras cheese were cut into small portions suitable to be fed through the inlet of a shredding machine (Braun mixer, Germany). The suitable amounts of ingredients including; fresh Ras cheese, mature Ras cheese, butter, water, maize starch, skimmed milk powder emulsifying salt and nisin were added consecutively in a laboratory style-processing kettle locally made in Egypt. Specifications of the cooking machine are mentioned by (Awad, 1996).

The ingredients were mixed for about 1 min before processing. The blends were adjusted to contain 58% moisture, 50% fat/dry matter, 3% emulsifying salt, 0.01% nisin in the finished product. The mixture was cooked for 10 min at 85-90°C using indirect steam at pressure 2-2.5 kg/cm^2. The melted processed cheese spread analogue of the different treatments was poured into wide mouth glass jars (150g) sealed with sterilized aluminum foil and capped directly after filling. The resultant processed cheese spread analogue was cooled at room temperature stored at 5°C for 3 months. Samples were taken and subjected to the different analyses when fresh and after 1, 2 and 3 months of storage.

Using a similar manufacturing process, a series of imitation cheese spread analogues were prepared by replacing 50% of butter fat with peanut cream fat and 100% of butter fat with peanut cream containing 45% fat.

2.2.3. Chemical analysis

2.2.3.1. Gross chemical composition

Moisture, fat, protein, pH and acidity contents of processed cheese spread and its analogue were determined according to (AOAC, 2006).

2.2.3.2. Fatty acids Analysis

Fatty acids were determination (as % of total). Fatty acids were extracted and determined as methyl esters (AOAC, 2006). The methylesters were prepared by weighing a 10 mg sample in a 2 ml test tube (with screw cap). The sample was dissolved in 1 ml hexane followed by the addition of 10 ml of 2 N potassium hydroxide. The tube was closed and vortex for 30 seconds. Analysis was carried out by Gas chromatography Ultra trace GC DSQ (Thermo scientific USA). TR-FAMe capillary column (Thermo scientific USA) (30 m length × 0.22 mm ID × 0.25 micron film thickness). For analysis fatty acid methyl esters, Cis/trans isomers, 70% cyano-propyl polysilphenylene – siloxane was used. Helium was employed as carrier gas, with constant flow 0.8ml/min. the temperature of injector was set at 200°C, then 1 µl was injected. The operating condition were as follow: oven temperature was held at 40°C for 1 min and then increased by 10°C/min to 180°C and held for 2 min increased again to 210°C at 4°C/min held for 3 min at 210°C, increased again to 250 at 10°C/min and finally isotherm at 250°C for 10 min. Scan mode was full scanning mass 50-650. Mass detector was used. Results were expressed as percent of relative area (Dabbou, Issaoui *et al*., 2009).

2.2.3.3. Texture profile analysis

Texture profile analysis of processed cheese spread analogue was determined according to (Bourne, 2002) by a universal testing machine

(Cometech, B type, Taiwan) provided with software. An Aluminum 25mm diameter cylindrical probe was used in a "texture profile analysis" (TPA) double compression test to penetrate to 50% depth, at 1 mm/ s speed test. firmness (N), gumminess (N), chewiness (N), adhesiveness (N.s), cohesiveness, springiness and resilience were calculated from the TPA graphic. Texture determinations were carried out in (40 x 40 x 30) mm-sized samples.

2.2.3.4. Micro-structural analysis

Micro-structural analysis of processed cheese spread analogue was determined using scanning electron microscopy (SEM).

Tissue pieces were fixed in 4% glutaradehyde in 0.2M sodium acodylate buffer (pH 7.3) for 4 hour, followed by post fixation in osmium tetra oxide SO4 for 2 hour. Then rinsed three times in the same buffer (sodium cacodylate buffer). The samples were dehydrated through a graded ethanol series from 10 to 100%, 10 minutes in each one except the finely one 100% for 30 minutes for three changes (each one for 10min.), then dehydrated using critical point dried instrument with liquid carbon dioxide (CO_2). The speciments were mounted on cupper stubs with double- sided adhesive tape, coated with gold using S150A Sputter Coated – Edwards – England. The specimens viewed in a scanning electron microscope JXA-840A Electron Probe Microanalyzer – JEOL – Japan.

2.2.3.5.Sensory evaluation of processed cheese spread analogue

Sensory analysis was performed according to the methodology described by (Meilgaard, Carr *et al*., 2006). The acceptance test was carried out using a 9-point structured hedonic scale, for the attributes of appearance, colour, flavour, creaminess, firmness, spreadability and overall impression. Based on 9 point hedonic scale; like extremely=9, like very much =8, like moderately =7, like slightly =6, neither like nor dislike =5, dislike slightly =4, dislike moderately =3, dislike very much =2, dislike extremely =1. About 20 g of each sample were served, at approximately 7°C, in white 50 ml plastic cups coded with random three digit numbers. A plastic knife and three cream cracker type biscuits were offered together with the sample, for the evaluation of spreadability. The differences between the scores were evaluated at the 5% level of significance, comparing the means using the Duncan's test.

2.2.4.Statistical analysis

Results were statistically analyzed using a computer program "SAS system for windows version 9.00 TS M0" (SAS 2008) for analysis of variance by one way (ANOVA) and comparison of means by Duncan's multiple comparison test where $P < 0.05$ was considered for significant difference.

3.Results and discussions

3.1. Changes of Chemical Composition of processed cheese analogue during storage.

The changes in chemical composition of cheese spread analogue samples prepared with different levels of peanut cream during storage for 3 months are shown in Table (2).

Table 2. Effects of replacing butterfat with peanut cream fat on the chemical composition of processed cheese spread analogue during storage at 5°C.

Characteristics (%)	Storage time (months)	Cheese spread analogue containing peanut cream		
		0% (control)	50 %	100%
Moisture	1	59.57±0.11^{f}	60.20±0.11^{d}	57.15±0.10^{h}
	2	62.32±0.10^{a}	60.29±0.16^{c}	59.78±0.11^{e}
	3	60.94±0.16^{b}	60.25±0.12^{c}	58.46±0.10^{g}
Ash	1	4.78±0.03^{a}	4.50±0.03^{cde}	4.50 ±0.03^{cde}
	2	4.56±0.03^{c}	4.43±0.03^{e}	4.55 ± 0.03^{c}
	3	4.67±0.03^{b}	4.46±0.03^{de}	4.52±0.03^{cd}
Protein	1	12.46±0.27^{b}	12.13±0.26^{e}	13.19±0.29^{a}
	2	12.35±0.27^{d}	12.09±0.26^{g}	11.49±0.25^{h}
	3	12.40±0.27^{c}	12.11±0.26^{f}	12.34±0.27^{d}
Fat/dry matter	1	50.82±0.14^{g}	52.11±0.14^{d}	50.78±0.12^{g}
	2	54.51±0.13^{a}	50.31±0.17^{h}	53.08±0.15^{b}
	3	52.24±0.10^{c}	51.22±0.16^{f}	51.68±0.17^{e}
pH	1	5.79±0.16^{bc}	5.86±0.10^{a}	5.88±0.14^{a}
	2	5.75±0.14^{c}	5.85±0.14^{a}	5.76±0.10^{bc}
	3	5.77±0.13b^{c}	5.85±0.13^{a}	5.80±0.11^{b}

Means having the same letters in the same row are not significantly different.

The obtained results showed that both the control cheese and cheese spread analogue containing peanut cream were found to be within the compositional range of processed cheese (Muir, Tamime *et al*., 1999). It is interesting to note that both levels of vegetable fat i.e., 50 and 100% exhibited little observable effects on the chemical composition of cheese analogues. There were significant differences between the control and only cheese analogue containing 100% peanut cream fat in all the chemical components (moisture, fat, protein and ash contents and pH value). These differences between cheese analogues and control cheese reflected the differences between formulations. The general trend of the chemical composition of control processed cheese and processed cheese analogues are in agreement with those reported by (Tamime, Muir *et al*., 1999). They found that the total solids and protein contents were higher in cheese analogues than the control, while the fat and ash contents and pH values were lower in cheese prepared with peanut cream than the control. The same trend was also mentioned by (Cunha, Dias *et al*., 2010), who reported that the analogue cheeses were higher in protein and lower in salt content and pH values than control.

The differences in protein levels, fat content, ash content, moisture and pH values were significant within all models of processed cheese analogue tested (including control samples). The differences in chemical profile between samples reflected differences between formulations.

3.2. Fatty acid composition of processed cheese spread analogue

Twenty fatty acids were detected in different processed cheese spread analogue, wherein palmitic and oleic acids were the main fatty acids. Both fatty acids accounted together more than 60% of total fatty acids. Myristic and stearic acids were detected also in high levels in different processed cheese spread analogues.

3.2.1.Volatile fatty acids

Fig. 1 shows the amounts of individual volatile fatty acids (C_4-C_{10}) in both control and processed cheese analogue spread containing 50 and 100% of peanut cream after 2 months of storage.

Considerable levels of short chain fatty acids were also detected in different cheese spread analogue Fig. (1) shows the effect of replacing butter fat with peanut cream fat on the volatile fatty acids in processed cheese spread analogue.

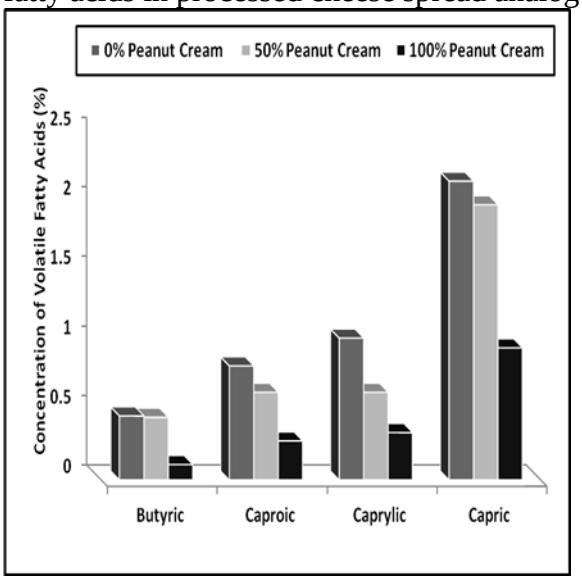

Figure 1. The effect of replacing butterfat with peanut cream fat on the volatile fatty acids in processed cheese spread analogue.

Free fatty acids (FFA) are major contributors to the flavour of cheese (Kilcawley, Wilkinson *et al*., 2006). The latter are released upon lipolysis and the short and intermediate-chain FFAs contribute directly to cheese flavour. These short and intermediate-chain fatty acids (C_4–C_{12}) have relatively low perception thresholds and each gives a characteristic flavour note. Butanoic acid (C_4) contributes "rancid" and "cheesy" flavours while hexanoic acid (C_6) has a "pungent", "blue cheese" flavour note (Collins, McSweeney *et al*., 2003). Depending on their concentration and perception threshold, volatile fatty acids can either contribute positively to the aroma of the imitation cheese or to a rancid defect. (Noronha,

Cronin *et al*., 2008) obtained similar results of volatile fatty acids in enzyme modified cheese.

The detected 4 volatile fatty acids were butyric, caproic, caprylic and capric acids. The higher concentration of volatile fatty acids could be explained on the basis that peanut cream did not contain any volatile fatty acids. The obtained results showed that the amounts of total and individual volatile fatty acids of control processed cheese spread were higher than processed cheese analogue spreads containing 50% or 100% peanut cream. The highest concentration of major short chain FFA (volatile fatty acids) was butyric acid, Capric acid, caprylic acid, caproic and in the control. Cheese containing peanut cream showed lower concentration of these fatty acids as compared to the control. This could be explained on the basis that milk contains short chain fatty acids (C_4-C_{10}) whereas peanut cream fat did not contain any volatile fatty acids.

Fig. (2) shows the non-volatile saturated fatty acids profile of different processed cheese spread analogue. It could be noted that different processed cheese spread analogues resulted in significant change in fatty acids profile of various processed cheese spread analogues. However, the changes were notably in the levels of the main fatty acids (palmitic, oleic acids, myristic and stearic), the same trend was also mentioned by (Cunha, Grimaldi *et al*., 2013).

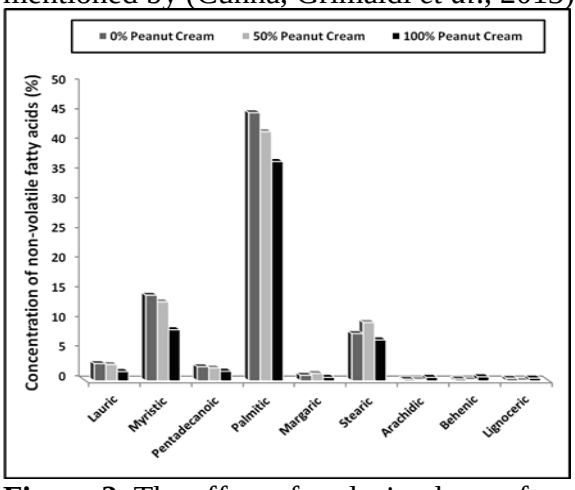

Figure 2. The effect of replacing butter fat with peanut cream fat on the non-volatile fatty acids in processed cheese spread analogue.

3.2.2.Mono Unsaturated fatty acids

Fig. (3) Shows the effect of replacing butter fat with peanut cream fat on the mono unsaturated fatty acids after two months of storage at room temperature.

The replacing of butter fat with peanut cream fat significantly affected the concentration of oleic acid as the major monounsaturated fatty acids in processed cheese spread analogue the same trend was also mentioned by (Cunha, Grimaldi *et al*., 2013).

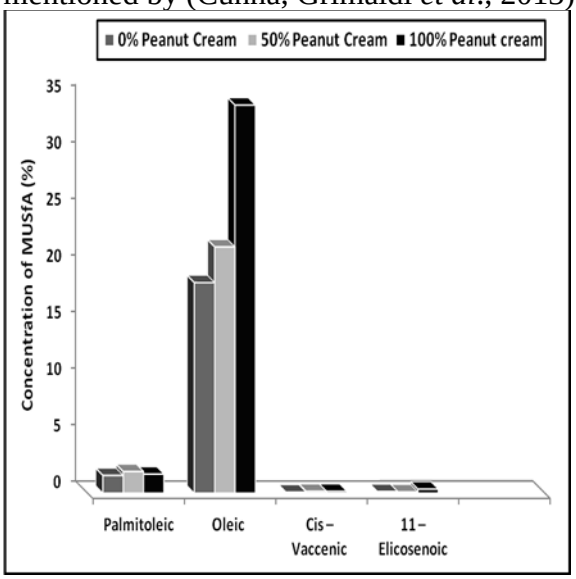

Figure 3. The effect of replacing butter fat with peanut cream fat on the monounsaturated fatty acids in processed cheese spread analogue.

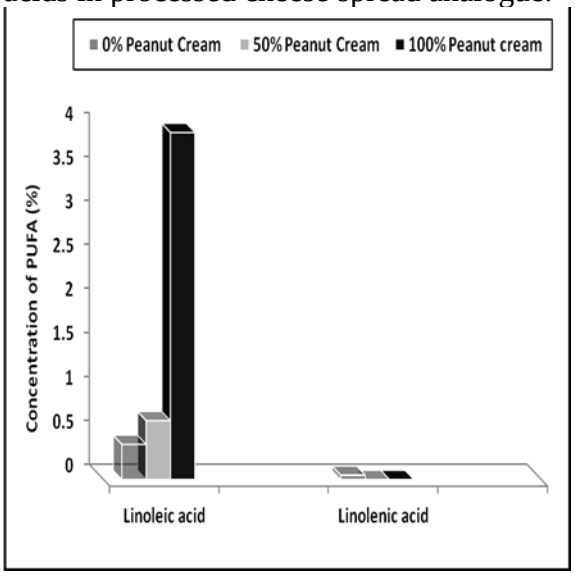

Figure 4. The effect of replacing butter fat with peanut cream fat on the polyunsaturated fatty acids in processed cheese spread analogue.

The level of these fatty acids in processed cheese analogue containing both 50 and 100% peanut cream was higher than the control.

The palmitoleic acid was also found in lower concentration in both control cheese and cheese containing peanut cream.

3.2.3.Polyunsaturated fatty acids

Fig. (4) Shows the effect of replacing of butter fat with peanut cream fat on the poly unsaturated fatty acids of processed cheese analogue after two months of storage at room temperature. Substitution of butter fat with peanut cream fat significantly affected the concentration of linoleic acid as the dominant fatty acids in polyunsaturated fatty acids of processed cheese spread analogue. The level of these fatty acids in processed cheese analogue containing both 50 and 100% peanut cream was higher than the control. This was more pronounced in processed cheese analogue spread containing 100% peanut cream.

3.2.4.Total saturated and unsaturated fatty acids

It is known that the excessive consumption of saturated fatty acids is related to the increase of the plasmatic cholesterol and the obesity (Sales, Costa *et al*., 2005). On the other hand, the consumption of polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA) it has been recommended to improve the lipidic profile in relation to the saturated fatty acids.

Fig. (5) Shows the effect of replacing of butter fat with peanut cream fat on the total saturated and unsaturated fatty acids of cheese analogue after two months of storage at 5° C. The substitution of butter fat with peanut cream fat significantly affected the concentration of linoleic acid as the dominant fatty acids in poly unsaturated fatty acids of cheese spread analogue.

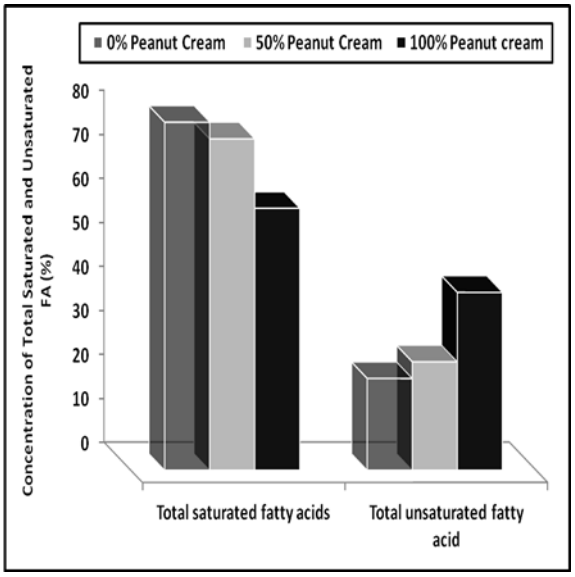

Figure 5. The effect of replacing butter fat with peanut cream fat on the total saturated and unsaturated fatty acids in processed cheese spread analogue.

The level of these fatty acids in cheese analogue containing both 50 and 100% peanut cream was higher than the control. This was more pronounced in cheese analogue spread containing 100% peanut cream.

3.3. Texture profile

Fig. (6) shows the profile texture of processed cheese spread analogue made by replacing 50 and 100% of butter fat with peanut cream fat in the formulation used in the preparation of imitation cheese spread analogue.

With respect to the firmness, the control cheese spread was firmer than the experimental processed cheese spread analogues, which is related to the presence of a high percentage of replacement of peanut cream fat. In the analogues, since the diameter of fat globules were greater, there was a greater protein mass per unit area of the fat. Such a high protein density has been associated with elevated firmness, since the protein matrix is the structural component conferring greater resistance to deformation (Bryant, Ustunol *et al*., 1995 and Lobato-Calleros, Vernon-Carter *et al.*, 1999).

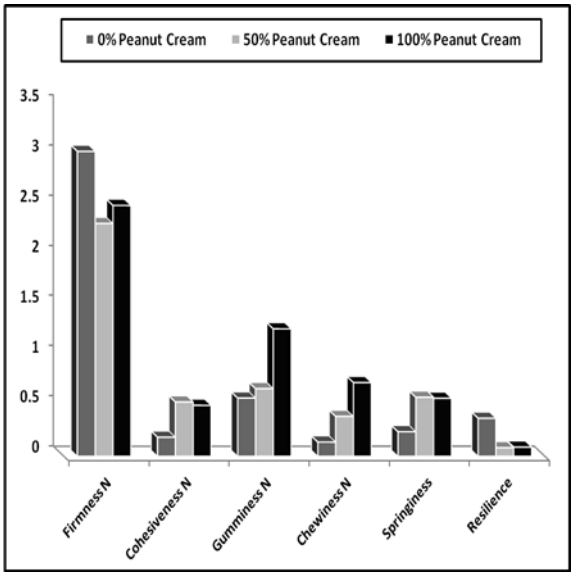

Figure 6. Effect of replacing butter fat with peanut cream fat on the texture profile of processed cheese spread analogue.

In this work, analogues of this cheese, made by substituting 50% and 100% of the dairy fat with vegetable fat, were studied with respect to physical–chemical composition, texture profile, microstructure and sensory acceptance. The substitution of part of the dairy cream by vegetable fat resulted in increased adhesiveness and hardness. The traditional cheese presented a uniform protein network, in which numerous small fat particles were dispersed, whilst in the analogues, the fat globules were present in smaller numbers and with greater diameters, a behavior intensified by increasing proportions of vegetable fat. In the sensory analysis, the traditional cheese and the analogue with 50% vegetable fat were better evaluated than the analogue with 100% vegetable fat.

The processed cheese analogues also showed greater values for cohesiveness than the traditional processed cheese spread analogue. Since there was no difference in the chemical compositions, the type of fat was probably responsible for this behavior. The differences in microstructure found were also probably associated with the increase in cohesiveness, since the protein network structure and extent of the interactions between the fat and the casein influence the degree of coherence between the product and the surface with which it is in contact. The results found are in agreement with the observations of (Lobato-Calleros, Vernon-Carter *et al.*, 1997 and Cunha, Dias *et al*., 2010). They found that the addition of soybean fat increased the adhesiveness and hardness of the processed cheese spread analogues.

Control had the lowest gumminess and chewiness values followed by the R1 and the R2 groups (Fig. 6). But, no significant differences were found with respect to springiness, cohesive-ness (P>0.05). (Lobato-Calleros, Vernon-Carter *et al.*, 1999) stated that soybean fat conferred hardness and adhesiveness to the processed cheese analogue however decreased cohesiveness and springiness. Hydrogenated cottonseed oil in-creased hardness but decreased cohesiveness in both imitation groups.

Microstructure Evaluation

The rheological properties of processed cheese spread analogue are of considerable importance as they affect: its handling, portioning and packing characteristics; its texture and eating quality, as they determine the effort required to masticate the processed cheese spread analogue or alternatively the level of mastication.

The partial or total substitution of butter fat by peanut cream fat into processed cheese spread analogue caused significant modification on the instrumental textural characteristics of hardness, adhesiveness, cohesiveness and chewiness, but no significant (p>0.05) variations in springiness were detected (Fig. 7).

Figures (7–9) show the photomicrographs of the microstructure of the control cheese spread and its analogues with 50% and 100% of peanut cream (vegetable fat), respectively, with the same magnitude (330x). In typical scanning electron micrographs of cheese spread analogue, a protein matrix is visible with various forms and sizes of open spaces, representing the fat globules that were extracted during sample preparation for analysis (Tamime and Robinson, 1999).

Fig. 7 illustrate the Scanning Electron Micrograph (SEM) of control processed cheese spread. This figure clearly shows that this type of structure could be observed in all the samples studied. The control processed cheese spread analogue presented numerous small particles of fat dispersed in a uniform protein network.

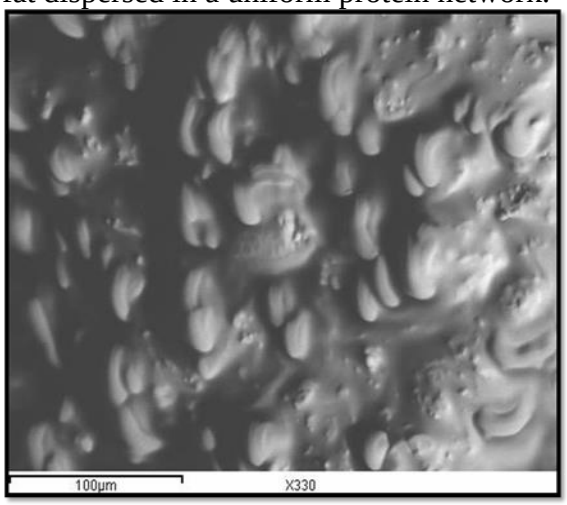

Figure 7. Scanning electron micrograph (SEM) of control processed cheese spread (Magnitude 330x at 100 µm).

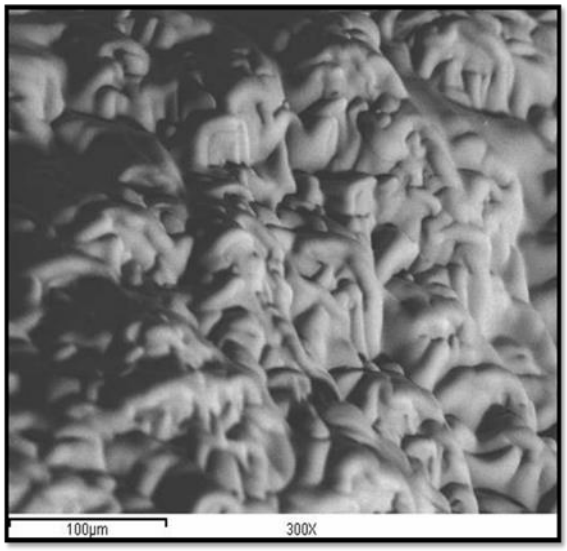

Figure 8. Scanning electron micrograph (SEM) of processed cheese spread analogue made by replacing 50% of butter fat with peanut cream fat (Magnitude 330x at 100 µm).

The fat globules are predominantly spherical and distributed uniformly throughout the protein matrix. The obtained results are in agreement with those obtained for processed cheese spread analogue by (Mistry and Anderson 1993 and Tamime, Kalab *et al*., 1990).

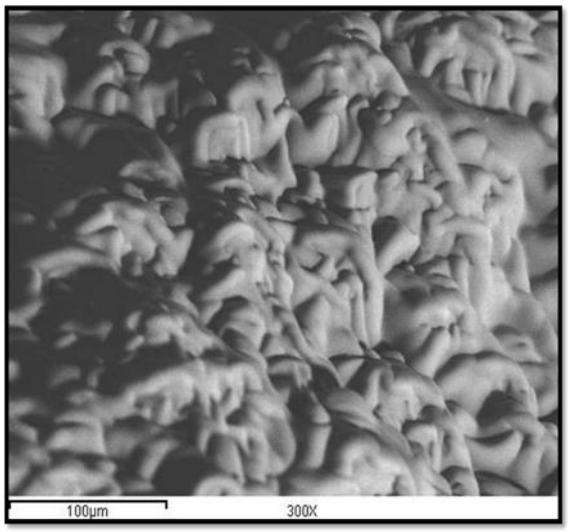

Figure 9. Scanning electron micrograph (SEM) of processed cheese spread analogue made by replacing 100% of butter fat with peanut cream fat (Magnitude 330x at 100 µm).

On the other hand, in the analogues the fat globules were greater in diameter. In addition, they were less uniformly distributed throughout the protein matrix than in the control processed cheese spread analogue.

Comparing the microstructures of the analogues with 50% (Fig. 8) and 100% (Fig. 9) of peanut cream, it can be seen that an increase in the percentage of vegetable fat resulted in a decrease in the number of fat globules and an increase in their diameters.

The vegetable fat used was composed mainly of fatty acids with more than 16 carbons (Table 2), conferring a highly hydrophobic characteristic and increasing the number of primary bonds, resulting in larger and more irregular crystals and globules. On the other hand, the milk fat presenting a greater proportion of saturated fatty acids contained a significant amount of low molecular weight, fatty acids, which reduced its non-polar nature, decreasing the attractive forces between the fat globules and increasing the affinity with the protein network.

In the control processed cheese spread, the fat droplets were small and uniformly distributed, while in the analogues fat globules presented a wider range of particle size. Increasing the percentage of peanut cream from 50% to 100% resulted in even larger fat droplets, which corroborates the results observed in the micrographs.

The textural characteristics of the processed cheese spread analogue are determined by the combined structural properties of the protein matrix and the fat droplets immersed in the former. As fat content is reduced, more non-interrupted protein zones compose the cheese spread analogue structure. In consequence, a high degree of crosslinking of protein molecules occurs resulting in three dimensional networks, exhibiting high resistance to the deformation (Bryant, Ustunol *et al*. 1995 and Lobato-Calleros, Robles-Martinez *et al*., 2001).

The main factor determining the textural characteristics in processed cheese spread analogue is the protein matrix structure, and it has been reported that high protein densities in processed cheese spread analogue are associated with high values of hardness (Bryant, Ustunol *et al*., 1995).

Milk fat contributed to the increase of all the textural characteristics, although its effect on cohesiveness was marginal, whereas on chewiness it was more pronounced (Figure 4). It has been demonstrated that butter milk globule sizes are larger and more heterogeneous than those for emulsified vegetable oils, so the number of the former per unit volume are lower than those of the latter.

On the other hand, milk fat has the highest melting point fatty acids than control conferring increased resistance to structure deformation, and thus an increased chewiness (Lobato-Calleros, Vernon-Carter *et al.*, 1999). Due to the slightly more lipophilic nature of EB, it had a relatively low effect on displacing protein from the oil in water interface (Euston, Singh *et al*., 1996) so that interactions between fat globules and the protein matrix were favored resulting in increased cohesiveness (Fig. 6).

Substitution of part of the butter (84% fat) with peanut cream (45% fat) as a vegetable fat promoted significant modifications in the texture and principally in the microstructure of the processed cheese spread analogue The size of the fat globules increased and the uniformity of their distribution in the protein matrix decreased with an increase in the percentage of vegetable fat used.

The porous network in the microstructure of the control process cheese spread analogue (Fig. 6) was similar to that of the internal structure of Edam and Gouda cheese spread analogue as reported by (Schmidt, 1982). Since both higher fat and water contents can lower the firmness of cheese spread analogue (Kalab, 1993).

3.4. Sensory evaluation

Table 3 shows the mean scores attributed to each of the parameters evaluated: appearance, color, flavour, creaminess firmness, spread ability and overall impression.

Table 3. Organoleptic scores for control processed cheese spread and processed cheese spread analogues containing 50 and 100% peanut cream fat.

Attributes	**Cheese spread analogue containing peanut cream**		
	0 %	**50 %**	**100 %**
Appearance	7.1[a]	6.7[a]	5.5[b]
Colour	6.9[a]	6.4[a]	5.7[b]
Flavour	7.8[a]	6.3[b]	5.6[c]
Creaminess	6.9[a]	6.9[a]	5.8[b]
Firmness	6.8[a]	6.7[a]	5.6[b]
Spreadability	7.4[a]	6.4[b]	5.9[c]
Overall Impression	7.3[a]	6.7[b]	5.5[c]

***** Means having the same letters in the same row are not significantly different.

All the products received means above 5.5 for all the attributes evaluated, showing that they were well accepted by the panelists. The analogue containing 50% of peanut cream presented acceptably by the panelists, and for the attributes of appearance, color, creaminess and firmness, this analogy was more preferred than all the other products tested, including the

control processed cheese spread analogue. Flavour was the only parameter for which the control processed cheese spread analogue received significantly higher ($p< 0.05$) mean scores than processed cheese spread analogue containing 50 and 100% of peanut cream fat, showing the importance of dairy fat on the flavor of dairy products.

Although neither of the analogues managed to imitate the flavour of the traditional processed cheese spread analogue, all showed gained acceptable quality. With respect to some of the attributes such as colour, firmness and creaminess, the analogue with 50% vegetable fat was better evaluated than 100% fat cheese spread analogue. This shows that there are good possibilities for successfully substituting traditional processed cheese spread (control) with an analogue manufactured with 50% peanut cream fat. (Abdel-Baky, *et al*., 1987) stated that the processed spread cheese made from Ras cheese revealed better flavour and appearance during storage at both refrigerator and at room temperatures. (Kebary, *et al.*, 1998) reported that the scores of organoleptic properties of spread cheese made from different blends decreased as storage period progressed except the scores of colour. (Razig and Yousif, 2010) showed that the best score of appearance, texture, flavour and overall acceptability was obtained in spread processed cheese containing 5% skim milk powder compared with others processed cheeses samples. They also found that 3 months were found to be quite satisfactory to attain good quality spread cheese.

4. Conclusions

Both levels of vegetable fat 50 and 100% exhibited significant small effects on the chemical composition of cheese analogues. The highest concentration of major short chain volatile fatty acids (VFA) was butyric acid, capric acid, caprylic acid and caproic acid in the control-processed cheese. Firmness of the control processed cheese spread was firmer than the experimental processed cheese spread analogues. Substitution of part of the butter fat with peanut cream led to promoted significant modifications in the texture, the sensory properties, the colour and, principally, in the microstructure of the processed cheese spread analogue. Regarded to the sensory analysis, the control processed cheese spread and the analogue with 50% vegetable fat had better characteristics than the analogue with 100% vegetable fat.

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OVEN COOKING AS ALTERNATIVE TO SMOKING: EVALUATION OF PHYSICOCHEMICAL, MICROBIOLOGICAL, TEXTURAL AND SENSORY PROPERTIES OF CIRCASSIAN CHEESE DURING STORAGE AND DETERMINATION OF PAH CONTENTS

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ABSTRACT
The aim of this work was to evaluate the physicochemical, microbiological, textural and sensory changes in three batches of Circassian cheese (non–smoked (AN), natural smoked (AS) and oven–baked (RO) cheese) during storage period of 90 days. Nine polycyclic aromatic hydrocarbons (PAHs) were also detected by HPLC-RD. Differences in cheese samples (total solids, fat, salt, titratable acidity, water-soluble, trichloroacetic acid-soluble and phosphotungstic acid-soluble nitrogen, hardness, gumminess, springiness, cohesiveness, chewiness and color properties) were observed depending on the manufacture procedure and smoking process. Microbial community in oven-baked cheese was found significantly lower than other samples. Use of oven for smoking enhanced the sensory properties compared to natural smoking, but batch AN had higher acceptability. Total and carcinogenic PAHs levels of oven-baked smoked cheese were remarkable lower than the levels of natural smoked cheese. Benzo[a]pyrene was detected only batch AS at the levels of 0.11 μg kg^{-1}. In this study, it was demonstrated that oven-baked smoking technique can be used as smoking technique instead of natural smoking for manufacture of Circassian cheese.

1.Introduction

Circassian cheese is a kind of traditional cheese produced from cow's, sheep's, goat's or skimmed buffalo's milk, or a mixture of these milks by circassian families in Anatolia especially the region of Sinop, Düzce, Bolu, Sakarya, Balıkesir, Bursa, Çanakkale, Biga, Hendek and Gönen (Kamber, 2008). It is also made in the ancestral regions of the Circassians in Caucasia. Circassian cheese can be consumed as fresh, ripened or dried (sundried or oven-dried) form (Ayar et al., 2015). It has typically a round shape, an average weight of 0.5 kg, and color varying from white to cream for the dried types. The change of color between the varieties can be explained with the smoked type of Circassian cheese has a light brown surface and a light yellow or cream interior, however fresh cheese has a light yellow color. The characteristic flavor profile of the cheese is slightly salty, cooked, creamy and fermented (Guneser and Yuceer, 2011) but the textural properties of the varieties show slight differences depending on soft consistency for fresh cheese and thin crust layer for dried type (Ayar et al., 2015).

Smoking process is applied to Circassian cheese in order to obtain long shelf life and higher organoleptic properties. Nowadays, Circassian cheese is mostly smoked with the aim of improving taste, color and textural properties rather than preservative impact (Ayar et al., 2015; Guneser and Yuceer, 2011). Different smoking processes have been developed to control of smoke concentration in foods, but traditional (natural) and liquid smoking techniques are mostly used for smoke of Circassian cheese (Aydinol and Ozcan, 2013; Kamber, 2008). According to Aydinol and Ozcan (2013), natural smoking method is better than liquid smoking due to better microbial quality and also natural smoked Circassian cheese received higher flavor scores. Since polycyclic aromatic hydrocarbons (PAHs) are formed during natural smoking, liquid smoking is preferred for industrial production. The number of cheesemakers using natural smoking method has gradually decreased because of highly presences of PAHs especially benzo[a]pyrene (B(a)P) and legal restrictions. PAHs are highly hydrophobic and organic lipophilic compounds with fused aromatic rings and they are formed during the incomplete combustion or pyrolysis of organic matter by different cooking process techniques such as roasting, barbecuing, grilling, frying, smoking, heating, drying, baking and cooking (Fasano et al., 2016; Singh et al., 2016). The Scientific Committee on Food (SCF) has evaluated the health risk associated with PAHs (SCF, 2002) and International Agency for Research on Cancer (IARC) has mentioned PAHs in priority pollutant list due to their carcinogenic and mutagenic properties (IARC, 2012). SCF suggested to use B(a)P as a marker of occurrence of the PAHs in foods (Lambert et al., 2012). However, four PAHs (PAH4) including B(a)P, chrysene (Chr), benzo(b)fluoranthene (B(b)F) and benzo(a)anthracene (B(a)A) has been established the most suitable indicators of PAHs in foods after September 2012 (EC, 2011) and the European Food Safety Agency (EFSA) suggested that PAH4 has been used as better indicator for the presence of PAHs in food instead of B(a)P (EFSA, 2008).

Circassian cheese is an acid–coagulated cheese and has different properties in comparison with enzyme–coagulated cheeses. Acid–coagulated cheeses, are generally used in natural smoking process, have risks of formation of PAHs. Therefore, Circassian cheese manufacturers have developed alternative practice and Circassian cheese is produced in an oven without any tree or smoked. In this case, enzyme–coagulated cheese is used at oven–baking treatment because of higher textural and sensory quality. There are limited studies about Circassian cheese (Ayar et al., 2015; Aydinol and Ozcan, 2013; Guneser and Yuceer, 2011) and there is no information about the oven–baked Circassian cheese. The aim of the present study is to investigate effects of oven–baking treatment instead of natural smoking treatment on physicochemical, microbiological, textural and sensory characteristics of Circassian cheese during storage, and PAHs contents of Circassian cheeses were also evaluated.

2. Materials and methods

2.1. Materials

Cheese productions were performed at the Karagöl Çiftliği Dairy Company (Sakarya, Turkey). Only natural smoking treatment was applied by a local cheesemaker according to traditional procedure. Calf rennet was obtained from Mayasan Company (Istanbul, Turkey) in production of enzyme–coagulated cheese. A total of 36 Circassian cheeses (three repetitions for each cheese) were manufactured as three batches independent Circassian cheeses. The samples were coded as follows: AN (acid and heat–coagulated, non–smoked cheese), AS (acid and heat–coagulated, natural smoked cheese) and RO (rennet–coagulated, oven–baked cheese). According to the experience of the producer dairy plant, the productions are restricted to these three batches because the cheese produced by the acid / heat coagulation technique is not suitable for oven-baking and if

the enzyme coagulation cheese is not baked, it is very similar to Kaşar cheese.

2.2. Manufacturing of cheeses

Raw cow's milk was filtered through a filter–line and transferred into process vat and then heated to 90 °C. Acidified whey (approximately 4%) was added into the milk, followed by obtaining of the curd formation, whey was drained. The curd was put into a plastic basket with holes then these baskets were stacked and kept for draining of the remaining whey for 12 h. After pressing, the cheese samples were put into brine solution (15% NaCl) for 24 h. Then the cheeses were kept with drying air for 24 h and after the drying treatment, non-smoked cheeses (batch AN) were vacuum–packaged. Batch AS samples were smoked with poplar tree for 36 h by using the traditional method. After cooling, the smoked cheeses were vacuum–packaged.

For manufacture of batch RO, raw cow's milk was filtered through a filter–line and pasteurized at 75 °C for 15 s. After cooling to 32 °C, the milk was coagulated with rennet enzyme. Then whey was removed from the curd and remaining whey is drained off by placing heavy blocks on the top of it. After pressing process, the curd was cut as large parts and then grated. Then, the curd was cooked at 75 °C for 3 min, and the cooked curd was kneaded and placed into molds. After waiting period for 24 h, cheeses were baked at 150 °C in a rotary electric–oven until enough browning of cheese surface and then cheese was cooled and vacuum–packaged.

2.3. Physicochemical analysis

Total solid, fat, salt, and titratable acidity (TA) were determined according to the methods described by Bradley et al. (1993). pH was measured using a pH meter (InoLab, Weilheim, Germany) calibrated with pH 4.0, 7.0 and 10.0 buffers. The total nitrogen was determined using the Kjeldahl method. The acid degree value (ADV) was determined as described by Deeth and Fitz-Gerald (1976) for evaluation of fat hydrolysis. For fat oxidation, lipid extraction from cheese was carried out according to the method of Kristensen et al. (2000) and the peroxide values were determined by a method described by Şengül et al. (2014).

2.4. Soluble nitrogen fractions

For preparation of water soluble extracts, 20 g of grated cheese was mixed with 40 mL of deionized water and homogenized with an Ultra Turrax homogenizer (WiseTis®HG–15D, Daihan, Korea) for 1 min. It was then centrifuged at 3000 x g for 30 min at 4 °C. The fatty layer was removed and the supernatant was filtered through Whatman 42 paper.

To analyses the content of water–soluble nitrogen (WSN), 10 mL of filtrate was taken and the Kjeldahl method was used (IDF, 1993). The 12% trichloroacetic acid–soluble nitrogen (TCA–SN) fractions were prepared by mixing 25 mL of the WSN fraction with 25 mL of 24% (w/v) TCA solution. The mixture was held at room temperature for 2 h and then filtered through Whatman 42 paper. The 5% phosphotungstic acid–soluble nitrogen (PTA–SN) fractions of the cheeses were prepared as follows: 3 mL of 33% (w/v) PTA solution and 7 mL of 3.95 M H2SO4 solution were added to 10 mL of the WSN fraction. The mixture was held overnight at 4 °C and filtered through Whatman 42 paper. The nitrogen contents were determined using the Kjeldahl method (IDF, 1993) and all WSN, TCA and PTA values were expressed as the percentage of the total nitrogen content of the cheese (Jarrett et al., 1982).

2.6. Microbiological analysis

Ten g of cheese samples were homogenized in 90 mL of 2% (w/v) solution of sodium citrate (Carlo Erba, Milano, Italy) for 1 min with a stomacher (AES Chemunex, Bruz, France). The dilutions of the suspensions were prepared with Ringer's solution (Merck, Darmstadt, Germany). The following media and incubation conditions were chosen to enumerate microbial counts: YGC agar (Merck) for yeasts and moulds at 30 °C for 72 h, KAA agar (Merck) for enterococci at 30 °C for 24 h, Rogosa agar

(Merck) adjusted to pH 5.5 with acetic acid for lactobacilli at 30 °C for 5 d under anaerobiosis, M17 agar (Merck) for lactococci at 30 °C for 72 h, MRS agar (Merck) supplemented with vancomycin (30 μg mL–1; Sigma–Aldrich, Steinheim, Germany) for leuconostocs at 30 °C for 72 h, VRB agar (Merck) for coliforms at 37 °C for 24 h.

2.7. Color properties

Surface and inner colors of cheese samples were measured with a Minolta Chroma Meter (CR-400, Minolta Camera Co., Osaka, Japan) and measurements were carried out in triplicate for each cheese samples. Color was expressed according to the Commission International de l'Eclairage (CIE) as L* (lightness; 100=white, 0=black), a* (redness; +, red; -, green), and b* (yellowness; +, yellow; -, blue), hue angle (ho) and chroma. In which the chroma and hue angle were calculated as follows:

$$Hue = h_{ab}^{*} = tan^{-1}\left(\frac{b^{*}}{a^{*}}\right) \quad (1)$$

$$Chroma = C_{ab}^{*} = (a^{*2} + b^{*2})^{0.5} \quad (2)$$

2.8. Texture profile analysis

For texture analysis, Circassian cheese samples were cut using a stainless–steel cylinder knife into cylindrical discs, nearly 20±0.5 mm height and 30±0.5 mm diameter and wrapped in plastic stretch film. The samples were adjusted to room temperature (20°C) before analysis and then Texture Profile Analysis (TPA) was performed with a TA.XT Plus Texture Analyser (Stable Micro Systems, Godalming, UK) in duplicate. An aluminum cylindrical probe (P/36R) with 36 mm of diameter was attached to a moving crosshead. Pre–test, test and post–test speeds of the probe were adjusted to 1 mm/s. Samples were compressed to 25% of the original height in two consecutive cycles. Compression time and trigger force were 5 s and 25 g, respectively. Data recording and analysis were carried out with the Texture Exponent Version 4.0.13.0 software (Stable Micro Systems).

2.9. Sensory properties

Circassian cheese samples were organoleptically evaluated after ripening at 4^{o}C for 7, 30, 60 and 90 days by a semi trained panelist group (twelve assessors) at the Ondokuz Mayis University, Food Engineering Department. The assessors were informed about the characterization of Circassian cheese and trained in relate evaluation to be familiar with attributes and scoring procedures of cheese samples under study. Score card was used to evaluate flavor-aroma (on a 10 point scale), body-texture, appearance and color (on a 5 point scales) with some criteria (salty, insipid, sour, bitter, tasteless, fermented, whey and smoke for flavor; tough and dry, soft, fragile and flexible for body-texture, rough, spotted, non-uniform, porous and fissure for appearance; mat and dingy for color) that lead to reduction of score. Before the testing, cheese samples were tempered for 30 min at room temperature inside a closed dish and cut into pieces of 1.5 x 2.0 cm base and 2-5 cm long. Cheese samples were identified with three numbers and randomly presented to assessors. Assessors used water and bread to clean their plates between samples.

2.10. Determination of PAHs

The extraction and clean-up procedures of PAHs from Circassian cheese was based on the method described by Anastasio et al. (2004) and Wegrzyn et al. (2006), respectively with some modifications. Five mL of 1 M KOH etanolic solution was added to 1 g of sample previously homogenized in a Teflon centrifuge tube and then tube placed in a water bath at 80°C for 3 h. After the cooling to room temperature, 5 mL of distilled water and 10 mL of cyclohexane were added and the mixture vortexed for 5 min and then centrifuged (Sigma, Model 3K30, OsterodeamHarz, Germany) at 4000 x g for 15 min. The supernatant was poured from the tube and the sample was re-extracted with 10 mL of cyclohexane as previously described. The combined extracts were concentrated by rotary vacuum evaporator at 40°C (Buchi Rotavapor R–200, Sweden) and then dried under a nitrogen

stream. After the drying process, residue was applied to a SPE cartridge after dissolving with 2 mL of acetonitrile. For the cleanup process, firstly SPE cartridge was washed and activated by passage of 10 mL of ultrapure water followed by 10 mL of methanol and dried with air by using syringe. Two mL of eluate was applied to a SPE cartridge and dried for 1 min at the air. For the elution of analytes from the cartridge, 10 mL of dichloromethane was used. After the concentrating step under a nitrogen stream, the residue was diluted with 500 µL acetonitrile and then injected to HPLC.

The separation and identification of PAHs were performed by HPLC system from Shimadzu (Tokyo, Japan), consisted of a fluorescence detector (FLD) RF–10XL, quaternary pump LC–20AT, degassing device DGU–20A5, column oven CTO–10ASVP, auto injector SIL–10A and system controller SCL–10AVP (data station LC–20AT). The HPLC column used was a Phenomenex Envirosep–PP column (125 mm x 4.6 mm, 4.6 µm, Phenomenex). The mobile phase used for HPLC analysis consisted of acetonitrile and water at a flow rate of 1 mL/min. The linear gradient elution program was set as 0 min–85% acetonitrile + 15% water, 9 min – 100% acetonitrile. The temperature of the column during chromatographic was set at 350C and settings for PAH detection were as follows (excitation/emission wavelength): λ1=216/336 for 3.9–4.4 min (Naph): λ2=240/320 for 4.5–4.9 min (Ace):λ3=248/404 for 4.9–5.5 min (Ant): λ4=236/384 for 6.2–7.1 min (Pyr): λ5=270/388 for 8.8–11.25 min (B[a]A): λ6=250/430 for 17.5–22.5 min (B[k]F, B[a]P): λ7=295/405 for 22.9–27.2 min (DB[ah]A, B[ghi]P).

The describe method was validated and performances were present in our primary study (Gul et al., 2015). Recoveries were calculated using spiked samples at three replicates each at three concentrations of PAH mix and they found in between 73.38% and 92.61%. The repeatability of method, expressed as relative standard deviation (RSD %), was comprised between 6.46% and 12.49% for all the considered individual PAHs. The limit of detection (LOD) and the limit of quantification (LOQ) for the method were lower than 0.09 and 0.27 µg kg^{-1}, respectively. The results of correlation coefficient (r^2) was better than 0.9991 for all PAHs.

2.11. Statistical analysis

Analyses of cheeses from the three experiments were performed in duplicate during storage period. Data analysis was performed with SPSS statistical package software with version 21.0 (SPSS Inc. Chicago, Illinois) and results were presented as mean ± standard deviation. One-way analysis of variance was applied to determine the differences between means with confidence level of 95% ($P<0.05$). When significant ($P<0.05$) main effect was found, the mean values were further compared using Duncan test.

3.Results and discussions

3.1. Compositional properties

The levels of total solid (TS), fat, fat in TS, salt, salt in TS, pH and titratable acidity (TA) of Circassian cheeses during storage period are shown in Table 1. Batch RO showed the highest TS values and the lowest values were determined in batch AN ($P<0.05$). Oven baking treatments in batch RO cheeses caused an increase in TS. TS values of the batch AS were higher than those of batch AN due to smoking treatment. Our TS results resemble the values of other researches (Aydinol and Ozcan, 2013; Guneser and Yuceer, 2011; Sıçramaz et al., 2017; Uysal et al., 2010). TS levels of the cheeses slightly decreased during storage period. Fat contents of the batch RO were the highest ($P<0.05$) and batch AS followed this batch depending on TS contents of the cheeses. The fat contents of cheeses didn't show significant changes during storage ($P>0.05$). The values of the fat in TS in batch RO and AN were similar and higher than those of batch AS. These results were agreed with the results of a previous report (Aydinol and Ozcan, 2013; Guneser and Yuceer, 2011). Salt contents of the

batches didn't change significantly during storage (P>0.05) and batch RO showed higher salt contents than those of other batches during storage period (P<0.05). The levels of salt in TS of the batch RO were also higher than the other batches (P<0.05). These results were similar to those of other studies (Aydinol and Ozcan, 2013; Guneser and Yuceer, 2011) and lower than results of Uysal et al. (2010). Batches AS and AN were similar in terms of slightly lower pH levels than batch RO. Similar results obtained by Ayar et al. (2015) who stated pH decreased for a greater extent for the cheese coagulated with acid whey. Additionally, Cais–Sokolińska et al. (2014) found that pH value of smoked Mozzarella cheese was significantly greater than that of unsmoked cheese. TA values of batches AN and AS were similar and they showed higher TA content than batch RO especially after day 60 due to acid and heat–coagulation of these cheeses. Sıçramaz et al. (2017) stated that the effect of smoking process on the TA value was not significant and the differences in the acidity were mainly as the result of the cheese compositions. However, in this study, batch RO was manufactured with rennet and hence, acidity value of this cheese was increased little during storage compared to acidity of batches AN and AS.

As seen in Table 1, the ADV of batch AN was significantly lower than in smoked cheeses and the highest value (2.13 meq KOH 100 g fat^{-1}) was found in batch RO (P<0.05). The higher temperature during smoking could have increased the ADVs of smoked cheeses. The ADVs increased in all cheeses during storage (P<0.05) and reached to 4.71 meq KOH 100 g fat^{-1} as the highest value in batch RO cheese.

Lipid oxidation is an important factor affecting the cheese quality especially those with a high fat content and during long periods of storage. Peroxide value of lipid extracted from samples varied from 0.86 to 1.63 meq O_2 kg fat^{-1} and the highest peroxide value detected in batch RO (P<0.05; Table 1). Smoking process caused the increase of peroxide value in cheeses and this may be associated to the increased oxidation of unsaturated fatty acids as a result of exposed to atmospheric oxygen and heating during smoking (Anvari et al., 2014). During the storage period, peroxide value was significantly increased for all cheese, however at the end of storage, it was found higher in batch RO (5.78 meq O_2 kg fat^{-1}) than others (P<0.05). The considerable differences between cheeses during storage were found due to different smoking and acid or enzyme coagulated types.

Table 1. Compositional properties of Circassian cheeses during storage.

Properties	**Samples**	**Storage time (days)**			
		7	**30**	**60**	**90**
TS (%)	*RO*	61.04 ± 0.38^{aA}	59.61 ± 1.01^{abA}	59.37 ± 0.81^{bA}	59.17 ± 1.13^{bA}
	AS	57.25 ± 0.87^{aB}	56.29 ± 0.64^{abB}	56.14 ± 0.97^{abB}	55.39 ± 0.79^{bB}
	AN	51.18 ± 1.66^{aC}	50.53 ± 0.33^{aC}	50.37 ± 0.38^{aC}	50.14 ± 0.23^{aC}
Fat (%)	*RO*	32.17 ± 1.15^{abA}	32 ± 1.01^{aA}	31.5 ± 0.25^{aA}	30.25 ± 0.25^{bA}
	AS	28 ± 0.66^{abB}	27 ± 0.5^{bcB}	26.83 ± 1.01^{aB}	26.75 ± 0.43^{cB}
	AN	25.83 ± 0.29^{abC}	26.92 ± 0.52^{aB}	25.25 ± 0.43^{bC}	26.5 ± 0.43^{aB}
Fat in TS (%)	*RO*	51.05 ± 1.57^{bA}	53.67 ± 0.84^{aA}	53.06 ± 0.37^{abA}	51.13 ± 1.23^{bA}
	AS	48.91 ± 0.87^{abB}	47.97 ± 0.71^{bB}	51.2 ± 2.58^{aA}	48.29 ± 0.49^{bB}
	AN	50.52 ± 2.06^{bA}	53.26 ± 1.23^{aA}	49.15 ± 0.61^{cB}	52.53 ± 0.86^{abA}
Salt (%)	*RO*	2.96 ± 0.18^{aA}	2.49 ± 0.26^{bA}	2.41 ± 0.22^{bA}	2.57 ± 0.37^{bA}

	AS	2.28 ± 0.1^{aB}	1.81 ± 0.17^{bB}	1.84 ± 0.08^{bB}	2.09 ± 0.08^{aAB}
	AN	1.77 ± 0.09^{aC}	1.97 ± 0.13^{aB}	1.81 ± 0.29^{aB}	1.91 ± 0.05^{aB}
Salt in TS (%)	*RO*	4.85 ± 0.27^{bA}	4.16 ± 0.39^{cA}	4.06 ± 0.42^{cA}	5.53 ± 0.53^{aA}
	AS	3.99 ± 0.15^{bB}	3.22 ± 0.29^{cB}	3.27 ± 0.17^{cB}	4.31 ± 0.1^{aB}
	AN	3.47 ± 0.13^{aB}	3.91 ± 0.28^{aA}	3.51 ± 0.6^{aAB}	3.79 ± 0.09^{aB}
pH	*RO*	5.77 ± 0.06^{aAB}	5.73 ± 0.05^{bAB}	5.71 ± 0.04^{bA}	5.7 ± 0.02^{bA}
	AS	5.58 ± 0.02^{aB}	5.44 ± 0.02^{bB}	5.35 ± 0.14^{bcB}	5.26 ± 0.08^{cB}
	AN	5.65 ± 0.04^{aA}	5.56 ± 0.03^{bA}	5.41 ± 0.07^{cB}	5.32 ± 0.02^{cB}
TA (%)	*RO*	0.54 ± 0.06^{bA}	0.64 ± 0.03^{bB}	0.66 ± 0.04^{abB}	0.79 ± 0.12^{aB}
	AS	0.62 ± 0.07^{cA}	0.81 ± 0.09^{bA}	0.95 ± 0.09^{aA}	1.01 ± 0.06^{aA}
	AN	0.59 ± 0.03^{dA}	0.77 ± 0.06^{cAB}	0.99 ± 0.08^{bA}	1.13 ± 0.14^{aA}
ADV (meq KOH 100 g fat^{-1})	*RO*	2.13 ± 0.36^{cA}	2.8 ± 0.13^{bA}	4.41 ± 0.21^{aA}	4.71 ± 0.35^{aA}
	AS	1.49 ± 0.19^{dB}	2.39 ± 0.21^{cAB}	2.96 ± 0.19^{bB}	3.58 ± 0.35^{aB}
	AN	0.86 ± 0.07^{dC}	1.94 ± 0.29^{cB}	2.87 ± 0.22^{bB}	3.88 ± 0.39^{aB}
Peroxide (meq O2 kg fat-1)	*RO*	1.63 ± 0.22^{dA}	2.65 ± 0.21^{cA}	4.03 ± 0.08^{bA}	5.78 ± 0.17^{aA}
	AS	0.86 ± 0.03^{dB}	1.53 ± 0.19^{cB}	2.34 ± 0.13^{bC}	3.29 ± 0.34^{aB}
	AN	0.86 ± 0.09^{dB}	2.46 ± 0.15^{cA}	3.57 ± 0.12^{bB}	5.15 ± 0.44^{aA}

TS : total solid; TA: titratable acidity; ADV: acid degree value.
$^{A-C}$ Different letters in same column indicates significant differences ($P<0.05$).
$^{a-d}$ Different letters in same line indicates significant differences ($P<0.05$).

3.2. Soluble nitrogen fractions

Proteolysis in the Circassian cheeses throughout storage period was evaluated by analyzing the proteolytic indices, including water-soluble nitrogen (WSN), trichloroacetic acid-soluble nitrogen (TCA-SN) and phosphotungstic acid-soluble nitrogen (PTA-SN). The values of WSN/TN (%), TCA–SN/TN (%), and PTA–SN/TN (%) are presented in Figure 1 during the storage of the Circassian cheeses. The values of WSN/TN (%) in cheeses showed significant increases in all stages ($P<0.05$) except batch AS on day 90. The mean WSN/TN (%) values of batches RO and AN were similar and higher than those of batch AS during storage. Guneser and Yuceer (2011) found that WSN/TN (%) levels in smoked Circassian cheese collected markets ranged between 2.30–29.35%. Uysal et al. (2010) found that the lower WSN/TN (%) was found in fresh Circassian cheese, but Circassian cheeses dried under the sun and in the stove had higher levels. S. Kalit et al. (2005) found 6.58% WSN/TN (%) in fresh Tounj cheese, which produced from raw milk and characterized by its distinct smoked flavor, and reached to 13.14% after a ripening time of 8 weeks.

The values of TCA–SN/TN (%) in cheeses were found between 0.58 to 0.78% and they showed significant increase in all storage stages ($P<0.05$). The highest value was observed in batch AN especially on day 60 and 90 (1.38 and 1.95%, respectively), whereas batch AS showed the lowest TCA–SN/TN (%) values during storage ($P<0.05$). M. T. Kalit et al. (2014), Guneser and Yuceer (2011) and S. Kalit et al. (2005) reported higher TCA–SN/TN (%) levels in Sir iz misine (cheese in a sack), Circassian and fresh Tounj smoked cheeses, respectively. The differences among the results obtained by researchers probably based on the differences in

acid sources and cheese-making procedures (Guneser and Yuceer, 2011).

Batch AN showed the highest PTA–SN/TN (%) value ($P<0.05$) and batches AS and RO were observed that they were similar. The highest value of PTA–SN/TN (%) at the end of storage reached to 0.79% for AN. The higher PTA–SN/TN (%) values (ranged between 3.33-6.26%) were found by Guneser and Yuceer (2011). In general, soluble nitrogen values of RO cheeses after the production were higher than those of other batches, and AS cheese showed the lowest soluble nitrogen values at the end of storage period. Smoking treatment caused increases in soluble nitrogen values for acid coagulated cheeses but decreases in values during storage of cheeses. Moreover, batch RO showed close values to batch AN, and higher values than those of batch AS.

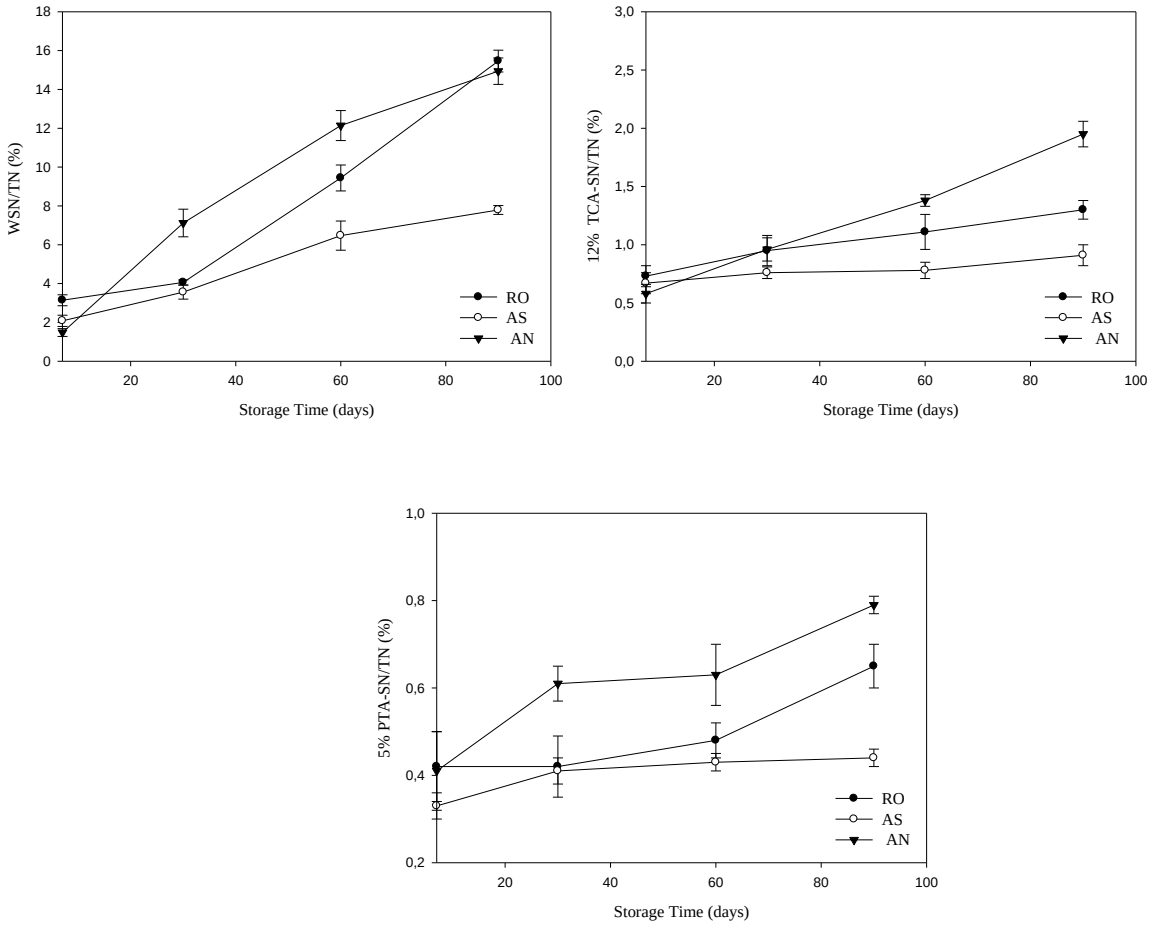


Figure 1. Soluble nitrogen fractions of Circassian cheeses during storage time

3.4. Microbiological characteristics

The counts of the lactobacilli in Circassian cheeses ranged from 5.27 to 9.12 log cfu g^{-1} during storage (Table 2). The counts of the lactobacilli showed a significant increase in only batch RO cheeses ($P<0.05$) during storage. The lactobacilli counts of batches AN and AS were similar and showed higher values than those of batch RO. The lactobacilli counts in all the batches remained constant during storage. The counts of the lactococci in batches AN and AS were also higher than those of the batch RO. Like lactobacilli and lactococci, leuconostoc had the lowest count in batch RO. The counts of the leuconostoc in all the batches did not show any important changes during storage ($P>0.05$). The counts of enterococci in batch AN were the highest ($P<0.05$), but enterococci were not found in batch RO during storage. Cooking and

oven baking treatments in batch RO cheeses caused decreasing in the lactobacilli, lactococci, leuconostoc and enterococci counts. Moreover, Majcher et al. (2011) reported that the number of bacteria inactivated owing to the increase in phenolic compounds formed during the natural smoking process, which are known to have bacteriostatic and/or bactericidal properties. The yeast and mould counts in the cheeses ranged from 3.27 to 5.42 log cfu g^{-1} during storage for all cheese samples and these results were agreed with the report of Aydinol and Ozcan (2013) and Sıçramaz et al. (2017). The yeast and mould counts in batch AN were higher than those of batches AS and RO and counts of batches AS and RO were similar (P>0.05). It was observed that cooking or baking and smoking treatments leaded to decrease in the counts of yeast and mould. While the yeast and mould counts of batches AS and RO showed tendency to decrease, batch AN exhibited increase during storage. Like enterococci, coliforms were not found in batch RO during storage due to probably cooking and baking processes. Batch AS showed decrease in the counts of coliforms during storage (P<0.05), but cheeses AN remained constant. Our coliform results were higher than those of Aydinol and Ozcan (2013) and lower than the results obtained by Sıçramaz et al. (2017).

Table 2. Microbiological composition of Circassian cheeses during storage time

Microbial groups	**Samples**	**Storage time (days)**			
		7	**30**	**60**	**90**
Lactobacilli	*RO*	5.27 ± 0.71^{cB}	6.13 ± 0.28^{bB}	7.01 ± 0.54^{aB}	7.38 ± 0.48^{aB}
	AS	9.12 ± 0.32^{aA}	8.54 ± 0.17^{aA}	8.61 ± 0.15^{aA}	8.81 ± 0.14^{aA}
	AN	8.54 ± 0.14^{aA}	8.24 ± 0.22^{aA}	8.51 ± 0.23^{aA}	8.71 ± 0.36^{aA}
Lactococci	*RO*	8.1 ± 0.62^{bB}	8.21 ± 0.57^{bB}	8.48 ± 0.65^{abB}	9.13 ± 0.32^{aA}
	AS	9.52 ± 0.29^{aA}	8.97 ± 0.17^{aA}	8.95 ± 0.53^{aAB}	9.14 ± 0.38^{aA}
	AN	9.54 ± 0.37^{aA}	9.42 ± 0.31^{aA}	9.51 ± 0.23^{aA}	9.61 ± 0.22^{aA}
Leuconostocs	*RO*	7.51 ± 0.62^{aC}	7.29 ± 0.48^{abB}	6.84 ± 0.31^{bB}	6.55 ± 0.32^{bC}
	AS	8.73 ± 0.44^{aB}	8.19 ± 0.11^{aA}	8.52 ± 0.16^{aA}	8.71 ± 0.43^{aB}
	AN	9.4 ± 0.24^{aA}	8.66 ± 0.21^{bA}	8.99 ± 0.19^{abA}	9.47 ± 0.41^{aA}
Enterococci	*RO*	<1	<1	<1	<1
	AS	4.52 ± 0.12^{cA}	4.98 ± 0.11^{bA}	5.46 ± 0.28^{cA}	5.07 ± 0.9^{bA}
	AN	4.46 ± 0.33^{aB}	4.35 ± 0.19^{aB}	4.47 ± 0.14^{aB}	4.19 ± 0.12^{aB}
Yeast and Moulds	*RO*	5.42 ± 0.15^{aA}	4.62 ± 0.45^{abAB}	4.17 ± 0.43^{bcB}	3.86 ± 0.74^{cB}
	AS	4.82 ± 0.5^{aB}	4.01 ± 0.09^{bB}	3.59 ± 0.24^{cB}	3.27 ± 0.25^{cB}
	AN	4.74 ± 0.46^{aB}	5.29 ± 0.54^{aA}	5.31 ± 0.31^{aA}	5.4 ± 0.19^{aA}
Coliforms	RO	<1	<1	<1	<1
	AS	3.24 ± 0.29^{aB}	2.28 ± 0.33^{bB}	1.55 ± 0.11^{cB}	1.54 ± 0.58^{cB}
	AN	4.39 ± 0.09^{aA}	3.72 ± 0.12^{bA}	3.81 ± 0.11^{bA}	4.01 ± 0.11^{abA}

$^{A-C}$ Different letters in same column indicates significant differences (P<0.05).
$^{a-c}$ Different letters in same line indicates significant differences (P<0.05).

3.5. Color properties

The rind and internal color properties of cheese types depend upon smoking and storage period are shown in Table 3. The color properties of rind and internal of cheese samples were mainly affected by smoking and storage period ($P<0.05$). Rind and internal L* values of batch RO were lower than other cheese samples due to cooking, kneading and oven baking process ($P<0.05$). While traditional smoking process significantly affected the rind L*value of cheese samples, there was no change at internal L* values in comparison non-smoked cheese. Similarly, Cais–Sokolińska et al. (2014) found that a significant difference related with lightness of color between non-smoked and smoked cheeses was shown only outer edge layer and at the center of non-smoked and smoked cheeses were equally light. The decrease of lightness for all cheese samples by the end of 60 d storage period was occurred and then both of rind and internal L* values of cheese samples were increased. It was probably related with the concentration of cheese components and differences of in dry matter content (Saldo et al., 2002). On the contrary, a marked increase in L* value was recorded for non-smoked Mozarella cheese but there was no significant changes of lightness for smoked cheese of the center (Cais–Sokolińska et al., 2014). The rind a* values for batch AS and RO were found more redness than AN ($P<0.05$), but internal a* values of all cheese samples were found similar. During storage, rind and internal a* values of all samples were slightly decreased. Moreover, the highest rind and internal b* value was recorded for batches AS and RO, respectively and the lowest for batch AN ($P<0.05$). During ripening period there was a little change in color of rind as more yellow by increases b* values for all cheese samples. The internal b* value only increased in batch AN during ripening, however these values of batches RO and AS were decreased at the end of 30 and 60 day of storage, respectively and then increased for both cheese samples. The degree of color saturation (chroma) was effected by a* and b* values, and the color of the rind and internal for batches AS and RO was more saturated than the others, respectively. Chroma values of cheese inside were not changed during storage period for all cheese samples. Color in the rind of smoked cheese became less saturated until the storage of 60 days and then increased. However, rind chroma value for batch AN was significantly increased during storage ($P<0.05$). The internal hue angle values for all cheese samples were found similarly and there was no difference during storage. But, the rind hue angle value for batch AS was found higher than batches AN and RO ($P<0.05$).

Table 3. Color properties of Circassian cheeses during storage time

Color Properties	Samples	Storage time (days)			
		7	30	60	90
Rind L*	*RO*	37.77 ± 1.57^{abC}	35.16 ± 1.76^{bcA}	34.62 ± 2.86^{cB}	38.84 ± 1.15^{aC}
	AS	44.11 ± 2.71^{aB}	44.38 ± 1.23^{aA}	41.15 ± 0.93^{bC}	43.35 ± 2.19^{abB}
	AN	87.71 ± 0.26^{aA}	82.8 ± 1.01^{bC}	81.87 ± 0.64^{bB}	83.39 ± 0.34^{bA}
Internal L*	*RO*	75.65 ± 1.19^{aB}	66.08 ± 0.89^{cA}	65.98 ± 0.78^{cA}	63.01 ± 0.44^{bB}
	AS	87.14 ± 0.37^{aA}	83.52 ± 0.72^{bcA}	83.05 ± 0.97^{cB}	84.76 ± 0.15^{bA}
	AN	87.08 ± 0.22^{aA}	84.06 ± 0.47^{bcB}	83.02 ± 0.78^{cA}	84.73 ± 1.13^{bA}
Rind a*	*RO*	14.54 ± 0.31^{aA}	12.22 ± 1.64^{bB}	12.37 ± 1.13^{bC}	12.41 ± 1.29^{bA}
	AS	11.52 ± 0.66^{aA}	10.99 ± 0.64^{aB}	10.87 ± 0.16^{aA}	10.42 ± 0.3^{aB}
	AN	-0.84 ± 0.07^{aC}	-1.44 ± 0.1^{aA}	-1.23 ± 0.12^{aB}	-1.66 ± 0.15^{aC}
Internal a*	*RO*	-0.24 ± 0.61^{aA}	-0.42 ± 1.02^{aA}	-0.55 ± 1.07^{aA}	-0.35 ± 0.28^{aA}

	AS	-0.91±0.07^{aB}	-1.27±0.24^{aB}	-0.95±0.39^{aA}	-1.12±0.29^{aB}
	AN	-0.92±0.14^{aB}	-1.44±0.1^{aA}	-1.44±0.29^{aB}	-1.63±0.23^{aB}
Rind b*	*RO*	17.05±1.12^{aB}	12.5±1.27^{bA}	13.24±0.48^{bC}	15.72±2.44^{aC}
	AS	19.62±1.01^{aA}	18.23±1.16^{abB}	16.61±0.56^{bB}	18.34±1.44^{abA}
	AN	13.37±0.18^{aA}	15.06±1.41^{aB}	15.07±0.24^{aA}	15.79±0.17^{aC}
Internal b*	*RO*	22.12±0.53^{aA}	22.59±0.94^{aB}	22.58±1.23^{aB}	22.72±0.11^{aA}
	AS	14.32±0.39^{bB}	15.77±0.61^{abB}	15.87±0.16^{aA}	15.39±0.35^{abB}
	AN	13.81±0.64^{bB}	14.34±1.52^{bA}	14.86±0.35^{aB}	14.21±1.26^{bB}
Rind Chroma	*RO*	22.42±0.89^{aA}	18.48±2.04^{bB}	18.13±1.03^{bA}	20.03±2.68^{bA}
	AS	22.78±0.75^{aA}	21.31±0.81^{abA}	19.83±0.79^{bA}	21.11±1.36^{abA}
	AN	13.39±0.18^{bB}	15.13±1.01^{aC}	15.13±0.24^{aB}	15.88±0.17^{aB}
Internal Chroma	*RO*	22.13±0.53^{aA}	22.62±0.94^{aA}	22.60±1.27^{aA}	22.72±0.11^{aA}
	AS	15.35±0.4^{aB}	15.82±0.63^{aB}	15.90±0.19^{aB}	15.45±0.34^{aB}
	AN	13.84±0.65^{aC}	14.41±1.52^{aB}	14.93±0.04^{aC}	14.31±1.27^{aB}
Rind Hue	*RO*	0.86±0.03^{aB}	0.80±0.02^{bB}	0.82±0.04^{bB}	0.90±0.03^{aB}
	AS	1.04±0.04^{abA}	1.03±0.05^{abA}	0.99±0.02^{bA}	1.05±0.03^{aA}
	AN	-1.51±0.01^{aC}	-1.47±0.01^{aC}	-1.49±0.01^{aC}	-1.46±0.01^{aC}
Internal Hue	*RO*	-1.55±0.01^{aB}	-1.5±0.01^{aA}	-1.55±0.02^{aA}	-1.56±0.01^{aB}
	AS	-1.51±0.01^{aA}	-1.49±0.01^{aA}	-1.51±0.02^{aA}	-1.48±0.02^{aA}
	AN	-1.50±0.01^{aA}	-1.47±0.02^{aA}	-1.48±0.02^{aA}	-1.46±0.01^{aA}

$^{A-C}$ Different letters in same column indicates significant differences (P<0.05).
$^{a-c}$ Different letters in same line indicates significant differences (P<0.05).

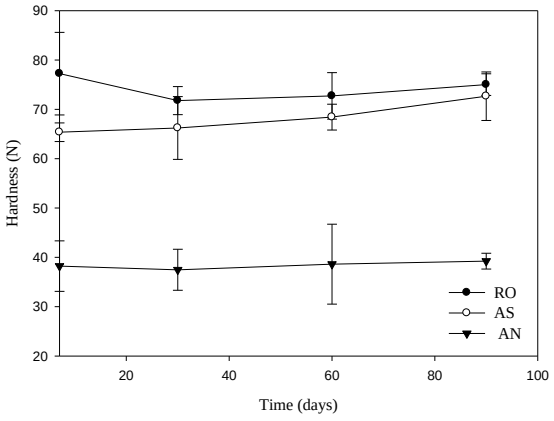
Table 4. PAH content of Circassian cheese

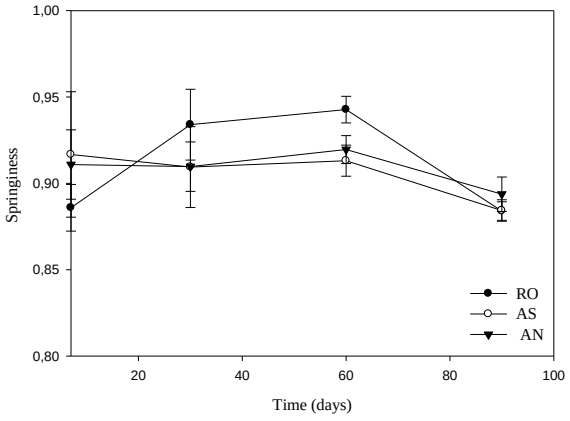
PAHs	**RO**	**AS**	**AN**
Naph	0.41±0.05	1.08±0.08	0.29±0.01
Ace	0.43±0.07	2.02±0.06	0.19±0.06
Ant	0.28±0.07	0.44±0.05	0.16±0.02
Pyr	0.29±0.06	0.86±0.04	ND
B[a]A	ND	0.15±0.01	ND
B[k]F	0.1±0.01	0.17±0.03	0.09±0.01
B[a]P	ND	0.12±0.01	ND
DB[ah]A	ND	0.1±0.01	ND
B[ghi]P	ND	ND	ND
Sum of PAHs	1.51±0.1^{b}	4.93±0.1^{a}	0.74±0.09^{c}
Sum of carcinogenic PAHS	0.1±0.01^{b}	0.53±0.04^{a}	0.09±0.01^{b}

ND: below the limit of detection
$^{a-c}$ Different letters in same line indicates significant differences (P<0. 05).

3.6. Textural properties

Textural properties of Circassian cheeses produced with different methods were evaluated for instrumental textural profile analysis (TPA) during the storage period for 90 days at 4 ^{o}C and shown in Figure 2. The batch RO had significantly highly hardness value than the others after cheese production and during storage ($P<0.05$). It may be explained by baking process for batch RO production due to applied high temperature and by higher TS contents of batch RO than other cheeses (see in Table 1). Moreover, Van Hekken et al. (2007) stated that the amount of fat and protein of the cheese also effects cheese texture. Additionally, Ayar et al. (2015) reported that the culture added and non-smoked Circassian cheese had the lowest hardness values. The hardness values decreased during storage of 30 days for the all cheese, however the decreasing of the value was significantly for only batch RO ($P<0.05$). After the storage of 30 days, hardness values significantly increased ($P<0.05$) and reached to 39.23, 72.66 and 75.02 N for batches AN, AS and RO, respectively. Similar result was obtained by García et al. (2016) found that hardness significantly increased during ripening period and this increases were probably related with the decrease in moisture content during storage. In our study, increase of hardness is probably explained by the increasing of protein in total solid (data not shown) that could affect the texture of the cheese, since moisture values of cheese samples were not significantly changed. Batch RO had higher gumminess value than other cheese samples and it was changed similar to hardness values during storage. The springiness and cohesiveness values of batch RO were found lower than other cheese samples and they were not significantly affected during storage period for all cheese ($P>0.05$). The highest chewiness value was observed in batch RO ($P<0.01$). An increasing trend for chewiness was observed during the 60 days of storage for all cheese and then it was significantly decreased ($P<0.05$). The resilience values of the all cheese samples were increased until storage of 30 days, but it was significantly decreased at the end of storage ($P<0.05$).





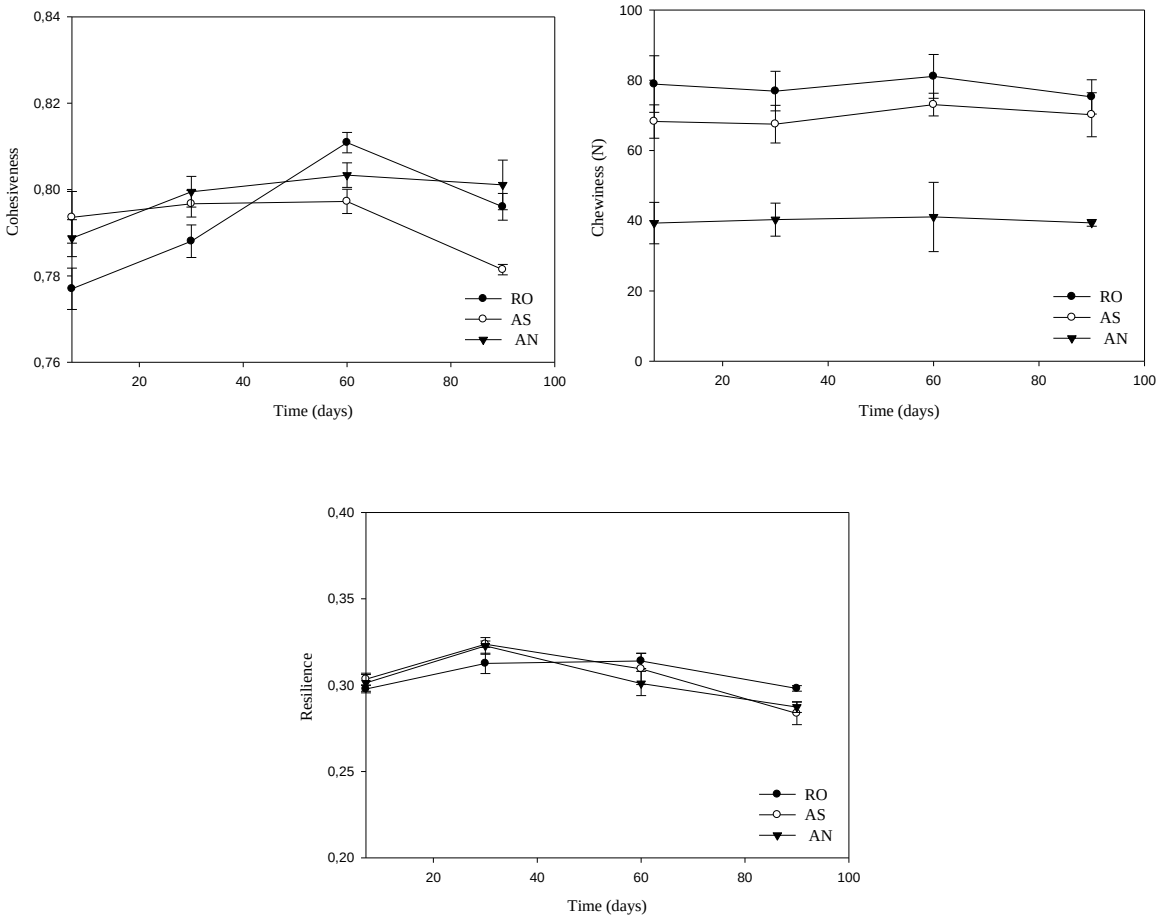


Figure 2. Textural properties of Circassian cheeses during storage time

3.7. Sensory properties

The results of sensory evaluation of Circassian cheeses are presented in Figure 3. The aroma scores of Circassian cheese samples were found higher in batch AN, followed RO and AS ($P<0.05$). Some panelists stated that higher incense aroma of batch AS was negatively affected the cheese aroma. Similarly, Atasever et al. (2003) showed that Kashar cheese smoked with traditionally had less aroma scores than non-smoked cheese and smoked with liquid smokes. However, Ayar et al. (2015) found that the incense aroma was achieved by applying the traditional method of fumigation. As expected, "whey" and "fermented" aroma, the most characteristic aroma terms of Circassian cheese, were defined for batch AN by panelists. Moreover, smoking process was decreased the "whey" or "fermented" aroma of smoked cheeses. On the contrary, Cais–Sokolińska et al. (2014) reported that smoked Mozzarella cheese was less acceptable than non-smoked cheese in related "whey" aroma. The aroma values of cheese samples were increased during storage except batch AN ($P<0.05$) and at the end of storage period, batches AS and RO were considered more acceptable than batch AN.

The texture scores were found highest for batch RO ($P<0.05$) and all texture scores of cheese samples were considered as "good". The texture of batch AN was identified as soft by panelists, but batches RO and AS were identified as slightly tough and flexible. Ayar et al. (2015) found that smoked cheeses were better than non-smoked cheeses according to average

structure scores. During storage period, texture values were increased for all cheese samples and finally batch RO was exhibited the most desirable texture.

The appearance and color properties of cheese samples were significantly affected by manufacture and smoking process ($P<0.05$). Smoking process was negatively affected the appearance and color properties. Similarly, Atasever et al. (2003) stated that Kashar cheese smoked with traditionally method had lower scores than non-smoked cheese samples. On the contrary, Ayar et al. (2015) reported that smoking process did not significantly affect to the visual properties of Circassian cheese. After the storage period, smoked cheese samples had similar scores to batch AN and all cheese samples were acceptable considering appearance and color scores.

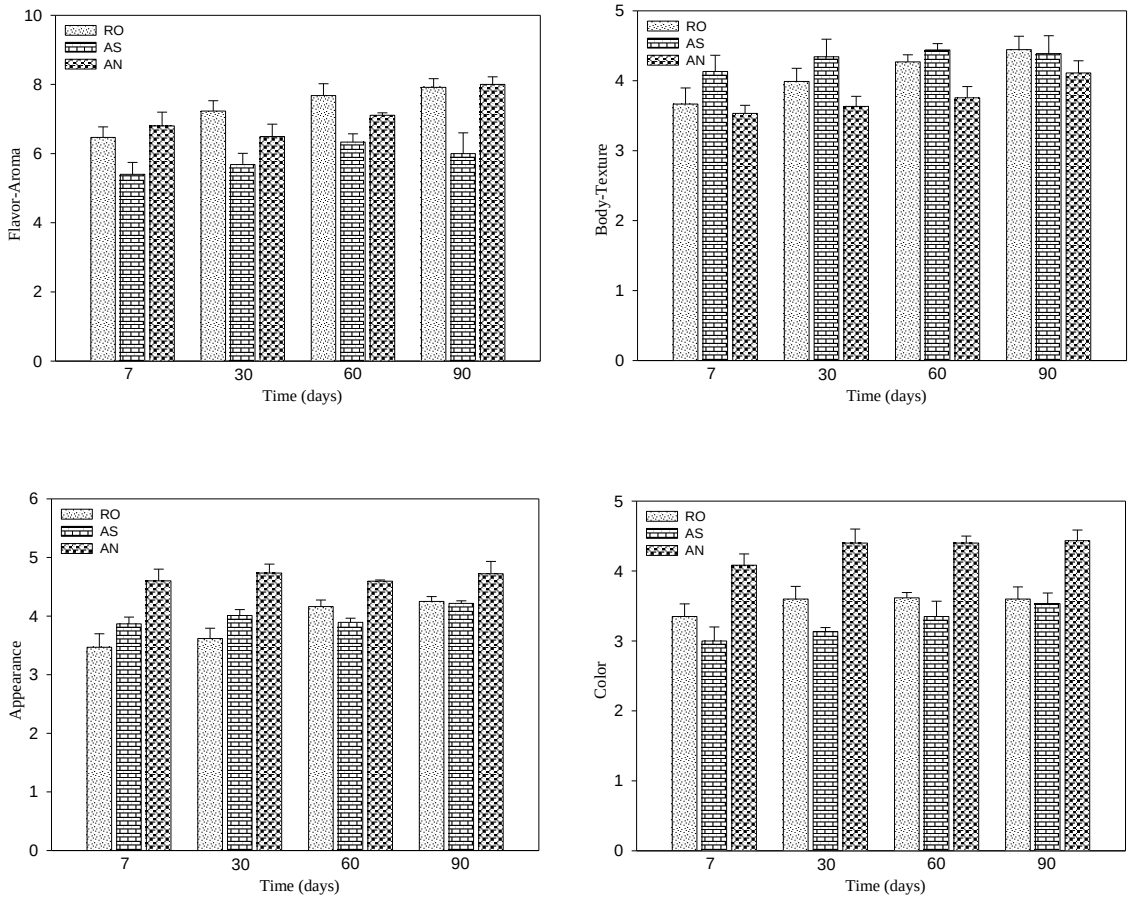


Figure 3. Sensory properties of Circassian cheeses during storage time

3.8. PAH contents

PAH contents of Circassian cheese produced and smoked with different process are shown in Table 4. The sum of nine PAHs (Naph, Ace, Ant, Pyr, B[a]A, B[k]F, B[a]P, DB[ah]A and B[ghi]P) and the sum of 5 carcinogenic PAHs (B[a]A, B[k]F, B[a]P, DB[ah]A and B[ghi]P) were found higher in batch AS (4.93 and 0.53 μg kg^{-1}, respectively) than AN and RO ($P<0.05$). Our results were much lower than other study carried out by Fasano et al. (2016) who reported that the total concentration of PAHs in the whole smoked cheese ranged from 1.1 to 176 μg kg^{-1} with mean value of 88 μg kg^{-1}. Naph and Ace showed the highest contributions of the total average PAHs as

53.25% for batch RO, 62.92% for batch AS and 66.1% for batch AN. Our results similar with those reported by Gul et al. (2015) for Circassian cheese since Naph and Ace were the most abundant of PAHs. While B[ghi]P was not detect in all samples (below the LOD), Naph, Ace, Ant and B[k]F were detected in all samples. B[a]P, could be used as marker for the occurrence of PAHs in foods (EC, 2006), was not detected (below the LOD) in batches AN and RO. But it was found in batch AS at the levels of 0.11 $\mu g\ kg^{-1}$ and the contribution of B[a]P was about 3.02%. Similarly, Aydinol and Ozcan (2013) reported that B[a]P was detected only in exterior part of the naturally smoked Circassian cheese at the level of 5 $\mu g\ kg^{-1}$. Most of studies were exhibited presence of B[a]P in naturally smoked cheese (Esposito et al., 2015; Fasano et al., 2016; Gul et al., 2015; Suchanová et al., 2008). It could be explained the deposition of PAHs containing solid particles on cheese surface during naturally smoking process (Suchanová et al., 2008). The B[a]P level of Circassian cheese in this study was below the maximum tolerable limits (5 $mg\ kg^{-1}$ for smoked meat) set by Commission Regulation (EC) No. 1881/2006 dated 19 December 2006 (EC, 2006) and the maximum permissible level of 1 $mg.kg^{-1}$ for smoked foods accepted by some European countries.

Smoking process carried out with electrically oven caused significantly increasing the level of Naph, Ace, Ant, Pyr and B[a] compared the PAH contents of non-smoked cheese. Moreover, traditionally smoking process caused significantly increase the all PAHs except B[ghi]P ($P<0.05$). Additionally, sum of nine PAHs and carcinogenic PAHs of batches AS and RO were found 2.14 and 6.67, and 1.99 and 6.12 times higher than batch AN, respectively. The traditionally smoking process could lead to the formation of toxic PAHs due to direct contact between the food and smoke (Esposito et al., 2015). However, increase of PAHs during smoking process with oven-baked could be explained by high temperature directly applied to cheese. Baking process has been increased the PAH contents in several foods such as breads, biscuits, cakes etc. reported by several studies (Iwegbue et al., 2014; Singh et al., 2016).

4. Conclusions

The results obtained from this preliminary study related with Circassian cheese smoked with different smoking processes (natural and oven) show that physicochemical, microbiological, textural and sensory properties were significantly affected the manufacture and smoking process. The use of oven for smoking caused significantly decrease the microbial counts and also decreased the PAH formation during smoking process. Carcinogenic PAHs and B(a)P detected in batch RO was considerable found lower than in batch AS. According to sensory evaluation, smoking of Circassian cheese used electrically oven was more acceptable than natural smoking process. Therefore, oven-baked smoking technique can be used as smoking process instead of natural smoking process in the dairy industry.

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EFFECTS OF SUPERHEATED STEAM DRYING ON THE ANTIOXIDANT AND ANTI-TYROSINASE PROPERTIES OF SELECTED LABIATAE HERBS

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ABSTRACT
In this study, the antioxidant and anti-tyrosinase properties of fresh, commercial dried (CD) and superheated steam-dried (SS-D) Labiatae herbs were analysed and evaluated. Superheated steam drying (SSD) was performed at 150°C and 200°C for 5, 10 and 20 min. Fresh and CD rosemary had the highest phenolic contents and the strongest primary antioxidant activities of free radical scavenging and ferric reducing power. Fresh spearmint, CD peppermint and CD oregano displayed the strongest secondary antioxidant activity of ferrous ion chelating ability. Based on total phenolic content and free radical scavenging, three broad categories of SS-D herbs were recognized i.e. herbs that showed declines for all the drying regimes (thyme and peppermint); those that showed declines or remained unchanged (marjoram and oregano); and those that showed all three traits of increment, declines or unchanged (rosemary, sage and spearmint). Tyrosinase inhibition was strongest in fresh sage, fresh rosemary, CD thyme and CD rosemary. Reported for the first time, SS-D rosemary, SS-D thyme and SS-D marjoram showed enhanced anti-tyrosinase properties for all the drying regimes. SS-D marjoram was the most exciting as tyrosinase inhibition was not detected in fresh samples. This study on the antioxidant and anti-tyrosinase properties of selected Labiatae herbs has provided some useful insights on the effects of SSD. The drying technique can be used for the production of tyrosinase inhibitors, which are increasingly used in medicines for treating pigmentation disorders, in cosmetics for skin whitening, and in food products for inhibiting browning.

1. Introduction

Drying and cooking methods and conditions can impart changes to the biochemical properties of culinary herbs and spices. For many herbs and spices, such studies are still lacking (Yi and Wetzstein, 2011). In our previous studies, the antioxidant, antibacterial and anti-quorum sensing (anti-QS) properties of Labiatae herbs, and the effects of microwave, blanching and boiling were analysed (Chan et al., 2012a, 2012b). In another study, we reported the antioxidant, anti-tyrosinase, antibacterial and anti-QS properties of selected spices, and the effects of microwave, blanching and boiling (Chan et al., 2015). In an earlier study, we reported the antibacterial and anti-QS activities of fresh, commercial dried and superheated steam-dried Labiatae herbs (Chan et al., 2017). In this study, the antioxidant and anti-tyrosinase properties of fresh and commercial dried Labiatae herbs were analysed and evaluated. The effects of superheated steam drying on their antioxidant

and anti-tyrosinase properties were reported for the first time.

Labiatae (also known as Lamiaceae) is a large family of plants with more than 200 genera and almost 4,000 species centred mainly in the Mediterranean region (Naghibi et al., 2010). The family comprises annual or perennial herbs that are aromatic and densely glandular (Kokkini et al., 2003). Leaves are simple and opposite, and stems are quadrangular in cross-section. Flowers are hermaphrodite and form whorls arranged in spikes, heads, racemes, or cymes. With chemical constituents of terpenoids, iridoids, flavonoids and phenolic acids, the different plant parts of Labiatae species are widely used for food flavouring and preservative, and as herbal teas or traditional medicines (Kokkini et al., 2003; Naghibi et al., 2010). Some species of Labiatae are planted as ornamentals and as sources of aromatic oils. Antioxidant, antimicrobial, analgesic, anti-inflammatory, hypotensive and cardiotonic activities are among their wide array of biological and pharmacological properties.

Superheated steam drying (SSD) is an emerging drying technique where saturated steam is heated beyond the boiling point at a given pressure (Law et al., 2013). Moisture from the food is removed by the temperature difference between the food and the superheated steam in a closed system. SSD offers several advantages (Karimi 2010; Law et al., 2013; Mujumdar 2014). It has a higher drying rate than air-drying. High steam temperature and free diffusion of water vapour are important factors for the high drying rate. The system is energy efficient as the exhausted steam can be recycled back to the system and only ~30 kJ of heat is needed to convert the saturated steam to superheated steam. No oxidation is involved and the system is free from fire or explosion hazards. SSD inactivates microbes and enzymes. Of interest to food processing is the non-oxidative reactions, ability to maintain colour and nutrients, and yielding products of higher porosity. Overall, SSD offers advantages such as, an oxygen-free environment, which lowers the percentage of oxidation and nutrient loss, improves thermal degradation due to the increase in heat transfer, improves energy efficiency, and accelerates drying rate (Cenkowski et al., 2007).

Studies have shown that some superheated steam-dried food products such as shrimp (Prachayawarakorn et al., 2002), oat groat (Head et al., 2009), rice (Rumruaytum et al., 2013), cocoa bean (Zzaman et al., 2014) and chicken sausage (Asmaa and Tajul, 2017) maintain good quality.

2. Materials and methods

2.1. Herbs studied

Seven fresh Labiatae herbs grown in Genting Highlands, Pahang, Malaysia were purchased in local supermarkets in Kuala Lumpur. They were rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.), oregano (*Origanum vulgare* L.), marjoram (*Origanum majorana* L.), thyme (*Thymus vulgaris* L.), peppermint (*Mentha piperita* L.) and spearmint (*Mentha spicata* L.). Their antioxidant and anti-tyrosinase properties were determined before and after SSD. Five commercial dried (CD) herbs of rosemary, sage, oregano, thyme and peppermint were also analysed for comparison.

2.2. Extraction of herbs

For the analysis of phenolic contents and antioxidant properties, fresh herbs (1 g) or dried herbs (0.3 g) were powdered with liquid nitrogen using a pestle and mortar. After powdering, the samples were poured into a conical flask and extracted using 50 mL of 70% methanol with continuous swirling for 1 h at 100 rpm using an orbital shaker at room temperature. The extracts were then filtered and stored at −20ºC in a freezer for further use.

For the analysis of anti-tyrosinase properties, fresh herbs (10 g) or dried herbs (3 g) were powdered with liquid nitrogen using a pestle and mortar. The powdered samples were then poured into conical flask and extracted using 100 mL of pure methanol with continuous swirling at 120 rpm using an orbital shaker. Extraction was repeated three times for 1 h each time. The filtered extracts were freeze-dried for 30 min to remove any liquid present before storage in a freezer at −20ºC for further analysis.

2.3. Superheated steam drying

Superheated steam drying (SSD) of fresh herbs was carried out at 150ºC and 200ºC for 5, 10 and 20 min. The superheated steam oven (Healsio, AX-1600, SHARP) was pre-heated at the required temperatures for 2 min before the herbs (20 g) were dried at the scheduled durations. On cooling, the dried herbs were weighed, sealed in an airtight bag and stored in a freezer at −20ºC for further analysis.

2.4. Phenolic contents

Herbs were analysed for phenolic contents of total phenolic content (TPC), total flavonoid content (TFC) and caffeoylquinic acid content (CQAC) using the Folin-Ciocalteu, aluminium chloride and molybdate assays, respectively (Chan et al., 2014, 2015).

In the Folin-Ciocalteu (FC) assay, extracts (300 µL) were introduced into test tubes wrapped with aluminium foil, followed by the addition of 1.5 mL of FC reagent (diluted 10 times) and 1.2 mL of sodium carbonate (7.5%, w/v). After incubating for 30 min in the dark, absorbance was read at 765 nm against a blank using a UV-vis spectrophotometer (Anthelie Advanced 5 Secoman). TPC was expressed as gallic acid equivalent (GAE) in mg/100 g of sample.

In the aluminium chloride assay, extracts (1 mL) were introduced into test tubes containing 4 mL of water. Then, 0.3 mL of 5% sodium nitrite was added, followed by 0.3 mL of 10% aluminium chloride, 2 mL of sodium hydroxide solution and 2.4 mL of water to make up to 10 mL. After mixing well and incubated at room temperature for 10 min, absorbance was read at 415 nm against a sample blank of 1 mL of the respective extracts with 9 mL of water. TFC was expressed as quercetin equivalent (QE) in mg/100 g of sample.

In the molybdate assay, the reagent was prepared by dissolving 16.5 g sodium molybdate, 8 g dipotassium hydrogen phosphate and 7.9 g potassium dihydrogen phosphate in 1 L of water. The reagent (2.7 mL) was added to the extract (0.3 mL), mixed and incubated at room temperature for 10 min. Absorbance was read at 370 nm against a sample blank of the extracts with 2.7 mL of water. CQAC was expressed as chlorogenic acid equivalent (CGAE) in mg/100 g of sample.

2.5. Antioxidant activities

Antioxidant activities of free radical scavenging (FRS) activity, ferric reducing power (FRP) and ferrous ion chelating (FIC) ability were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, potassium ferricyanide and ferrozine assays, respectively (Chan et al., 2014; 2015). In the DPPH assay, different dilutions of extracts (1 mL) were added to 2 mL of DPPH (5.9 mg in 100 mL methanol). Absorbance was read at 517 nm after 30 min. The IC_{50} was expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg ascorbic acid (AA)/100 g of sample, which was calculated as $IC_{50\ ascorbic\ acid}$ / $IC_{50\ sample}$ x 10^5 where the IC_{50} of ascorbic acid was 0.0039 mg/mL.

In the potassium ferricyanide assay, different dilutions of extracts (1 mL) were added to 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide

(1%, w/v). The mixture was incubated at 50°C for 20 min. After adding trichloroacetic acid solution (2.5 mL, 10%, w/v), the mixture was separated into aliquots of 2.5 mL, and diluted with 2.5 mL of water. Ferric chloride solution (500 mL, 0.1%, w/v) was added to each diluted aliquot and absorbance was read at 700 nm against a blank using a UV-vis spectrophotometer (Anthelie Advanced 5 Secoman) after 30 min. FRP was expressed as mg GAE/100 g of sample. The calibration equation for gallic acid (GA) was y = 16.767x (R^2 = 0.9974), where y is the absorbance and x is the GA concentration in mg/mL.

In the ferrozine assay, different dilutions of extracts (1 mL) were mixed with $FeSO_4$ (0.1 mM, 1 mL) and ferrozine (0.25 mM, 1 mL). Absorbance was read at 562 nm after 10 min. FIC ability was calculated as ($1 - A_{sample} / A_{control}$) x 100% and expressed as chelating efficiency concentration (CEC_{50}) in mg/mL or the effective concentration of extract needed to chelate ferrous ions by 50%.

2.6. Anti-tyrosinase activity

Tyrosinase inhibition of herbs was determined using the dopachrome assay with L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate (Tan and Chan, 2014; Chan et al., 2015). Samples were prepared by dissolving 10 mg of dried extracts in 1 mL of methanol and 1.5 mL of 50% dimethyl sulphoxide (DMSO). The assay was conducted in a 96-well microtiter plate using a plate reader (BIOTEK PowerWave XS Microplate) to measure absorbance at 450 nm with 630 nm as reference. Each well was filled with 40 µL of sample with 80 µL of phosphate buffer (0.1 M, pH 6.8), 40 µL of tyrosinase (31 units/mL) and 40 µL of L-DOPA (2.5 mM). Each sample was accompanied by a blank with all the components except L-DOPA. Results were compared with a control consisting of DMSO in place of sample. Tyrosinase inhibition in percent was calculated as ($A_{control} - A_{sample}$) / $A_{control}$ x 100%. The concentration of extracts used for determining tyrosinase inhibition was 0.25 mg/mL.

2.7. Statistical analysis

Experiments were conducted in triplicate (n = 3) and results were expressed as means ± standard deviations. Analysis of variance was analysed using the Tukey Honestly Significant Difference (HSD) test based on significant difference of $p < 0.05$.

3. Results and discussion

3.1. Antioxidant properties of fresh herbs

Of the seven fresh herbs analysed (Table 1), rosemary displayed the highest phenolic contents of TPC (1280 mg GAE/100 g), TFC (215 mg QE/100 g) and CQAC (467 mg CGAE/100 g), and the strongest antioxidant activities of AEAC (1890 mg AA/100 g) and FRP (1210 mg GAE/100 g). Spearmint and peppermint had the lowest phenolic contents and weakest antioxidant activities.

The potent antioxidant properties of rosemary may be attributed to its phenolic constituents. Carnosol, carnosic acid, rosmanol and rosmarinic acid are the most important constituents that account for most of the antioxidant activity of rosemary (Ho et al., 2000; Etter, 2004). From rosemary, rosmanol and carnosol exhibited antioxidant activity more than four and two times higher than butylated hydroxytoluene (BHT), respectively (Nakatani, 2000).

In contrast, the chelating efficiency concentration (CEC_{50}) of spearmint (2.6 mg/mL) was the strongest and the weakest in rosemary (6.3 mg/mL) (Table 1). In contrast to the ranking based on phenolic contents of TPC, TFC and CQAC which showed similarity with antioxidant activities of AEAC and FRP, the ranking based on antioxidant activity of CEC_{50} was almost the reverse.

Rosemary had the highest phenolic contents of TPC, TFC and CQAC, and the

strongest antioxidant activities of AEAC and FRP but the weakest CEC_{50}. On the other hand, spearmint had the lowest phenolic contents and the second lowest antioxidant activities of AEAC and FRP but was the strongest in CEC_{50}. AEAC and FRP are measures of the hydrogen- and electron-donating abilities of primary or chain-breaking antioxidants (Antolovich et al., 2002). They prevent oxidative damage by directly scavenging free radicals (Lim et al., 2007). FIC measures the ability of secondary or preventative antioxidants to chelate metal ions. They act indirectly by preventing the generation of hydroxyl radicals *via* the Fenton's reaction. Findings of this study support our earlier report that herbal teas with strong FIC ability usually display moderate or low primary antioxidant activities (Chan et al., 2012a). This indicates that plants with potent primary antioxidant activities may not have strong secondary antioxidant activities.

The antioxidant properties of fresh non-Labiatae herbs have reported earlier by Chan et al. (2014) using the same protocols. This presented an opportunity to broadly compare between the antioxidant properties of Labiatae herbs in this study (Table 1) and those of non-Labiatae herbs. In general, non-Labiatae herbs with the strongest antioxidant properties (e.g. *Anacardium occidentale*, *Persicaria hydropiper* and *Cosmos caudatus*) surpass those of rosemary. At the same time, there is a host of non-Labiatae species with antioxidant properties weaker than or comparable to those of Labiatae herbs.

3.2. Antioxidant properties of CD herbs

Out of the five commercial dried (CD) herbs analysed, rosemary displayed highest phenolic contents (TPC, TFC and CQAC) while lowest values were observed in oregano (Table 2). Rosemary also had the strongest primary antioxidant activities with AEAC of 5710 mg AA/100 g and FRP of 3510 mg GAE/100 g. Peppermint had the weakest activities with AEAC of 3720 mg AA/100 g and FRP of 1930 mg GAE/100 g. In terms of CEC_{50}, the strongest chelating ability was observed in peppermint (1.0 mg/mL) followed by oregano (1.3 mg/mL). Both rosemary (3.0 mg/mL) and thyme (2.9 mg/mL) had the weakest activities. The ranking of secondary antioxidant activity of FIC based on CEC_{50} was in the reverse order of the ranking in phenolic contents of TPC, TFC and CQAC, and primary antioxidant activities of AEAC and FRP.

To find out if herbs or spices have stronger antioxidant properties, data of CD herbs (Table 2) were compared to those of dried spices reported by our research group earlier using the same protocols (Chan et al., 2015). Interestingly, some distinct contrasts emerged. There was evidence that spices have a much greater range of values compared to herbs. In terms of phenolic contents of TPC, TFC and CQAC, the highs of spices (e.g. clove and cinnamon) were much higher than those of rosemary, and the lows of spices (e.g. poppy and cardamom) were much lower than those of oregano. The same trend was evident for primary antioxidant properties of AEAC and FRP. Based on secondary antioxidant activity of CEC_{50}, herbs generally had stronger chelating ability than spices.

3.3. Superheated steam drying of herbs

Average moisture loss of the herbs following superheated steam drying (SSD) at 150^{o}C for 5, 10 and 20 min was 61%, 77% and 84%, and the loss following SSD at 200^{o}C for the same durations was 71%, 83% and 86%, respectively. Acknowledging that variations exist between herbs, changes in colour are briefly described. Following SSD at 150^{o}C, the herbs turned from dark green (5 min) to dark green with slight browning (10 min), and to browning and shrivelling of leaves (20 min). After SSD at 200^{o}C, the herbs turned from dark green with slight

browning (5 min) to browning and shrivelling of leaves (10 min), and to browning, shrivelling and slight scorching of leaves (20 min).

3.4. Antioxidant properties of SS-D herbs

When subjected to drying at 150ºC and 200ºC for 5, 10 and 20 min, superheated steam-dried (SS-D) herbs showed variations in antioxidant properties when compared with those of fresh herbs. Based on TPC and AEAC, three broad categories of effects can be recognized (Table 3). They are herbs that showed declines in TPC and AEAC for all the drying regimes (thyme and peppermint); those that showed declines or remained unchanged (marjoram and oregano); and those that showed all three traits of increments, declines or remained unchanged (rosemary, sage and spearmint). In the first category, declines of 26−60% were observed in thyme and 28−73% in peppermint. In the second category, declines were 20−64% in marjoram and 21−48% in oregano. In the third category, gains of 23−52% and declines of 26−44% were displayed by rosemary, 24−35% and 25−50% by sage, and 40−60% and 21−58% by spearmint. Most of the declines in TPC and AEAC were observed following SSD for 20 min. Overall, findings of significant declines in TPC and AEAC following SSD were consistent with our earlier study on the effects of oven drying on Labiatae herbs (Chan et al., 2012a, 2012b). Earlier studies using other drying methods also showed variable effects. Compared to fresh herbs, the phenolic content of air-dried oregano and peppermint increased significantly but their radical scavenging activity declined significantly and remained unchanged, respectively (Capecka et al., 2005). The phenolic content and antioxidant activity of sun-dried rosemary and peppermint were enhanced while those oven-dried at 40ºC and 70ºC remained unchanged (Yi and Wetzstein, 2011).

Studies on other plant products also showed declines in antioxidant properties following SSD. Although the phenolic content of SS-D mate leaves was 1.9 times higher than leaves dried with conventional hot air, they were about 3 times lower than fresh leaves (Zanoelo et al., 2006). The higher phenolic content of SS-D mate leaves was credited to lower phenol oxidase activity induced by an oxygen-free atmosphere. SS-D cocoa beans at 150ºC, 200ºC and 250ºC for 10−50 min resulted in significant declines of phenolic contents of TPC and TFC, and antioxidant activities of DPPH radical scavenging and FRP with increasing time and temperature (Zzaman et al., 2014). Similarly, SSD resulted in the loss of antioxidant properties of mangosteen rinds (Suvarnakuta et al., 2011). Although SS-D avocado pulp had significantly higher TPC and TFC, and stronger DPPH radical scavenging activity than freeze-dried samples, no comparisons were made with unprocessed avocado pulp (Husen et al., 2014).

Processing methods are known to have variable effects on the phenolic content and antioxidant activity of plant samples. Effects include little or no change, significant losses or enhancement (Nicoli et al., 1999). Food processing can induce the formation of new compounds so that the overall antioxidant properties increase or remain unchanged (Tomaino et al., 2005). Declines in antioxidant properties of plant samples have been attributed to thermal degradation of phytochemicals and to loss of antioxidant enzyme activities during heat treatments (Larrauri et al., 1997; Lim and Murtijaya, 2007; Chan et al., 2013).

3.5. Anti-tyrosinase properties of fresh herbs

Of the fresh herbs analysed for tyrosinase inhibition, rosemary (51%), sage (52%) and peppermint (47%) were significantly the strongest (Table 4). The

weakest tyrosinase inhibition was observed in spearmint and oregano (both 17%) while that of marjoram was not detected. Ranking of fresh herbs based on tyrosinase inhibition was rosemary ~ sage ~ peppermint > thyme > spearmint ~ oregano > marjoram. In support of findings of this study, Lin et al. (2011) studied the anti-tyrosinase activity of 48 herbs and found that oregano, marjoram, rosemary, sage and peppermint were observed to have tyrosinase inhibition, with exception of marjoram. Contrary to findings of this study, *O. majorana* was observed to exhibit the strongest tyrosinase inhibitory activity among 28 species of Lamiaceae plants, with an IC_{50} of 0.11 mg/mL (Lee et al., 2011).

Table 1. Phenolic contents and antioxidant activities of fresh herbs

Fresh herb	Phenolic content			Antioxidant activity		
	TPC	TFC	CQAC	AEAC	FRP	CEC_{50}
Rosemary	1280±164^{a}	215±40^{a}	467±74^{ab}	1890±333^{a}	1210±133^{a}	6.3±0.7^{c}
Thyme	875±109^{b}	127±10^{c}	384±45^{b}	871±119^{c}	594±50^{c}	4.7±0.1^{b}
Marjoram	735±48^{b}	170±15^{b}	503±49^{a}	1620±166^{a}	816±62^{b}	3.3±1.2^{a}
Sage	732±108^{b}	241±19^{a}	348±37^{bc}	886±120^{c}	870±54^{b}	5.5±0.4^{bc}
Oregano	524±49^{c}	86±18^{d}	281±42^{c}	1100±19^{b}	471±41^{d}	4.4±0.7^{b}
Peppermint	367±55^{d}	83±10^{d}	160±29^{d}	345±47^{e}	203±40^{f}	3.9±0.5^{ab}
Spearmint	294±14^{d}	65±2^{e}	148±22^{d}	457±18^{d}	275±7^{e}	2.6±0.3^{a}

Abbreviations: TPC = total phenolic content (mg GAE/100 g), TFC = total flavonoid content (mg QE/100 g), CQAC = caffeoylquinic acid content (mg CGAE/100 g), AEAC = ascorbic acid equivalent antioxidant capacity (mg AA/100 g), FRP = ferric reducing power (mg GAE/100 g), CEC_{50} = median chelating efficiency concentration (mg/mL), GAE = gallic acid equivalent, QE = quercetin equivalent, CGAE = chlorogenic acid equivalent and AA = ascorbic acid. Data on antioxidant properties based on phenolic contents and antioxidant activities in fresh weight are means ± standard deviations. Lower CEC_{50} values indicate stronger ferrous ion chelating (FIC) ability. Within each column, values with different superscript letters (a−e) are significant at $p < 0.05$ using the Tukey HSD test.

Table 2. Phenolic contents and antioxidant activities of commercial dried herbs

CD herb (brand)	Phenolic content			Antioxidant activity		
	TPC	TFC	CQAC	AEAC	FRP	CEC_{50}
Rosemary (McCormick)	6030±305^{a}	973±31^{b}	2490±41^{a}	5710±284^{a}	3510±286^{a}	3.0±0.2^{d}
Thyme (McCormick)	4670±331^{b}	1450±116^{a}	1660±194^{c}	5820 ±363^{a}	2460±118^{b}	2.9±0.5^{cd}

Sage (Heritage)	4390±269^{b}	944±88^{b}	2220±152^{b}	5100 ±308^{b}	2090±265^{c}	2.5±0.2^{c}
Peppermint (Boh)	3100±115^{c}	1510±76^{a}	1240±58^{e}	3720 ±184^{d}	1930±60^{c}	1.0±0.1^{a}
Oregano (McCormick)	2260±64^{d}	971±44^{b}	1400±55^{d}	4670 ±215^{c}	2420±129^{b}	1.3±0.1^{b}

Abbreviations: CD = commercial dried, TPC = total phenolic content (mg GAE/100 g), TFC = total flavonoid content (mg QE/100 g), CQAC = caffeoylquinic acid content (mg CGAE/100 g), AEAC = ascorbic acid equivalent antioxidant capacity (mg AA/100 g), FRP = ferric reducing power (mg GAE/100 g), and CEC_{50} = median chelating efficiency concentration (mg/mL), GAE = gallic acid equivalent, QE = quercetin equivalent, CGAE = chlorogenic acid equivalent and AA = ascorbic acid. Data on phenolic contents and antioxidant activities in dry weight are means ± standard deviations. Lower CEC_{50} values indicate stronger ferrous chelating ability. Within each column, values with different superscript letters (a−e) are significant at $p < 0.05$ using the Tukey HSD test.

Table 3. TPC and AEAC of superheated steam-dried herbs at 150°C and 200°C based on % gain or % loss compared to fresh herbs

Herb	Fresh TPC/FRS	SS-D at 150°C ($SS\text{-}D_{150}$)			SS-D at 200°C ($SS\text{-}D_{200}$)		
		5 min	10 min	20 min	5 min	10 min	20 min
Rosemary	1280±164 1890±333	+52 +37	+38 UC	UC UC	+38 +23	UC UC	−26 −44
Marjoram	735±48 1620±166	−56 −64	−29 −53	−20 −38	UC UC	UC −20	UC −44
Oregano	524±49 1100±19	UC UC	UC UC	UC −28	UC −21	−30 −28	−42 −48
Thyme	875±109 871±119	−36 −41	−49 −53	−55 −60	−41 −42	−26 −27	−32 −38
Sage	732±108 886±120	+27 UC	+35 +24	−25 −50	UC UC	UC +32	−31 UC
Spearmint	294±14 457±18	UC −41	+59 UC	−21 −58	+40 UC	+60 UC	UC −53
Peppermint	367±55 345±47	−61 −73	−46 −51	−63 −68	−41 −42	−30 −31	−30 −28

Abbreviations: TPC = total phenolic content, FRS = free radical scavenging expressed as ascorbic acid equivalent antioxidant capacity (AEAC), SS-D = superheated steam-dried and UC = unchanged. Data on TPC (mg GAE/100 g) and AEAC (mg AA/100 g) of SS-D herbs are based on fresh weight equivalent. They show significant percentage gain (+) or significant percentage loss (−) or unchanged (UC) compared to those of fresh herbs at $p < 0.05$ using the Tukey HSD test.

Table 4. Tyrosinase inhibition (%) of superheated steam-dried herbs at 150°C and 200°C compared to fresh herbs

Herb	Fresh	SS-D at 150°C ($SS\text{-}D_{150}$)			SS-D at 200°C ($SS\text{-}D_{200}$)		
		5 min	10 min	20 min	5 min	10 min	20 min
Sage	52±6	48±2	61±4↑	73±6↑	71±4↑	53±7	49±4
Rosemary	51±3	68±2↑	88±4↑	93±5↑	90±5↑	84±6↑	69±2↑
Peppermint	47±6	47±4	44±6	54±4	50±3	42±5	51±5
Thyme	31±2	59±2↑	40±2↑	39±1↑	45±2↑	51±2↑	60±4↑
Spearmint	17±6	33±5↑	39±5↑	47±6↑	42±4↑	25±5	47±5↑
Oregano	17±6	29±7	39±4↑	48±4↑	41±5↑	46±4↑	25±3
Marjoram	ND	34±6↑	16±2↑	35±5↑	21±6↑	11±6↑	22±5↑

Abbreviations: SS-D = superheated steam-dried and ND = not detected. Tyrosinase inhibition (%) of SS-D herbs with significant gain (↑) at $p < 0.05$ or remaining unchanged compared to fresh herbs.

3.6. Anti-tyrosinase properties of CD herbs

Of the five commercial dried (CD) herbs, tyrosinase inhibition was the strongest in thyme (61%), followed by rosemary (51%), peppermint (38%), sage (31%) and oregano (27%). Out of six dried spices, Chan et al. (2015) reported the strongest anti-tyrosinase activity in cinnamon (45%) and cumin (42%). This would mean that CD herbs have stronger anti-tyrosinase properties.

3.7. Anti-tyrosinase properties of SS-D herbs

Three of the SS-D herbs, namely, rosemary, thyme and marjoram, showed significantly enhanced anti-tyrosinase properties compared with their fresh counterparts for all the drying regimes (Table 4). Of these, rosemary had the largest gains of 17−42% for SSD at 150°C and 18−39% for SSD at 200°C. The anti-tyrosinase properties of SS-D marjoram represented the most exciting discovery as tyrosinase inhibition was not detected in fresh samples. The anti-tyrosinase properties of sage, spearmint and oregano showed significant gains or remained unchanged while those of peppermint remained unchanged for SSD at 150°C and 200°C. This is the first report on the enhancement effects of SSD on the tyrosinase inhibition of Labiatae herbs. Previous studies on the anti-tyrosinase properties have been based on fresh plant samples. Phenolic acids such as rosmarinic acid and caffeic acid, and flavonoids such as luteolin, quercetin and kaempferol found in Labiatae herbs have been reported to possess anti-tyrosinase activity (Ulubelen et al., 2005; Kim and Uyama, 2005; Fujimoto et al., 2011). Increase in bioactivity of plant materials after heat treatment has been attributed to bioactive compounds released due to the rupture of the cell matrix or produced by thermal chemical reaction, and to thermal inactivation of oxidative enzymes (Jimenez-Monreal et al., 2009; Chan et al., 2013). These reasons can also be applied for the enhanced anti-tyrosinase activity of the SSD herbs. In the literature, enhanced anti-

tyrosinase activity of plant extracts after thermal treatments has been reported. The anti-tyrosinase activity of microwave-treated cashew leaves of 49% was significantly higher than fresh leaves of 40% (Tan and Chan, 2014). Similarly, Chan et al. (2015) reported that microwave-treated cardamom (24%) also showed significant increase in tyrosinase inhibition compared to fresh cardamom (17%).

4. Conclusion

This study on the antioxidant and anti-tyrosinase properties of selected Labiatae herbs has provided some useful insights on the effects of SSD, which are reported for the first time. SS-D herbs included those that showed declines in antioxidant properties for all the drying regimes; those that showed declines or remained unchanged; and those that showed all three traits of increments, declines or unchanged. Of the SS-D herbs, rosemary, thyme and marjoram showed enhanced anti-tyrosinase properties following all drying regimes with marjoram being the most exciting as tyrosinase inhibition was not detected in fresh samples. SSD may therefore be a promising drying technique of Labiatae herbs for the commercial production of tyrosinase inhibitors, which are increasingly used in medicines for treating pigmentation disorders, in cosmetics for their skin-whitening effects, and in food products for inhibiting browning. However, further research is needed in optimising the drying conditions to maximise their anti-tyrosinase potentials for use as pharmaceutical and cosmeceutical products, and as food preservatives.

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THE EFFECTS OF SAUSAGE CASING WITH POTASSIUM SORBATE ON COLOUR, MICROBIOLOGICAL PROPERTIES AND FORMATION OF BIOGENIC AMINES OF DRY FERMENTED SAUSAGE (SUCUK)

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ABSTRACT
The sausage casings were dipped into potassium sorbate (PS) solutions (PS0; 0% (Control), PS5; 5%, PS10; 10%, PS20; 20%) before filling and the effects of this treatment were investigated on the formation of biogenic amine (BA), microbiological and physical properties of sausage during the ripening (12 days) and refrigerated storage (4 °C, 30 days). The effect of PS treatment was not found to be singificant on the pH values of the sausages. Yeast-mold and *Enterobacteriaceae* were detected only in the PS0 samples. The effect of PS treatment on the counts of total aerobic mesophilic bacteria, lactic acid bacteria and *Micrococcus/Staphylococcus* was found to be significant. Moreover, this effect on BAs except for spermine and spermidine was found to be significant. While PS treatment restricted the increase in tryptamine, 2-phenylethyl amine, putrescine, cadaverine, tyramine contents of sausages, it caused an increase in the formation of histamine. As the PS concentration increased, the redness (*a*) and yellowness (*b*) values of the sausages decreased while the lightness (*L*) values decreased in the PS20 samples.

1. Introduction

The quality of fermented sausages is closely related to the species and count of microorganisms in the flora (Tekinşen et al., 1982). During the fermentation and ripening stages, particularly development of some microorganisms is desired whereas the development of some microorganisms is not desired (Kurt, 2006). Moreover, the presence of biogenic amines (BAs), known to be toxic for humans and animals (Shalaby, 1996), in meat and meat products is considered to be indicator of undesirable microbiological activity (Lu et al., 2010). For this reason, it is important to control the formation of biogenic amines in fermented meat products (Kurt 2006).

Dry fermented sausages contain high levels of BA (Lu et al., 2010) because of that they have appropriate preconditions for BA (Komprda et al., 2009). The most important BAs in fermented sausages are putrescine, cadaverine, phenylethylamine, spermine, spermidine, histamine, tryptamine and tyramine (Lu et al., 2010) and these are main products of microbial decarboxylation of amino acids (Xie et al., 2015). Decarboxylase activity in meat products generally has been attributed chiefly to *Enterobacteriaceae*, lactic acid bacteria, *Micrococcaceae* species (Ruiz-Capillas and Jimenez-Colmenero, 2004). Furthermore, Yeasts such as *Debaryomyces* and *Candida* species isolated from fermented meats have also been reported to have histidine decarboxylase activity (Ercoşkun et al., 2005). The relationship between BAs and microbial load has been proven and it is stated that BA formation can be

restricted by controlling the count of microorganisms (Ercoşkun et al., 2000).

On the other hand, one of the most important problem in fermented sausage production is mold growth (Öztürk, 2015) due to their tolerances to low water activity and pH (Castellari et al., 2010). Molds and yeasts usually develop on the surface and near the surface of sausages (Gökalp et al., 1999). The development of undesirable mold species may adversely affect the quality properties of the product, furthermore cause the formation of toxic secondary metabolites such as mycotoxin. For this reason, mold growth on sausage surface is regarded as a toxic hazard for human health (Chaves-Lopez et al., 2012). To avoid this risk, various chemical preservatives such as potassium sorbate (PS) and sodium benzoate are added to the product formulations (Chaves-Lopez et al., 2012).

Sorbic acid and its salts, especially PS, are widely used worldwide in various foods (Davidson et al., 2005). Sorbates can be used in various forms such as direct addition to the product, spraying with sorbate solution or dipping in sorbate solution (Sofos and Busta, 1981). Sorbates are effective on many microorganisms including yeast, mold and bacteria (Davidson et al., 2005). The inhibitor effect PS on the bacteria is lower than the inhibitor effect on yeast-mold (Tekinşen et al., 1999). PS is metabolized in the human body in similar to fatty acids (Doğruer et al., 1996). It is on the GRAS list (Hoang and Vu, 2016; Robach and Sofos, 1982). There are some studies (Bozkurt and Erkmen, 2002a; Jastrzębska et al., 2016; Shalaby and El-Rahman, 1995) using PS to limit the formation of some BA in meat products. They have investigated the effect of PS by adding to meat product formulation. Any study which sausage casings are treated with PS solution before filling to limit the formation of BAs in fermented sausage production was not encountered in the literature. In addition, it is necessary to investigate the color changes that can occur in the color values due to the application of PS to the sausage casing. This study was to investigate the effects of PS treatment of sausage casing before filling on the colour values, microbiological properties and BA formation.

2. Materials and methods

One day after slaughter, beef, beef subcutaneous fat and sheep tail fat were supplied from a local meat processor (Adıyaman, Turkey). Potassium sorbate was obtained from Carlo Erba. A collagen casing (35-36 mm diameter, Naturin Darm, Germany) was used to fill the sausage batter.

2.1. Sausage preparation

To prepare the sausage, beef (72%), beef fat (10%) and sheep tail fat (10%), spices (3.6%), salt (1.9%), garlic (2%), sugar (0.5%) and sodium nitrite (150ppm) were mixed and minced in a grinder with 3 mm diameter hole plate (Alveo, Turkey). Resulting batche of sausage batter was rested for 12 hour at 4°C. Collagen casing dipped into the potassium sorbate solution for 10 min before the batter was filled into the casings. Batter stuffed into collagen casings by using hydraulic filling machine (Mainca EM-30, Spain). Samples were ripened for 12 days in a climatic cabin (Qualitec Biosan, Konya). Ripening temperature was adjusted to 23 °C and then first 6 days decreased 1°C for every day. The relative humidity was started from 60±3% for 5 hours and was then increased to 90±3% and decreased every day by 1 unit. At the end of the ripening period, sausages were packed in a polyethylene bag and stored at 4°C for 30 days.

2.2. pH and microbiological analysis

Ten grams of sample was homogenized in 100 ml distilled water and the pH was determined using a pH meter (Hanna 2215, USA). For the microbiological analysis, twenty grams of sample was taken from each sample and homogenised in 180 ml of sterile salt solution (0.85% NaCl). The count of moulds-yeasts was determined on Dichloran Rose Bengal Chloramphenicol Agar (DRBCA)

incubated at 25°C for 5 days (APHA 1992), while total aerobic mesophilic bacteria on Plate Count Agar (PCA) were incubated at 37°C for 48 hours (APHA 1992) and Enterobacteriaceae was determined on Violet Red Bile Glucose Agar (VRBGA) incubated at 30 °C for 24 h (Harrigan, 1998). Lactic acid bacteria (LAB) was cultured on DeMan Rogosa Sharpe Agar (MRS) incubated at 30°C for 72 h (APHA, 1992).

2.3. Determination of biogenic amines

Determination of biogenic amines: a chromatographic method (Eerola et al., 1993) was used for the determination of cadaverine, tyramine, histamine, putrescine, tryptamine, 2-phenylethylamine, spermine and spermidine. High performance liquid chromatography (HPLC; Shimadzu, Japan) was used to separate the amines. The seperation was carried out by a gradient elution with 0.1 M ammonium acetate/acetonitrile on a reverse phase column (ODS-2; 5 µm, 125 x 4mm, Teknoroma, Spain) at the flow rate of 1 ml/min using a diode-array-detector (SPD-M20A, Shimadzu, Japan) at 254 nm.

250 µl internal standard (1.7 diaminoheptane) and 10 ml 0.4M perchloric acid solution were added to a 4 g sample and homogenised with a homogenisator (Wisd HG-15, Daihan, Korea). Then, sample was centrifuged (Universal 32R, Hettich, Germany) at 2400 g for 10 min. and the supernatant was transfered in to 25 mL bottle through filter paper. Extraction was repeated with 10 ml of 0.4 M perchloric acid solution in the remaining sample. Supernatants were adjusted to 25 ml with 0.4M perchloric acid solution.

A 500 µl extract was adjusted to alkaline using 100 µl 2N sodium hydroxide solution. Then, a 150 µl sodium bicarbonate (saturated), 1 ml dansyl chloride solution (10 mg/1 ml acetone) were added and incubated at 40 °C for 45 min. The residual dansyl chloride was then removed by addition of 50 µl of ammonia (25%). The dansylated extract was allowed to stand for 30 minutes, then adjusted to 5 ml with 0.1 M ammonium acetate/acetonitrile (1/1) and filtered through a 0.45 µm syringe filter.

The amount of biogenic amine in the sample was calculated using the following formula:

$C_u = 250 \times RF \times (H_A / H_i) \times C_i / W_S$

where C_u: mg unknown/kg sample, 250: dilution factor, RF: response factor, C_i: concentration of internal sandard, H_A: peak height of unknown, H_i: peak height of internal standard, W_S: weight of sample.

2.4. Instrumental colour analysis of sausages

Instrumental colour analysis of sausages: a portable colorimeter (Minolta CR400, Osaka, Japan) was used to measure the colour values of sausage samples. The instrument was standardized on a white standardization plate prior to each measurement. The colour was determined as *L* (lightness), *a* (redness) and *b* (yellowness) values according to CIELAB systems. Each sausage colour was measured eight times, from different places and averages of eight data were taken.

2.5. Statistical analysis

Statistical analysis: this study was carried out two replicates. Variance analysis (ANOVA) was used for the data and the results were given as mean ± standard deviation (SD). Differences between values of the samples were compared using Duncan's multiple range tests; a $p<0.05$ probability value was considered significant.

3. Results and discussions

3.1. The effects of PS on the pH

The changes in the pH values of the sausages during ripening and storage were shown in Table 1. The effect of PS on the pH values of the sausage was not found to be significant, whereas the effect of ripening and storage stages was significant. In all samples, the pH value decreased with the reason of lactic acid formation during ripening. The decrease in pH during ripening period is very important in terms of the desired flavour, colour formation and inhibition of undesired microorganisms in fermented sausage (Bozkurt, 2006). On the 30th

day of storage, the pH values showed a slightly increase in all samples. This increase in pH during the storage time was thought to be due to the formation of components such as amine and ammonia (Gök, 2006) or the increase in lactic acid degradation (Kurt, 2016).

Table 1. The effect of ripening and storage stages on the pH values of sausages[1]

Potassium Sorbate (%)	Ripening			Storage
	0th day[2]	6th day	12th day	30th day
0	5.84±0.01 ^{A}	4.63±0.04 ^{B}	4.41±0.01 ^{C}	4.60±0.01 ^{B}
5	5.84±0.01 ^{A}	4.60±0.05 ^{B}	4.40±0.01 ^{C}	4.60±0.04 ^{B}
10	5.84±0.01 ^{A}	4.62±0.05 ^{B}	4.38±0.02 ^{C}	4.58±0.05 ^{B}
20	5.84±0.01 ^{A}	4.65±0.04 ^{B}	4.39±0.06 ^{C}	4.60±0.02 ^{B}

[1]The effect of PS treatment on pH values of sausages was not significant ($p>0.05$).

[2] 0th day analyses were done on samples taken before sausage filling. They were independent for PS effect and were given for statistical analysis.

$^{A-D}$Different uppercase letters in a row show significant differences ($p<0.05$) between the groups.

3.2. The effects of PS on the microbiological properties

The effect of PS on the count of yeast-mold was found to be significant (Table 2). Yeast-mold was only detected in the PS0 sample, whereas they were not found in other samples. This is thought to be due to wide spectrum inhibitory property of PS on yeast-mold (Vasakou et al., 2003). It is suggested that sorbic acid prevents molds development by inhibiting dehydrogenase enzyme activity which involved in the oxidation of fatty acids (Sofos and Busta, 1981). PS is an effective antifungal agent used to prevent or reduce mold growth in fermented sausages (Öztürk, 2015). Ergüzel (1988) stated that the effect of dipping of sausage into 15% PS solution on the yeast-mold growth during the ripening and storage was significant. Chaves-Lopez et al., (2012) reported that dipping sausages in 20% PS solution inhibited mold growth on sample surface. Bozkurt and Erkmen (2002a) stated that the addition of some additives, including PS, inhibited yeast-mold growth and also stated that higher rate PS decreased the count of yeast-molds in fermented sausage. In another study, the addition of nitrite/nitrate or PS decreased the count of yeast-molds in fermented sausage (Bozkurt and Erkmen, 2007). Matos et al., (2007) showed that PS had an inhibitory effect on some mold species isolated from Portuguese dried-smoked sausages.

The effect of the ripening stage on the count of yeast-mold of PS0 sample was significant (Table 2). The count of yeast-mold in the PS0 sample decreased during ripening, whereas it did not change during storage. There are different findings about changes in the counts of yeast-mold in fermented sausages during ripening and storage (Bozkurt and Erkmen, 2002a; Bozkurt and Erkmen, 2007; Kaban, 2013).

PS significantly influenced the development of *Enterobacteriacea* (Table 2). These microorganisms could not be detected in PS treated sausages, it was merely detected in the PS0 sample. The absence of *Enterobacteriaceae* in PS treated sausages was thought to be due to the antimicrobial effect of PS (Sofos and Busta, 1981). It has been stated that the dipping of some meat products in PS solution was effective on the count of *Enterobacteriacea* (Gençcelep et al., 2014; Güner et al., 2004). Güner et al., (2004) reported that dipping into 2.5% and 5% PS solution of some poultry (chicken, turkey, quail, partridge) caused the decrease in the count

of *Enterobacteriaceae* during cold storage and this effect continued during storage.

The effect of ripening and storage stages on the count of *Enterobacteriacea* of PS0 sample was significant (Table 2). In the PS0 samples, *Enterobacteriaceae* showed a decrease during ripening stage, but it was not detected in the last days of storage time. Lizaso et al., (1999) stated that *Enterobacteriacea* could not be detected in the Spanish-type dry cured sausage with the development of ripening. Fernandez-Lopez et al., (2008) obtained similar results in Spanish-type dry fermented sausage and the decrease in the count of *Enterobacteriaceae* has been associated with reduction in pH occurred as a result of activity of lactic acid bacteria. Yalınkılıç et al., (2012) expressed that the *Enterobacteriaceae* in fermented sausage easily disappeared with ripening owing to the low water activity and pH susceptibility.

Table 2. The microbiological changes of sausages during the ripening and storage stages

Microorganism and Potassium Sorbate (%)	**Ripening**			**Storage**
	0th day[1]	**6th day**	**12th day**	**30th day**
Yeast-Mold[2] (log cfu g^{-1})				
0	3.90±0.08 ^{A}	3.52±0.04 ^{B}	3.32±0.04 ^{C}	3.31±0.08 ^{C}
Enterobacteriaceae[2] (log cfu g^{-1})				
0	2.93±0.14 ^{A}	2.35±0.26 ^{B}	2.24±0.29 ^{B}	ND
Aerobic mezophilic bacteria (log cfu g^{-1})				
0	5.35±0.03 ^{C}	8.78±0.01 ^{aA}	8.66±0.10 ^{aA}	8.17±0.07 ^{aB}
5	5.35±0.03 ^{C}	8.71±0.02 ^{aA}	8.55±0.09 ^{aA}	8.01±0.08 ^{abB}
10	5.35±0.03 ^{D}	8.57±0.14 ^{abA}	8.33±0.04 ^{bB}	7.86±0.04 ^{bcC}
20	5.35±0.03 ^{C}	8.42±0.06 ^{bA}	8.19±0.02 ^{bA}	7.66±0.18 ^{cB}
Lactic acid bacteria (log cfu g^{-1})				
0	4.35±0.04 ^{C}	8.20±0.06 ^{aA}	7.87±0.11 ^{aB}	8.11±0.03 ^{aA}
5	4.35±0.04 ^{C}	8.15±0.04 ^{aA}	7.77±0.08 ^{aB}	8.06±0.00 ^{aA}
10	4.35±0.04 ^{D}	8.10±0.01 ^{aA}	7.72±0.03 ^{aC}	8.00±0.02 ^{bB}
20	4.35±0.04 ^{C}	7.89±0.01 ^{bA}	7.49±0.01 ^{bB}	7.93±0.01 ^{cA}
Micrococcus-Staphylococcus bacteria (log cfu g^{-1})				
0	4.97±0.04 ^{A}	4.91±0.07 ^{aA}	4.43±0.06 ^{aB}	4.20±0.18 ^{aB}
5	4.97±0.04 ^{A}	4.74±0.06 ^{bA}	3.94±0.21 ^{bB}	3.67±0.04 ^{bB}
10	4.97±0.04 ^{A}	4.60±0.04 ^{cB}	3.75±0.04 ^{bcC}	3.65±0.05 ^{bC}
20	4.97±0.04 ^{A}	4.51±0.01 ^{cB}	3.54±0.04 ^{cC}	3.52±0.05 ^{bC}

[1]0th day analyses were done on samples taken before sausage filling. They were free from PS and were given for statistical analysis.
[2]Not detected in samples with PS.
ND: Not detected
$^{A-D}$Different uppercase letters in a row show significant differences between the groups ($p<0.05$).
$^{a-b}$Different lowercase letter in a column show significant differences between the groups($p<0.05$).

Another significant effect of PS was found to be on the count of TAMB (total aerobic mezophilic bacteria). As shown in Table 2, at the 6th and 12th days of ripening, TAMB counts of PS10 and PS20 samples were lower than other samples. At the storage stage, counts of TAMB

of all samples with PS were lower than PS0 sample. This change in counts of TAMB is thought to be due to the antimicrobial properties of PS (Holley, 1981) and its interaction with the enviroment conditions. There is no exact agreement among scientists about the action mechanism of sorbates against microorganisms (Sofos and Busta, 1981). However, it is suggested that the effect of sorbic acid on microorganisms is based on the inactivation of some enzymes (Sofos and Busta, 1981). Sorbates has been thought to be effective against numerous microorganisms such as gram negative and positive, catalase negative and positive, aerobic, anaerobic, mesophilic and thermophilic microorganisms (Dinçoğlu, 2002). Wareltzis et al., (1984) stated that addition of 0.20% PS instead of sodium nitrite to frankfurter sausage caused a better inhibition on TAMB. In another study (Jin et al., 2015), the count of TAMB of emulsified sausage with 0.2% PS was lower than the control sample after 4 weeks of storage.

It was observed that the effects of ripening and storage stages on the counts of TAMB of the sausages with or without PS were found to be significant (Table 2). The counts of TAMB of all samples increased during ripening. In the last days of ripening, a slight decrease was observed TAMB counts of samples. Furthermore, at the storage stage, there was a decrease in the counts of TAMB of all sausages. The results are similar to those previously reported by some researchers (Aksu and Kaya, 2004; Gök et al., 2011; Kurt, 2016). Gök et al., (2011) reported that the count of TAMB of fermented sausage increased in the first 7 days of ripening and then decreased. Kurt (2016) stated the count of these microorganisms in fermented sausage decreased during the storage.

One of the important microorganism cultures in fermented sausage is LAB. PS treatment had significant effects on the counts of LAB. On 6th and 12th days of ripening, the LAB counts of PS20 sample was lower than other samples (Table 2). At the storage time, the LAB counts of PS10 and PS20 samples were less than other samples. This change in LAB counts might be due to the antimicrobial properties of PS (Davidson et al., 2005). Another study (Öztürk, 2015), reported that the effect of dipping of 15% PS solution of sausage on the count of LAB of fermented sausage was not found significant. Howeover, Jin et al., (2015) has stated that emulsified sausages with PS contained less LAB than the control samples after cold storage.

The counts of LAB changed significantly during ripening and storage (Table 2). The count of LAB increased from 4.35 log cfu g^{-1} to 8.20 log cfu g^{-1} at the first days of ripening and decreased to 7.49 log cfu g^{-1} at the last days of ripening. This has been associated with adaptability to the meat environment of LAB (Fernandez-Lopez et al., 2008). Moreover, the counts of LAB in all samples increased during storage stage. Ercoşkun and Özkal (2011) stated that LAB count in sausage showed a rapid increase at the first days of ripening, but then, this increase was stopped.

Another important microorganism cultures in fermented sausages are *Micrococcus/Staphylococcus* species and they can improve color by reducing nitrate to nitrite and contribute to the development of specific aroma with lipolytic and proteolytic activities (Gökalp et al., 1998). *Micrococcus/Staphylococcus* counts of sausages were significantly affected by PS application (Table 2). There was a decrease in *Micrococcus/Staphylococcus* counts depending on PS concentration. During ripening and storage, the count of *Micrococcus/Staphylococcus* of PS0 sample was higher than other samples. Öztürk (2015) stated that dipping of the fermented sausages into 15% PS solution was a significant effect on the count of *Micrococcaceae*. Moreover, our findings were similar with the results of other meat products using PS (Doğruer et al., 1996; Güner et al., 2004). Tekinşen et al., (1999) reported that count of *Micrococcus/Staphylococcus* increased depends on garlic reduction rate in cemen of pastrami, but this increase was limited by PS addition.

The effect of ripening stage on *Micrococcus/Staphylococcus* count was significant, whereas the effect of storage stage on *Micrococcus/Staphylococcus* count was not found to be significant (Table 2). There was no significant difference between the *Micrococcus/Staphylococcus* counts of PS0 and PS5 samples in the 0th days and 6th of ripening. On the other hand, *Micrococcus/Staphylococcus* counts of PS10 and PS20 samples decreased at the first 6 days of ripening. After 6th day of ripening, the count of *Micrococcus/Staphylococcus* decreased in all samples. Kaban and Kaya (2009), Yalınkılıç et al., (2012) reported that the effect of ripening stage on the count of *Micrococcus/Staphylococcus* of fermented sausage was important. Another study (Kaban and Kaya, 2009), has been reported that the count of *Micrococcus/Staphylococcus* increased at the first days of fermentation and then their counts remained relatively stable throughout the ripening. Moreover, no increase was observed in these microorganisms at the first days of fermentation in our study (Table 2). Kaban (2013) reported that the growth and viability of these microorganisms was depending on acidity during fermentation. However, in some cases, it has been reported that the rapid growth of lactic acid bacteria may cause inhibition against these slowly growing microorganisms (Kaban and Kaya, 2009).

3.3. The effects of PS on the biogenic amine (BA) formation

The BA contents of the samples during the ripening and storage stages were given in Table 3. The use of PS treated casings in the sausage was found to be effective considering BAs with the exception of spermine and spermidine. However, PS treatment was not caused significant changes in spermine and spermidine contents of sausages.

The effect of PS treatment on tryptamine contents was found to be significant. The contents of tryptamine of all samples has been shown an increase at the ripening and storage. The highest tryptamine content was determined in the PS0 sample during ripening and storage stage. At the 6th day of ripening, tryptamine contents of PS10 and PS20 samples were considerably lower than PS0 and PS5 samples. After the 6th day of ripening, all PS concentrations provided a reduction in tryptamine content compared to the PS0 sample. Shalaby and El-Rahman (1995) reported that the content of tryptamine in fermented sausages with PS and starter culture increased during fermentation and then decreased to undetectable level. In another study (Bozkurt and Erkmen, 2002a), it was stated that the use of additives, including PS, did not affect the content of tryptamine in fermented sausages.

PS treatment caused significant differences in 2-phenylethylamine contents of samples. As shown in Table 3, the contents of 2-phenylethylamine of sausages with or without PS were increased at the ripening and storage. The effect of PS treatment on contents of 2-phenylethylamine were not found significant at 6th day of ripening. However, the contents of 2-phenylethylamine of PS10 and PS20 samples were lower than other samples at the 12th day of ripening. During the storage stage, the contents of 2-phenylethylamine of all samples were found the less than PS0 sample. Bozkurt and Erkmen (2002b) reported that 2-phenylethylamine could not be detected in fermented sausage produced with additives including PS. In another study (Gençcelep et al., 2014), it was reported that 2-phenylethylamine content of *pearl mullet* which dipping into 1-5% PS solution increased during cold storage. It has been reported that *Micrococcus/Staphylococcus* in the microflora of dry fermented sausages can produce 2-phenylethylamine (Kurt, 2006). In addition, some yeast species can generate 2-phenylethylamine (Ercoşkun et al., 2005). It is not desirable that the content of 2-phenylethylamine is above 30 ppm in terms of "good manufacturing practices" (Shalaby, 1996). In our study, the contents of 2-phenylethylamine were below this level. In a research (Gençcelep et al., 2008), 2-

phenylethylamine was detected maximum 25 mg kg^{-1} in 17 of 30 fermented sausage collected from local markets in Turkey.

Putrescine was the most abundant biogenic amine in PS treated samples (Table 3). The contents of putrescine of all samples increased at the ripening and storage. PS treatment limited the increase of putrescine contents from the first days of ripening and the content of the highest putrescine was determined in the PS0 sample. Shalaby and El-Rahman (1995) investigated the effect of PS on the formation of BA in fermented sausage, and stated that putrescine cannot be determined. The putrescine content of fresh meat is related to the count of total aerobic organism (Ruiz-Capillas and Jimenez-Colmenero, 2004). It is also reported that *Enterobacteriaceae* species produce considerable amounts of putrescine and there is a relation between the counts of these bacteria and the content of putrescine (Kurt, 2006). Ba et al., (2016) reported that one of the two highest biogenic amines in fermented pork sausage produced was putresin. Komprda et al., (2004) reported that the two highest concentration of BAs were putrescine and tyramine.

Table 3. Biogenic amine concentrations of sausages during the ripening and storage stages

Biogenic amines (mg kg^{-1})	Samples	Ripening			Storage
		0th day[1]	6th day	12th day	30th day
Tryptamine	PS0	0.54±0.01 ^{C}	47.51±4.94 ^{aB}	70.28±4.82 ^{aA}	72.56±6.25 ^{aA}
	PS5	0.54±0.01 ^{C}	39.07±4.40 ^{aB}	53.42±1.51 ^{bA}	55.89±1.86 ^{bA}
	PS10	0.54±0.01 ^{D}	25.58±0.94 ^{bC}	40.64±2.08 ^{cB}	47.04±3.23 ^{bA}
	PS20	0.54±0.01 ^{C}	18.85±1.58 ^{bB}	21.16±2.67 ^{dAB}	23.71±0.95 ^{cA}
2-phenylethyl amine	PS0	2.79±0.05 ^{C}	4.80±0.88 ^{aB}	7.81±0.43 ^{aA}	9.22±0.32 ^{aA}
	PS5	2.79±0.05 ^{C}	4.28±1.52 ^{aBC}	6.33±0.91 ^{aAB}	7.75±0.89 ^{abA}
	PS10	2.79±0.05 ^{C}	3.86±1.27 ^{aAB}	4.71±0.35 ^{bAB}	5.90±0.86 ^{bcA}
	PS20	2.79±0.05 ^{B}	3.38±0.68 ^{aB}	3.76±0.35 ^{bB}	5.09±0.48 ^{cA}
Putrescine	PS0	136.78±0.35 ^{D}	841.52±48.91 ^{aC}	1168.20±45.69 ^{aB}	1365.90±63.79 ^{aA}
	PS5	136.78±0.35 ^{D}	790.82±5.64 ^{abC}	1097.90±33.33 ^{abB}	1337.40±64.96 ^{aA}
	PS10	136.78±0.35 ^{D}	736.65±3.68 ^{bC}	1004.40±24.34 ^{bcB}	1220.00±58.68 ^{abA}
	PS20	136.78±0.35 ^{D}	611.39±34.27 ^{cC}	912.94±34.82 ^{cB}	1151.70±16.22 ^{bA}
Cadaverine	PS0	0.00±0.00 ^{D}	16.10±0.23 ^{aC}	61.61±0.98 ^{aB}	78.28±2.42 ^{aA}
	PS5	0.00±0.00 ^{D}	16.01±0.26 ^{aC}	58.85±2.25 ^{aB}	70.96±2.35 ^{bA}
	PS10	0.00±0.00 ^{D}	14.60±0.71 ^{bC}	56.19±2.12 ^{aB}	67.58±0.90 ^{bA}
	PS20	0.00±0.00 ^{D}	9.43±0.01 ^{cC}	40.85±5.35 ^{bB}	51.89±2.60 ^{cA}
Histamine	PS0	2.46±0.01 ^{B}	2.74±0.08 ^{bB}	3.54±0.00 ^{cA}	3.73±0.22 ^{bA}
	PS5	2.46±0.01 ^{D}	2.72±0.09 ^{bC}	3.63±0.04 ^{bcB}	3.88±0.04 ^{bA}
	PS10	2.46±0.01 ^{C}	2.89±0.02 ^{abB}	3.86±0.13 ^{abA}	4.06±0.09 ^{abA}
	PS20	2.46±0.01 ^{C}	3.00±0.05 ^{aB}	4.04±0.18 ^{aA}	4.38±0.19 ^{aA}
Tyramine	PS0	2.52±0.06 ^{C}	158.54±2.39 ^{aB}	252.10±16.41 ^{aA}	274.96±5.72 ^{aA}
	PS5	2.52±0.06 ^{D}	139.86±5.93 ^{bC}	236.05±12.50 ^{aB}	260.90±1.30 ^{aA}
	PS10	2.52±0.06 ^{C}	128.62±10.56 ^{bcB}	220.24±6.46 ^{aA}	246.05±16.52 ^{aA}
	PS20	2.52±0.06 ^{C}	115.89±0.57 ^{cB}	172.58±15.94 ^{cA}	189.91±17.40 ^{bA}
Spermidine	PS0	7.41±0.11 ^{B}	9.90±0.93 ^{aA}	11.17±0.26 ^{aA}	11.30±0.46 ^{aA}
	PS5	7.41±0.11 ^{C}	9.72±0.69 ^{aB}	11.16±0.26 ^{aA}	11.29±0.53 ^{aA}

	PS10	7.41±0.11 ^{C}	8.98±0.48 ^{aB}	11.01±0.22 ^{aA}	11.03±0.16 ^{aA}
	PS20	7.41±0.11 ^{B}	8.62±0.85 ^{aB}	10.95±0.27 ^{aA}	11.01±0.16 ^{aA}
Spermine	PS0	55.21±0.33 ^{B}	57.70±8.88 ^{aAB}	70.85±3.31 ^{aA}	58.93±0.03 ^{aAB}
	PS5	55.21±0.33 ^{B}	52.30±8.25 ^{aAB}	65.66±1.70 ^{aA}	58.74±0.19 ^{aAB}
	PS10	55.21±0.33 ^{AB}	41.86±9.90 ^{aB}	62.42±1.14 ^{aA}	58.68±0.29 ^{aA}
	PS20	55.21±0.33 ^{AB}	39.74±10.52 ^{aB}	57.97±5.01 ^{aA}	58.82±0.05 ^{aA}

[1]0th day analyzes were done on samples taken before sausage filling. They were free from PS and were given for statistical analysis.

0.00±0.00: Not detected

$^{A-D}$Different uppercase letters in a row show significant differences between the groups ($p<0.05$).

$^{a-b}$Different lowercase letter in a column show significant differences between the groups($p<0.05$).

PS also significantly affected the formation of cadaverine (Table 3). Cadaverine was not detected before the filling, however an increase in cadaverine contents was detected together with ripening. Whereas, in some studies (Ba et al., 2016; Bozkurt, 2002; Shalaby and El-Rahman, 1995), it has been reported that cadaverine cannot be detected in fermented sausage. In our study, the highest cadaverine content was determined in the PS0 sample. It was found that PS10 and PS20 samples contained less cadaverine than the PS0 during ripening. Also, samples with PS contained less cadaverine than the PS0 sample during storage. Particularly during the storage, the cadaverine content of the PS20 was found significantly lower than the other samples. Previous studies (Shalaby and El-Rahman, 1995; Bozkurt and Erkmen, 2002a) have reported that cadaverine was not detected in fermented sausages produced using PS.

Although PS treatment limited the formation of tryptamine, 2-phenylethylamine, putrescine and cadaverine, it increased histamine formation during ripening and storage. As shown in Table 3 shows that histamine contents slightly increased with PS treatment. The highest content of histamine during ripening and storage stages was determined in the PS20 sample. Shalaby and El-Rahman (1995) reported that histamine content initially increased in fermented sausages with PS addition, but fell below undetectable level with ripening. Taylor and Speckhard (1984) reported that 0.5% PS concentration inhibited the development of certain histamine-producing bacteria. It was reported that histamine in fermented sausages occurs more between 2th and 4th weeks and it depends on the count of LAB rather than ripening conditions (Stratton et al., 1991). It was also stated that *Enterobacteriaceae* species can produce histamine in significant amounts in addition to cadaverine and putrescine production.[8] Debaryomyces and Candida species isolated from fermented meats have been reported to exhibit high decarboxylase activity (Ercoşkun et al., 2005). Histamine and tyramine are known to be the most toxic amines that cause intoxication (Anastasio et al., 2010). However, it is known that histamine is found in fermented sausages at a lower level than other Bas (Latorre-Moratalla et al., 2012). 50-100 ppm of histamine is considered acceptable for "good production practices" (Ekici et al., 2004). In some studies (Ekici et al., 2004; Gençcelep et al., 2008) reported that the histamine contents of dry fermented sausages were less than this level.

Tyramine contents of sausages had been shown to increase with ripening. The highest tyramine is detected in PS0 sample during the ripening and storage. It can be said that all PS concentrations were effective on the formation of tyramine at the first days of ripening. However 20% PS concentration was effective on tyramine formation at the 12th day of ripening and storage stage. The most abundant BA following putrescine was tyramine. It is stated that the most commonly BA in fermented sausages is tyramine and is produced mostly by LAB (Latorre-Moratalla et al., 2012). Moreover,

this BA is generated more rarely by coagulase negative *Staphyloccoccus* (Latorre-Moratalla et al., 2012).

Although significant differences were not found in spermidine and spermine contents of the samples, the effect of ripening and storage stages on contents of spermidine and spermine was significant. Spermidine slightly increased during ripening and it also remained nearly stable throughout the storage. In addition, spermine contents of sausages apart from PS0 decreased during the first days of ripening and then spermine contents of all samples increased. Also during the storage, the spermine contents of sausages except for PS20 sample slightly decreased. It has been known that fresh meat contains considerably spermidine and spermine (Hernandez-Jover et al., 1996) and that the formation of these amines does not originate from factors such as food deterioration, fermentation process (Hernandez-Jover et al., 1997). Shalaby and El-Rahman (1995) stated that spermidine and spermine were not detected in fermented sausag es with PS.

3.4 The effects of PS on the colour values of sausages

The colour values of sausages were given in Table 4. The effects of PS treatment on *L*, *a* and *b* values were found to be significant. The effect of only 20% PS treatment on the *L* (lightness) value was significant during ripening and storage stages. From the 6th day of ripening, it was determined that the *L* value of the PS20 sample was lower than the other samples. Öztürk (2015) stated that the *L* value of sausage which was dipped into PS solution, decreased during ripening. In another study (Jin et al., 2015), the *L* value of sausage with PS was found to be similar to the *L* value of control sample. The effect of ripening and storage stages on the *L* values of the sausages was found to be significant. *L* values of all samples decreased during ripening stage and it was too little increased during the storage stage. In previous studies (Bozkurt, 2006; Ercoşkun and Özkal, 2011), it has been stated that the *L* value of the sausage is decreased during the ripening stage. Üren and Babayiğit (1997) reported that the decrease in the *L* value represents the dark coloration of sausage after drying.

Table 4. Colour values of sausages during the ripening and storage stages

Instrumental Colour	Sample	Ripening			Storage
		0th day	6th day	12th day	30th day
L	PS0	36.06±0.03 ^{aA}	27.45±0.00 ^{aB}	26.03±0.57 ^{aC}	27.08±0.11 ^{aB}
	PS5	35.17±0.57 ^{aA}	26.96±1.24 ^{aB}	25.70±0.47 ^{aB}	26.84±0.25 ^{aB}
	PS10	35.34±1.21 ^{aA}	27.12±0.04 ^{aB}	25.63±0.58 ^{aB}	26.65±0.33 ^{aB}
	PS20	35.24±0.47 ^{aA}	24.61±0.12 ^{bBC}	24.41±0.23 ^{bC}	25.38±0.25 ^{bB}
a	PS0	6.76±0.36 ^{aA}	6.77±0.55 ^{aA}	4.99±0.11 ^{aB}	5.31±0.21 ^{aB}
	PS5	5.40±0.28 ^{bAB}	5.76±0.15 ^{abA}	4.33±0.08 ^{bC}	5.04±0.23 ^{aB}
	PS10	5.62±0.58 ^{bA}	5.22±0.38 ^{bAB}	4.12±0.08 ^{bB}	4.71±0.40 ^{aAB}
	PS20	5.38±0.00 ^{bA}	3.74±0.41 ^{cB}	3.02±0.18 ^{cC}	3.37±0.17 ^{bBC}
b	PS0	5.94±0.53 ^{aA}	4.61±0.24 ^{aB}	3.55±0.01 ^{aC}	3.66±0.01 ^{aC}
	PS5	4.90±0.21 ^{aA}	4.39±0.54 ^{aA}	3.26±0.06 ^{abB}	3.53±0.03 ^{aB}
	PS10	5.67±0.61 ^{aA}	4.06±0.11 ^{aA}	3.10±0.04 ^{bC}	3.32±0.19 ^{aBC}
	PS20	6.14±0.58 ^{aA}	2.86±0.18 ^{bB}	2.59±0.22 ^{cB}	2.81±0.26 ^{bB}

[A-D]Different uppercase letters in a row show significant differences between the groups ($p<0.05$).

[a-b]Different lowercase letter in a column show significant differences between the groups($p<0.05$)

The *a* (redness) values of samples decreased with the PS treatment. Moreover, the effect of only 20% PS concentration on the *a* values of sausages during the storage was found to be significant. PS0 sample had highest *a* value and the *a* values of sausages decreased with the increasing PS concentration during the ripening. In the storage stage, *a* value of PS20 sample was found to be lower than the other samples. Öztürk (2015) reported that sausage which was dipping into PS had the lowest *a* value. It was determined that the effect of ripening and storage stages on the *a* values of sausages was significant (Table 4). The *a* values of all samples showed a decrease during the ripening stage. During the storage stage, the *a* values of all samples slightly increased. Ercoşkun and Özkal (2011) stated that the *a* value of sausage decreased from the 4th day of ripening. Bozkurt and Bayram (2006) reported that the *a* value of sausage increased during the first 5 days of ripening but then declined. It has been reported that this decrease in *a* value is due to the myoglobin denaturation caused by lactic acid (Ercoşkun and Özkal, 2011; Perez-Alvarez et al., 1999).

PS treatment also significantly affected the *b* (yellowness) values of sausages (Table 4). At 6th day of ripening, *b* value of PS20 sample was significantly lower than the other samples. However, all PS concentrations caused decrease in *b* value during 12th day of ripening. Only 20% PS concentration was found to be effective on *b* value during the storage stage. Moreover, *b* values in all samples decreased during ripening. Karabacak and Bozkurt (2008) stated that the *b* value of sausage decreased during the ripening stage. In another study (Perez-Alvarez et al., 1999), it was reported that *b* value of Spanish type dry-cured sausage decreased during ripening. This decrease in *b* value was associated with a decrease in the oxymyoglobin, which is contributed by the yellow color to the oxygen consumption by the microorganisms (Perez-Alvarez et al., 1999).

4. Conclusion

PS treatment to sausage casings decreased the counts of LAB, TAMB and *Micrococcus/Staphylococcus*. It was effective when applied at higher concentrations on LAB. No yeast-mold and *Enterobacteriaceae* were detected in sausages using PS-treated casings. Moreover, PS application to the casing significantly prevented the formation of biogenic amines with the exception of histamine. While the effect of PS on spermin and spermidine was not significant, it significantly increased histamine concentration. However, PS adversely affected red color, and with the application of more than 15% PS, lightness and yellowness were adversely affected. This process which casings were dipped into PS solution before filling, can be used effectively to inhibit microbial activity and to limit the formation of BA in sausage production. When the color values are taken into consideration, this treatment can be carried out at low PS concentrations such as 5-15%.

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ULTRASOUND ASSISTED EXTRACTION OF POLYPHENOLS WITH HIGH ANTIOXIDANT ACTIVITY FROM OLIVE POMACE (*Olea europaea* L.)

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ABSTRACT

Olive pomace is an industrial by-product resulted from the olive oil production process. This study was carried out to optimize the extraction of polyphenols with high antioxidant activity from olive pomace using different techniques. The extraction was performed using homogenization and ultrasonic techniques at different solvent/pomace ratios till polyphenols reached a plateau. Total phenolic content was determined with the Folin-Ciocolteau method. Extracts were analyzed by HPLC for polyphenol and flavonoid contents. Scavenging activity of the extracts was determined against 1,1-diphenyl-2-picryl-hydrazyl and hydrogen peroxide radicals. The highest yield of the polyphenols (86.13±0.80 mg gallic acid equivalents/g dried defatted pomace) was recorded after 30 min of extraction using ultrasonic technique and 40/1 methanol (80%)/pomace (v/w) ratio. Extracts obtained by the methanol/sample ratio of 20/1 and high ultrasonic intensity for 7 min possessed higher antioxidant activity than the synthetic antioxidant, butylated hydroxytoluene.

1. Introduction

Olive pomace is the main agricultural by-product of the olive industry. It represents a particular environmental problem. The types and concentrations of polyphenols in olive pomace depend on the cultivar, agro-climatic conditions, fruit maturity, fruit storage, and extraction method. About 90% of the total phenolic compounds in olive products are present in the free form (Alu'datt *et al*., 2010). A two-phase extraction system accumulated metabolites in the solid pomace (Boskou, 2015). The majority of polyphenols present in olive pomace are hydroxytyrosol, oleuropein, and tyrosol, in addition to, caffeic acid, ρ-coumaric acid, vanillic acid and rutin (Ciriminna *et al*., 2016). Many of the phenolic compounds showed antioxidant activity. Recent investigations are focussed on the isolation and evaluation of antioxidant phenolics from plant wastes. Acoustic cavitation of ultrasound-assisted extraction (UAE) technique causes molecular movement of solvent and sample. Advantages of the UAE include high efficiency, reduced extraction time and low solvent consumption versus conventional extraction techniques (Jerman *et al*., 2010; Wong Paz *et al*., 2015).

Most methods of extracting polyphenols from olive pomace use a solvent/material ratio of 5/1 up to 25/1 and an extraction time of 15 min to 12 hours , with temperatures from 25 to 70 °C or higher. These different techniques have a yield that ranges from 1.29 to 60 mg gallic acid equivalent/g dried pomace (Alu'datt *et al*., 2010; Lafka *et al*., 2011; Aliakbarian *et al*., 2012; Ramos *et al*., 2013). Accordingly, this study was carried out to optimize the extraction conditions of the polyphenols from olive pomace using homogenization, and ultrasound techniques and

to evaluate the antioxidant activities of the obtained extracts.

2. Materials and methods

2.1. Materials

2.1.1. Samples

Olive pomace Two-phase Maraki variety used in the experiment was collected from an olive oil factory by-product (Mini Frantoio Oliomio-50-60 Centrifuge, Italy) located in Agricultural Research Centre, Giza, Egypt. The obtained pomace was dried in an oven at 70 °C under vacuum (70 mm Hg). Solid samples were ground using a laboratory mixer. Dried samples were extracted with petroleum ether (b.p. 40-60 °C) as a solvent to remove the residual oil using a Soxhlet apparatus for 4 h.

2.1.2. Reagents and standards

HPLC-grade solvents were purchased from Merck (Darmstadt, Germany). Folin–Ciocalteau phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), polyphenol reference standards: syringic acid, gallic acid, pyrogallol, 4-aminobenzoic acid, 3-hydroxytyrosol, protocatechuic acid, catechin, chlorogenic acid, catechol, epicatechin, caffeine, 4-hydroxybenzoic acid, caffeic acid, vanillic acid, ρ-coumaric acid, ferulic acid, oleuropein, ellagic acid, benzoic acid, coumarin, naringin, rutin, hesperidin, rosmarinic acid, quercitrin, quercetin, naringenin, hesperetin were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Methods

2.2.1. Extraction of polyphenols

Dried, defatted and milled pomace samples (2.0±0.05 g) were extracted with aqueous methanol (methanol/water, 80/20, v/v) to pomace ratios (v/w) of 20/1 and 40/1, at room temperature. Extraction used UAE technique according to Jerman *et al.* (2010). Homogenization technique was performed according to Arslan and Ozcan (2011). Alcoholic extracts were centrifuged using Hettich Universal Centrifuge D7200, Germany at 327 *g*/min for 5 min.

2.2.1.1. Homogenization extraction technique

Homogenization (T1 and T2 used solvent/pomace ratios (v/w) of 20/1 and 40/1, respectively) was conducted using Heidolph type ST1 homogenizer (Germany) at the maximum power (264 W) for 10-60 min, at intervals of 10 min.

2.2.1.2. Ultrasonic-assisted extraction (UAE) technique

UAE (T3- T6, a solvent/pomace ratio (v/w) of 20/1) used a Fisher-Sonic, Dismemberator Model 300, USA at 10, 20, 40 and 50% of the maximum output power (300 W), respectively, for 10 min, at one minute intervals. In T7, extraction was performed using a solvent/pomace ratio of 40/1 at 50% of the maximum output power for 10 min, at one minute intervals and continued for 60 min, at intervals of 10 min.

2.2.2. Determination of total polyphenols

Total polyphenols (TP) content was determined using the Folin-Ciocalteu reagent and Unico UV-2000 Spectrophotometer, USA at 750 nm according to Zhou *et al.* (2017). A standard calibration curve was prepared using gallic acid (50–800 mg/L). Total polyphenol concentration was calculated from a calibration curve (r^2 = 0.9951). Spectrophotometric analysis was carried out for each extract in triplicate. Results were expressed as mg gallic acid equivalents (GAE)/g dried defatted pomace (ddp) ± standard deviation (SD) and μg GAE/mL of the olive pomace extract (OPE) ± SD.

2.2.3. HPLC analyses of polyphenols

HPLC was used for the identification and quantification of polyphenols for the extract with the highest yield that obtained by each extraction technique. The assays were performed with Agilent Technologies 1200 HPLC series, USA equipped with Agilent

1200 Series quaternary pump, vacuum degasser, and Agilent UV-VIS detector. Five microliters of the extract were injected into a column (Zorbax ODS, 250 mm×4.6 mm inner diameter, Agilent, USA) at room temperature. The solvent system used a gradient of A (8% CH_3COOH) and B (acetonitrile). The separation was obtained with the following gradients: at 0 min, 5% A and 95% B; at 5 min, 25% A and 75% B; at 10 min, 45% A and 55% B; at 15 min, 65A and 35% B; at 20 min, 85% A and 15% B; and from 25 to 30 min, 99% A and 1% B. The solvent flow rate was 1 mL/min, and separation was performed at 35°C. Wavelength of the UV-VIS detector was set at 330 nm for polyphenols and 280 nm for flavonoids. Identification was accomplished by comparing the retention time of the analyte with that of a reference standard. The results were expressed as mg/g ddp. Quantification of the identified compounds was performed using the calibration curves of the reference standards.

2.2.4. Antioxidant assays

2.2.4.1. DPPH radical scavenging activity

DPPH free radical-scavenging activity of the extracts was determined, according to Zhou *et al.* (2017). The absorbance was measured at 517 nm against methanol (blank) using Unico UV-2000 Spectrophotometer, USA. The synthetic antioxidant BHT was used as a reference compound (positive control) at 50, 100, 150 and 200 µg/mL. The inhibition percentage of DPPH radicals was calculated according to the following formula:

$$\% \text{ inhibition} = [A_b - A_s / A_b] * 100 \quad \text{Eq. (1)}$$

Where A_b and A_S stand for the absorbance of blank and sample or reference, respectively. The concentration of the test extract or reference providing 50% inhibition (IC_{50}, expressed in µg/mL) was calculated from the graph plotted with inhibition percentage against the concentration. Assays were carried out in triplicate and the results were expressed as mean values ± SD.

2.2.4.2. Hydrogen peroxide scavenging activity

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Amessis-Ouchemoukh *et al*. (2017). The absorbance of the reaction mixture was recorded after 10 min at 230 nm using Unico UV-2000 Spectrophotometer, USA against a blank solution containing the phosphate buffer without hydrogen peroxide. The inhibition percentage of H_2O_2 was calculated according to Eq. (1)

2.2.5. Statistical analyses

Polyphenol extraction and evaluation of the antioxidant activity of the extracts were carried out in triplicate. The data were analyzed using Costat statistical software version 6.4. The significance of the differences of the means at a 5% level used one-way analysis of variance (ANOVA) and Duncan's multiple-range test. The IC_{50} values were obtained with Origin 2016 software (Origin Lab Corporation, USA).

3. Results and discussions

3.1. The effect of extraction conditions on the yield of total polyphenols

Aqueous methanol was an efficient solvent to extract lower molecular weight polyphenols (Pintać *et al*., 2018). The effects of solvent/pomace ratio, time and technique on the TP of OPE are shown in Fig. 1.

The contents of polyphenols extracted by homogenization at a solvent/pomace ratio (v/w) of 20/1 (T1) reached a maximum level (21.3±0.30 mg GAE/g ddp) after 30 min (Fig. 1a). Further increases in extraction time did not significantly (p>0.05) increase the yield of the extracted polyphenols. Increasing the solvent/pomace ratio to 40/1, (T2) increased significantly (P < 0.05) polyphenol yields to 74.35±0.93 mg GAE/g ddp during the same extraction time (Fig. 1a). Beyond 30 min, the yield of the polyphenols decreased sharply (p<0.05) and reached a minimum at 60 min, possibly because of the decomposition of the active compounds during the prolonged

homogenization time. These results are in agreement with the findings of Zhu *et al*. (2016).

On the other hand, increasing the ultrasonic intensity during the extraction of polyphenols from 10% to 50% of the maximum ultrasonic output power using a methanol (80%)/pomace ratio 20/1 (v/w) caused a remarkable increase in the recovery of polyphenols (T3-T6, Fig. 1b). The extracted polyphenols using 50% of the maximum ultrasonic output power were twice as high as those at 10% at identical extraction times. The significantly (p<0.05) highest yield of the polyphenols (86.13±0.80 mg GAE/g ddp) was recorded after 30 min of extraction using UAE at 50% of the maximum output power and 40/1 methanol (80%)/pomace (v/w) ratio (T7, Fig. 1c). The yield of T7 was higher than that obtained by T6 that conducted for a short time (7 min) using low solvent/pomace ratio (20/1). This could be due to the distribution of pomace in the solution is rather low and diluted, since it needs more time before decomposition of polyphenols by oscillation.

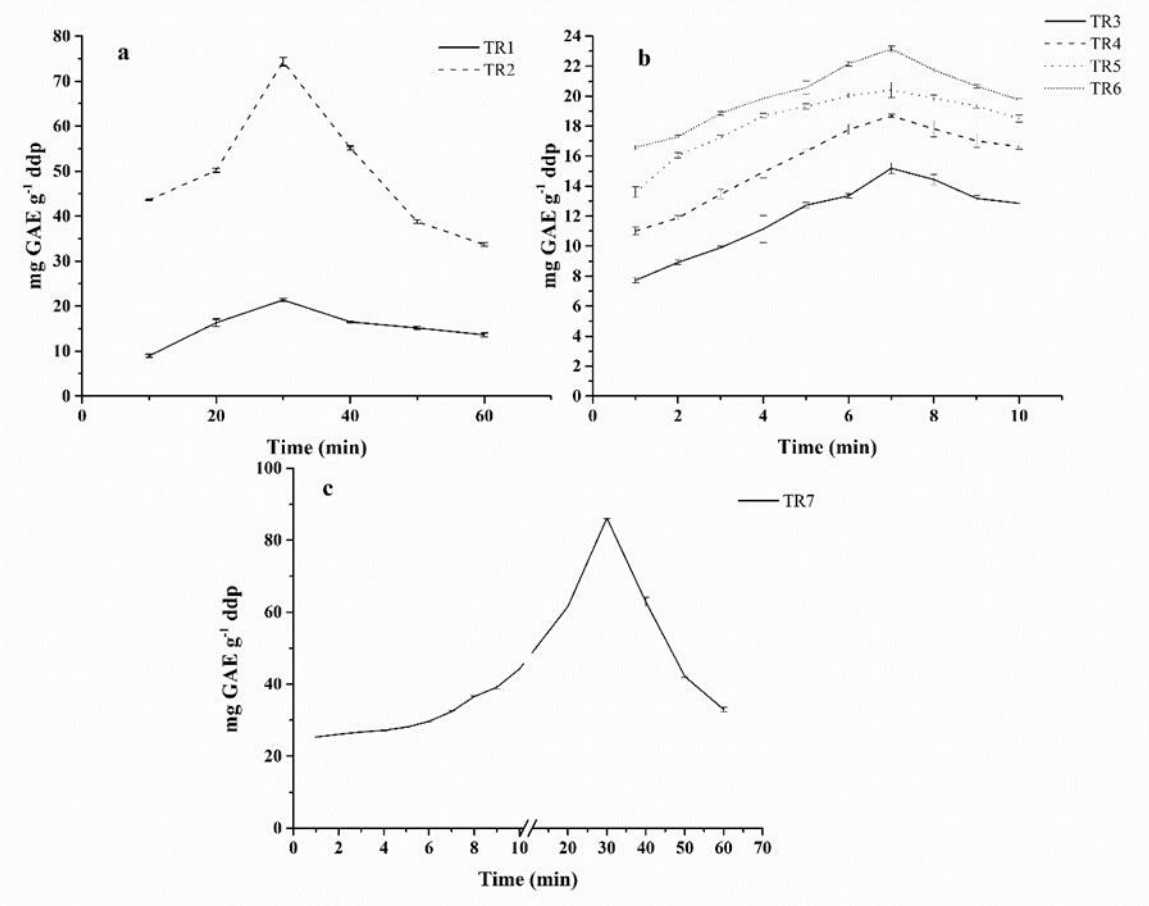

Figure 1. Yield of the polyphenols (mg GAE/g ddp ± SD) during (a) homogenization using solvent/ pomace ratios 20/1 (T1) and 40/1 (T2); (b) ultrasonic assisted extraction using solvent/ pomace ratio 20/1 at ultrasonic intensity of 10%, 20%, 40% and 50% of the maximal output power (T3-T6, consecutively); (c) ultrasonic assisted extraction using solvent/pomace ratio 40/1 at ultrasonic intensity of 50% of the maximal output power (T7). Error bars indicate the standard deviation of triplicate values (p<0.05)

These results exceeded those obtained by other investigators (Aliakbarian *et al*., 2012; Neviani *et al*., 2019) who found that the TP yield of OPE ranged from 9.1 to 68 mg/g dried pomace. The results indicated that extending UAE time, under T7 conditions, to 60 min was accompanied by a significant (p<0.05) reduction in the yield of TP to 30.13±0.15 mg GAE/g ddp (Fig. 1c). This could be due to the degradation of polyphenols by excessive Ultrasonic. These results are in agreement with previous studies (Zhang *et al*., 2015; Sun *et al*., 2016). They found that increasing the extraction time increased the recovery of TP until it reached a plateau, but further increases in extraction time caused a drop in the extracted polyphenols. Long extraction time increased the chances of polyphenol oxidation. Selecting an efficient extraction method to maintain the stability of the polyphenols is critically important because conventional extraction methods such as maceration have low efficiency and require long extraction times (Plazzotta and Manzocco, 2018).

Increasing solvent/pomace ratio (v/w) from 20/1 to 40/1 provided significantly (p<0.05) higher yield of extracted polyphenols at each extraction time regardless of the extraction technique used (Fig. 1). This could be due to mass transfer principles. The extraction efficiency of analytes in the sample depends on the intensity of the ultrasound transmitted to the medium and the number of cavitation bubbles produced. Ultrasonic waves create expansion-compression cycles in extracting media. These generate strong liquid jets that rupture the cells (Rodsamran and Sothornvit, 2019). During high-intensity ultrasound waves, the implosion of gas bubbles in liquid generates intense pressure within the material, causes plant tissue disruption, enhances penetration of the solvent into cellular materials, facilitates the transfer of components from the cell into the solvent and improves the mass transfer rate (Boskou, 2015). This could explain why ultra-sonication is more effective in extractability than homogenization.

3.2. HPLC analyses of polyphenols

HPLC analyses of the resulting extracts of each extraction technique with the highest yield of polyphenols are illustrated in Figures 2, 3 and tabulated in Table 1.

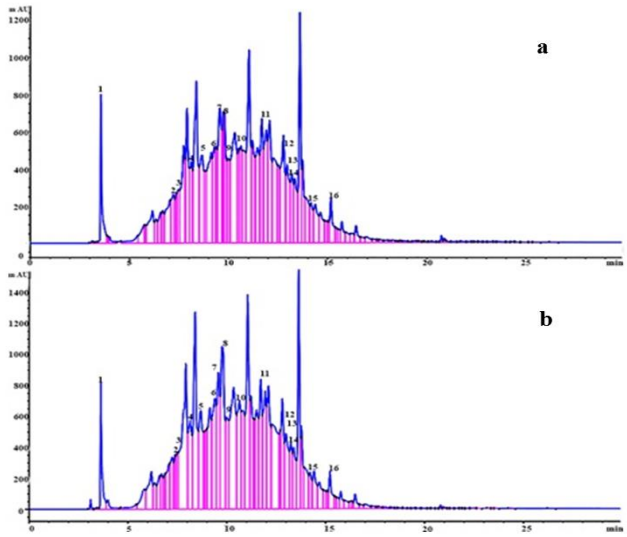

Figure 2. HPLC chromatogram of olive pomace extract polyphenols using (a) homogenization; (b) ultrasonic techniques with solvent/pomace ratio of 40/1. Peaks:1, syringic; 2, gallic acid; 3, pyrogallol; 4, 3-OH-Tyrosol; 5, protocatchuic; 6, catechins; 7, chlorogenic; 8, catechol; 9, caffeine; 10, 4-OH-benzoic; 11, vanillic; 12, ferulic acid; 13, oleuropein; 14, ellagic acid; 15, benzoic acid; 16, coumarin.

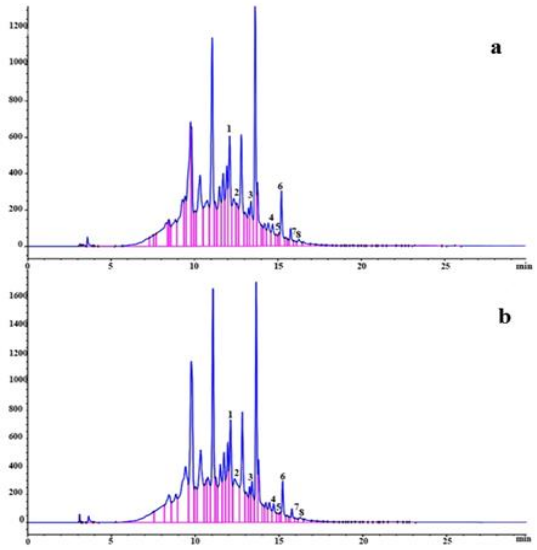

Figure 3. HPLC chromatogram of olive pomace extract flavonoids using (a) homogenization; (b) ultrasonic technique with solvent/pomace ratio of 40/1. Peaks:1, naringin; 2, rutin; 3, hesperidin; 4, rosmarinic acid; 5, quercetrin; 6, quercetin; 7, narengenin; 8, hespertin.

Table 1. Identified phenolic compounds (mg/g dried defatted pomace) of olive pomace extracts obtained by different extraction techniques

Compounds	Extraction Techniques	
	Homogenization	Ultrasonic
Polyphenols		
Syringic acid	0.368	0.574
Gallic acid	0.150	0.196
Pyrogallol	8.607	12.834
4-Aminobenzoic acid	0.441	0.484
3-Hydroxytyrosol	7.894	13.028
Protocatechuic acid	0.961	1.213
Catechin	0.310	1.347
Chlorogenic acid	0.058	0.909
Catechol	2.67	4.976
Epicatechin	0.531	1.675
Caffeine	0.419	0.254
4-Hydroxybenzoic acid	2.867	3.991
Caffeic acid	0.311	0.179
Vanillic acid	0.808	1.862
ρ-coumaric acid	0.547	0.833
Ferulic acid	0.544	1.181
Oleuropein	9.139	13.112
Ellagic acid	1.88	2.374
Benzoic Acid	8.37	2.992

Coumarin	0.159	0.169
Salicylic acid	1.912	0.630
Flavonoids		
Naringin	2.390	2.342
Rutin	0.266	0.570
Hesperidin	3.760	4.413
Rosmarinic acid	0.161	0.178
Quercitrin	0.310	0.031
Quercetin	0.337	0.230
Naringenin	0.214	0.274
Hesperetin	1.811	1.671
Kaempferol	0.060	0.064
Rhamnetin	0.069	0.065
Apigenin	0.047	0.031

Many polyphenols were detected in the OPE and include oleuropein, hydroxytyrosol, pyrogallol, catechol, ellagic acid and benzoic acid. The OPE obtained via homogenization was characterized by higher concentrations of benzoic acid, caffeine and caffeic acid versus other extracts. Sonication resulted in the highest recoveries for the other polyphenols. Sonication extracted ≥1.5 fold more syringic acid, pyrogallol, 3-hydroxytyrosol, chlorogenic acid, catechol, epicatechin, 4-hydroxybenzoic acid, vanillic acid, ρ-coumaric acid, ferulic acid and oleuropein versus other technique.

The most common flavonoids in the extracts were hesperidin, naringin, and hesperetin. Rutin, quercetin, rosmarinic acid, naringenin, and quercitrin were also found in considerable concentrations. The sonicated extract had the highest concentrations of rutin, hesperidin, and rosmarinic acid. On the other hand, the homogenized extract was characterized by higher concentrations of quercitrin and naringin versus the other investigated extracts. These results are consistent with those reported by other researchers (Gomez-Rico *et al*., 2009; Boskou, 2015).

3.3. Radical scavenging activity of pomace extracts

Polyphenols and flavonoids are the most common antioxidants in olives. Two assays based on different radicals (DPPH and hydrogen peroxide) assessed the antioxidant activity of the extracts during the upward part of the polyphenolic yield for each extraction technique. Hydrogen peroxide activity comes from its potential to produce a highly reactive hydroxyl radical through Fenton reaction (Kerins and Ooi, 2018). The DPPH and H_2O_2 radical-scavenging activities were recorded in terms of % inhibition (Fig. 4-5); IC_{50} values were deduced from the graphs. The results were compared to BHT as a reference standard in concentrations from 50 to 200 μg/mL to deduce the IC_{50} for BHT (Fig. 5f).

The lowest IC_{50} values for DPPH and H_2O_2 (98 μg GAE/mL and 105.8 μg GAE/mL, respectively) of the homogenized extracts were recorded by T1 (Fig. 4a). However, the highest antiradical activity was recorded for the homogenized extracts obtained by T2 (75.39 ± 0.99% and 71.07 ± 0.5% against DPPH and H_2O_2 radicals, respectively) (Fig. 4b).

The extract obtained by UAE (T3) at 10% of the maximum output power (300 W) showed a lower ability to reduce free radicals (Fig. 5a). On the other hand, the extracts obtained by UAE (T4, Fig. 5b) exhibited lower IC_{50} values against DPPH and H_2O_2 radicals than those of T5 (Fig. 5 c).

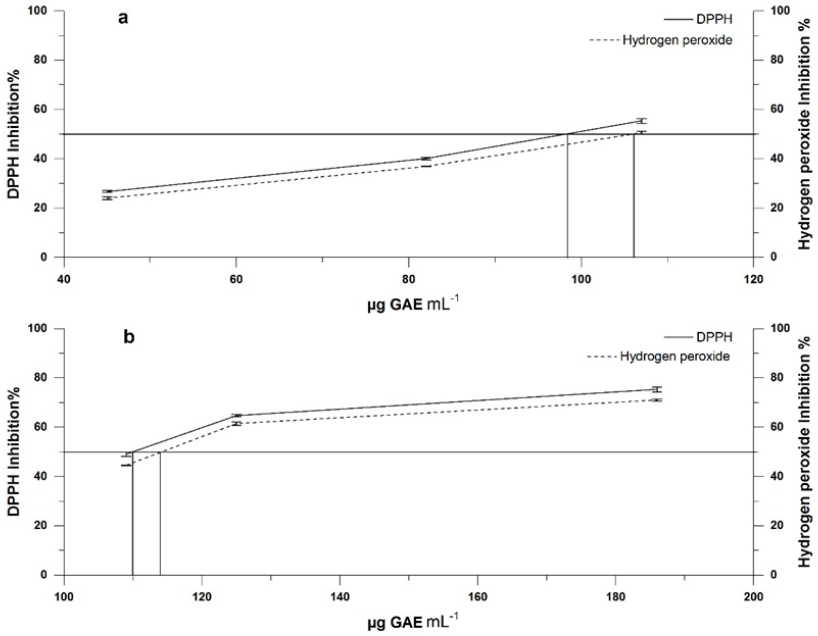

Figure 4. Inhibition percentage of DPPH and H_2O_2 radicals ± SD and IC_{50} values of extracts obtained by homogenization as a function of solvent/pomace ratio (20/1 (a); 40/1(b)) and performed at different polyphenol concentrations. Error bars indicate the standard deviation of triplicate values ($p<0.05$)

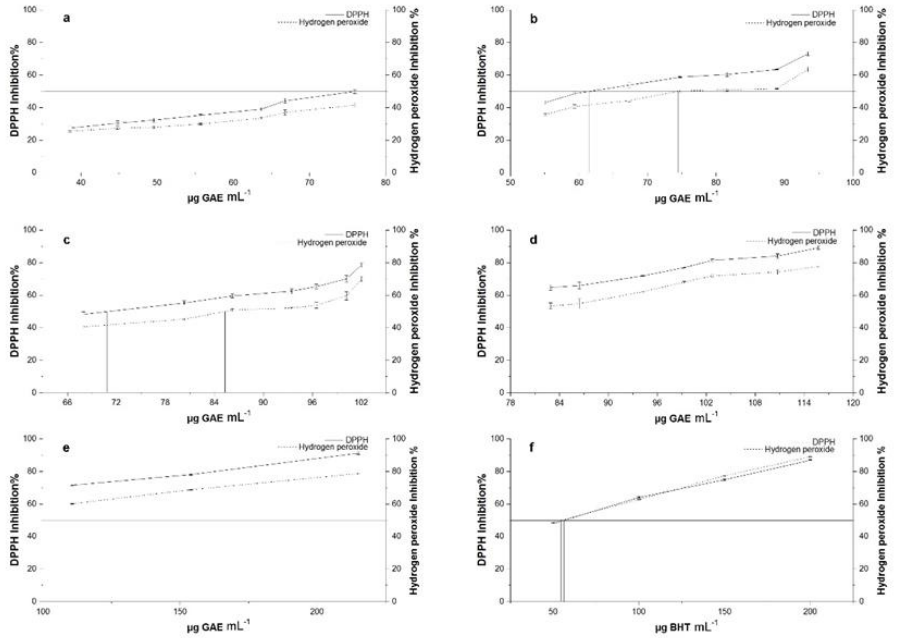

Fig 5. Inhibition percentage of DPPH and H_2O_2 radicals ± SD and IC_{50} values of extracts as a function of ultrasonic intensity (10%, 20%, 40% and 50%, (a-d)) at solvent/pomace ratio of 20/1, and at 50% of ultrasonic intensity and solvent/pomace ratio of 40/1 (e) and performed at different polyphenol and BHT (f) concentrations. Error bars indicate the standard deviation of triplicate values ($p<0.05$)

At high ultrasonic intensity, the sonicated extracts (T6, 7 min, 115.75 μg GAE/mL, Fig. 5d) and (T7, 30 min, 213.3 μg GAE/mL, Fig. 5e) displayed the significantly highest (p<0.05) antioxidant activity against DPPH radicals (DPPH % inhibition 89.20% and 91.30%, respectively) among all the investigated extracts. However, the sonicated extract (T6) showed a significantly higher (p<0.05) level of free radical-sequestering activity than the sonicated extract of T7, at the same concentration. This result may be attributed to the individual polyphenols present in each extract. The efficacies of the extracts could be classified in the following order: extract obtained by UAE > extract obtained by homogenization. The sonicated extract was rich in hydroxytyrosol, oleuropein, pyrogallol and catechol as illustrated in Table 1. These polyphenols have a significant DPPH-quenching ability (Xie and Schaich, 2014). The scavenging ability of olive pomace extracts obtained by homogenization or UAE at low intensity (< 50%) was found to be polyphenols concentration dependent.

The IC_{50} values of DPPH and H_2O_2 of the reference antioxidant BHT were 52.96 μg/mL and 53.56 μg/mL, respectively (Fig. 5f). These values for BHT agree with those reported by Xu *et al*. (2009). The results illustrate that olive pomace sonicated extract (T6) possessed higher antioxidant activity than that of the synthetic antioxidant BHT (Fig. 5f). At 115.75 μg GAE/mL of OPE (T6), 89.20% of the DPPH radicals were inhibited. The same effect required ~200 μg BHT/mL.

4. Conclusions

This study showed that extraction of the polyphenols from pomace is not favored at times longer than 30 min during homogenization or UAE. The UAE maximized the extracted polyphenols from olive pomace and increased the antioxidative activities in the extract. The scavenging activities against DPPH and H_2O_2 radicals reflected the unique antioxidant activity of the olive pomace extract obtained by the UAE.

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CHEMOMETRIC APPROACH BASED ON FATTY ACID COMPOSITION AND $\delta^{13}C$ ANALYSIS FOR VERIFICATION OF ORGANIC RAW MILK FROM COWS WITH DIFFERENT DIET

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Chemometry .

ABSTRACT

The growing interest in organic farming, which involves a significant percentage of "green grass" and hay in the animal diet and increasing the output of milk labeled "100% grass-fed," induces an urgent necessity for authentication such value-added products. The aim of this study was to develop the integrated approach for discrimination of organic milk from farms with silage-haylage and grass-hay cow diet. The research was based on the characterization of fatty acid composition and carbon stable isotopes ratios (δ13C), and included data processing with chemometric approach.

It has been shown that absolute value ranges of most fatty acids didn't allow to discriminate the milk from different organic farms, although the difference in certain fatty acids content between separate seasons has been proven to be statistically significant. This integrated approach with application of the principal components analysis envisaged that the analyzed parameters were normalized by the maximum value and the data matrix consisted of not only absolute values of the fatty acids content, but also additional derivative parameters (the sum of cis-, trans-isomers etc.). The approach proved the significance of content values of C16:1 trans-9, C18:1 trans-11, C18:3 cis-9,12,15, C18:2 trans-9,12 and conjugates of linoleic acid for milk samples discrimination. Thus, the analysis of the total data set of absolute and derivative normalized parameters by the method of principal components analysis allows to distinguish completely the organic milk with different share of green grass and hay in cows diet both the stalling and pasture periods.

1.Introduction

Increasing of consumers' interest in organic products leads to the growth of their producing. According to the Organic Milk Suppliers Cooperative (www.omsco.co.uk) the world production of organic milk will amount to ≈$13 billion by the end of 2019. Also, the interest in the so-called "pasture dairy management", which involves cows grazing on pastures in summer and feeding by hay with minimum usage of concentrates in winter, is renewed.

The certification requirements for organic dairy farms are different. Thus, the requirements for feedstuff on organic farms range from simple recommendations for pasturing (Japanese Organic Standard) to the certain values of the roughage percentages in the feed dry matter – from 60% (EU regulations, "Bioland") to 90% ("Biosuisse",

Switzerland). Meanwhile, in the United States, the world biggest organic products market (Organic Milk Market Report, 2017) a number of organic manufactures has approved own animal grass-fed cattle standard under the auspices of the American Grassfed Association. It establishes such requirements for the production of dairy and meat raw materials, so that the labelling of "100 % grassfed" of the corresponding dairy and meat products is legitimate. This certification exists in parallel with the additional certification for organic producers – "Pennsylvania Certified Organic 100% Grassfed certification" (www.paorganic.org/grassfed).

Besides, in the United Kingdom market (www.arlafoods.co.uk) organic milk from cows with grazing for more than 200 days per year is labelled as "free range" and in the Netherlands market organic milk and dairy products labelled as "meadow milk" from cows grazing at least for 120 days per year and 6 hours per day are available (www.weidemelk.nl). Accordingly, the price of such type of milk is much higher than the price of both organic and conventional ones. Thereby it is necessary to develop the approaches for authentication of such dairy products.

Cattle diet is one of the decisive factors which affects physical, chemical and biochemical parameters of milk, especially the milk fat composition. It is known that the higher percentage of fresh green grass and hay in the cow's diet leads to higher concentration of polyunsaturated fatty acids, in particular ω-3-acids and conjugates of linoleic acid (CLA) (Średnicka-Tober, 2016; Adler, 2013; Butler, 2008). However, it is usually difficult to distinguish the feed base of milk origin and dairy management type by the absolute values of fatty acids content in milk. Therefore, the combination of methods has recently been used to solve this problem.

Thus, the processing of triglyceride database of milk fat allowed to distinguish organic milk from farms certified according to European standards (organic, biodynamic) from conventional milk without labelling. However, the effectiveness of this approach decreased from ≈90% to 72% when analysing the samples has taken in early spring and summer (Capuano, 2013). Also, it was noted the complexity of identification of organic milk, "pasture" milk and milk from farms with biodynamic type of management only by triglyceride composition (Capuano, 2013).

The principal components analysis (PCA) of milk fatty acid composition discriminated the samples from farms with different types of cattle diet ("fresh-cut green grass", "pasture", organic, biodynamic and conventional). Reliable differences were obtained in summer for conventional and "fresh-cut green grass" milk. However, the differences in winter were less reliable (Capuano, 2014). Also, it was possible to discriminate organic and biodynamic milk from conventional by fatty acid composition. However, it was impossible to distinguish organic milk from biodynamic one by fatty acid composition both in summer and winter (Capuano, 2014).

It has also been shown that comparing the data of fatty acid and triglyceride composition gives the possibility to distinguish retail organic milk, conventional milk and retail milk from farms with the grazing duration at least 120 days per year. At the same time, this approach has not established the significant difference between the last and conventional types (Capuano, 2015). Thus, the absolute values of fatty acids content in milk could indicate the proportion of fresh green grass and hay in the cows' diet, but only in certain periods of the year in the cases of significant differences in feeding.

It is also possible to determine the composition of cattle diet by the ratio of stable isotopes $^{13}C/^{12}C$ ($\delta^{13}C$) in milk. Grass and hay on the one hand, and corn silage and grains on the other hand, are different in the type of plant photosynthesis (C_3 and C_4 respectively) and, accordingly, in the carbon isotopes profiles, which affects $\delta^{13}C$ values in milk. There is a positive correlation between the $\delta^{13}C$ values and the percentage of corn silage in the cows' diet (Camin, 2008; Zhukova, 2017). As a result, the $\delta^{13}C$ values in milk, as well as features of fatty acid composition, could reflect the proportion of grass in the cows' diet.

In addition, the lipids of green grass are characterized by the high content of unsaturated fatty acids, which are localized in cells chloroplasts, so their composition depends on the type of photosynthesis. At low seasonal temperatures the plants with C_3-type photosynthesis dominate on pasture, and 50 – 75% of all fatty acids of green grass are linolenic acid and CLA (Elgersma, 2006; Elgersma, 2003; Dhiman, 1999, Kochubey, 1996). At the same time, in plants with C_4-type photosynthesis, which are predominantly distributed in warm climate, linolenic acid is less than 40% of all fatty acids (White, 2001). Also, in the pastures C14:0 and C16:0 acids dominate in the grass at the end of summer, and the C18:3*cis*-9,12,15(ω-3) and CLA content decreases as compared to the spring period and the first weeks of summer (Loor, 2002).

Thus, the analysis of correlation of fatty acid composition with $\delta^{13}C$ values could be the effective method for the authentication of dairy products and milk obtained from cows with a high content of grass in the diet. At present, there is a shortage of similar studies. The available results showed that the content of C18:3*cis*-9,12,15(ω3), which minimal value in organic milk products was 0.50%, correlated with $\delta^{13}C$ values, which maximal value was –26.5‰ (Molkentin, 2013). It was also noted that such an approach should take into account the region, feed, climatic conditions etc. (Molkentin, 2009; Petrov, 2016).

The purposes of our study were: 1) analysis of the fatty acid composition and ratios of carbon stable isotopes $^{13}C/^{12}C$ in milk from organic farms with different volumes of green grass and hay in the cattle diet; 2) multivariate analysis of milk fatty acid composition and $\delta^{13}C$ values database; 3) development of the integrated approach to the authentication of milk from grass-fed farms throughout the year.

2. Materials and methods

2.1. Sampling

The organic milk samples were taken from 2 organic farms in Zhytomyr region with silage- and haylage-based cattle diet (n = 30) (O1) and from 2 organic farms in Chernihiv region with grass- and hay-based cattle diet (O2) certified according to Council Regulation (EC) №834/2007 and Commission Regulation (EC) №889/2008 (n = 24). The research was carried out during the indoor period (November – April) and outdoor period (May – October) in 2015 – 2017. The samples of milk were taken from a tank on farms, transported in plastic bottles with a screw cap at +4ºC. Data on cow's feeding diet and daily consumption of dry matter (kg/cow) were received from farmers.

2.2. Instrumental analysis

For the milk fat fraction extraction, the milk sample was heated to 35°C in a water bath ("RVO-400", «Ingos, s.r.o. Czech Republic), stirred and cooled to 20±2°C. 100 ml of milk were mixed in a separating funnel, then were added 80 ml of ethanol, 20 ml of ammonium aqueous solution (14.0 mol/dm^3), 100 ml of diethyl ether, mixed vigorously for 1 min, then was added 100 ml of petroleum ether, gently mixed, then the liquid fraction was poured. Then was added 100 ml of 10%

solution of sodium sulfate to the liquid fraction, stirred, filtered through a filter paper and evaporated on a rotary evaporator ("RVO-400", «Ingos, s.r.o. Czech Republic). This method was applied according to ISO 14156:2005.

The mixture of methyl esters of fatty acids were prepared by methanolysis of glycerides. 0.1 g of fat was transferred to a test tube, 5.0 ml of hexane was added, mixed thoroughly. 0.2 ml of sodium methylate solution (2 mol / dm 3) was added to the test tube, carefully stirred for 2 min, and filtered through a paper filter with added $Na_2HSO_4 \times H_2O$. This method was applied according to ISO 15884/IDF 182:2008

The fatty acid composition was analysed using the gas chromatograph Crystallux ("Analityka", Ukraine) with the flame ionization detector, SP 2556 column (Supelco, USA), 100 m × 0.25 mm I.D., 0.20 μm film layer, with the help of "Analityka" software (SPC "Analityka", Ukraine) for system monitoring and data processing. The mixture of 37 methyl esters of fatty acids FAME ("Sigma-Aldrich", USA) was used as the standard.

Parameters of the measurement: the initial temperature of the column was 60°C; isothermal period - 15 minutes; temperature rise to 186°C with rate of 10°C/min; isothermal period - 20 min; the temperature rise to the final column temperature - 220°C, with rate of 5 °C/min. The total analysis time was 120 minutes. The temperature of the detector was 260°C, the temperature of the vaporizer was 250°C. This method was applied according to ISO 15885/IDF 184:2008.

The ratio of $^{13}C/^{12}C$ ($\delta^{13}C$) isotopes was analysed in milk fatty phase using mass spectrometer "MI-1201SG", ("Electron", Ukraine) at the Isotope Geochemistry Lab of the Institute of Environmental Geochemistry. The measurements were made by using the international standard polyethylene foil (PEF-1) and transferred to the international standard Vienna Pee Dee Belemnite (VPDB) according to Gerstenberg and Herrman, 1983. The isotopes ratio is given in ‰ by δ scale and calculated by equation (1):

$$\delta C = \frac{R_1 - R_2}{R_2} \cdot 1000‰ , \quad (1)$$

where C – Carbon, R_1 – $^{13}C/^{12}C$ ratio in test sample, R_2 – $^{13}C/^{12}C$ ratio in internal standard PEF-1.

2.3. Statistical analysis

The data statistical processing was carried out using the univariate dispersion analysis in MS Excel 2010. The multivariate data analysis was performed using PCA in PAST software (Hammer et al., 2001) at the Analytical Chemistry Department of the National University of Pharmacy.

3.Results and discussions

3.1. Analysis of cattle diet

Organic dairy farms regulate the composition of cattle diet according to the herd size, the possibilities of fodder procurement, the planned milk productivity and other factors. The analysis of livestock feeding diets has shown the differences depending on farming type (Table 1).

Thus, during the outdoor period, the diet on O1 farms with silage and haylage ration and herd size near 400 heads, was characterized by a greater feed variety. In structure of feed dry matter intake (DMI) a significant part was occupied by haylage, silage and hay, that meets the requirements of organic certification standard – not less than 60 % of DMI.

At the same time, the concentrated feed accounts for about one-third of DMI. On O2 farms with the grass and hay diet of cows and with the herd size of ≈115 heads the consumption of DMI comprises more than 90% of fresh grass of annual and perennial

plants and concentrated fodder without corn silage addition.

During the indoor period on O1 farms the feed structure differed from the outdoor period by increasing the proportion of haylage and reducing the proportion of roughage. At the same time the values of other groups of feed were without significant differences. Rough, juicy and concentrated feeds without corn silage were used on O2 farms during the indoor period.

In addition, local climatic peculiarities of the farms affect diet forming and herd size. The farms with silage and haylage feeding type are located in Zhytomyr region, where the average temperature during the outdoor period is 21.7°C with the absolute maximum of 36.2°C, however, the rainfall is minor – 74.6 mm. The farms with grass and hay feeding type were located in Chernihiv region, where the average temperature during the outdoor period equals 22.3°C with the absolute maximum of 41.1°C, the rainfall is 66.3 mm. For comparison, in Switzerland and Germany during grazing period the average temperature is 25.3°C and 20.9°C respectively with the absolute maximum of 37.8°C and 36.0°C, however, rainfall is 183.2 mm and 86.2 mm, that causes the greater possibilities for cows feeding with fresh grass.

Table 1. Cows' diets on organic farms

Parameter	Organic farms with silage-haylage diet (O1)		Organic farms with grass-hay diet (O2)	
	Outdoor period	Indoor period	Outdoor period	Indoor period
Milk productivity of cows, kg/day*	22.0-23.0	22.0-23.0	11.0-12.0	8.0-9.0
Feed dry matter intake (DMI) per day, kg/cow*	18.1-24.0	20.3-25.4	8.0-9.0	13.5-14.0
Diet composition, % DMI*				
Green fodder (grass on pasture, fresh-cut grass)	11.9-20.5	-	88.0-93.0	-
Roughage (hay, straw)	14.3-23.0	8.5-23.7	-	21.8-24.3
Juicy feed, % DMI*				
- haylage	23.2-33.4	34.4-43.1	-	55.0-63.2
- corn silage	16.4-22.8	10.6-16.0	-	-
Concentrated feed (grains of cereals and beans; sunflower cake)	30.3-34.2	31.3-32.5	7.0-10.0	15.0-18.0

* data is presented in the form of range of values obtained during 2015 – 2017.

3.2. Fatty Acid Analysis

The analysis of fatty acids composition of milk from the farms during outdoor period has shown that absolute values of individual short chain fatty acids varied slightly (Table 2). However, the average annual content of C4:0 – C12:0 in milk from O1 farms was higher by 17.3% than in milk from O2 farms, C14:0 and C16:0 content – by 17.9% and 21.5% respectively. The average annual content of C18:0, on the contrary, was higher by 33.7% in milk from O2 farms than from O1 farms.

Variations between indoor and outdoor periods for milk from O1 and O2 farms within each farm were 1.9% and 5.5% for C4:0 – C12:0, 5.0% and 1.8% for C14:0, 4.5% and 7.5% for C16:0, respectively. Variations in C18:0 content between indoor and outdoor periods were 2.9% and 11.7% for O1 and O2 milk respectively (Table 2).

More significant differences have been revealed between O1 and O2 milk fatty acid composition. The content of C4:0 – C12:0 was higher by 21.1% and 14.8% for O1 milk in outdoor and indoor periods than for O2 milk; the C14:0 content was higher by 15.4% and 20.9% respectively, the C16:0 content was higher by 23.7% and 21.5% respectively than for milk from O2 farm (Table 2).

However, the C18:0 content in milk from O1 farms was lower by 24.5% and 43.4% respectively in outdoor and indoor periods than in milk from O2 farm.

Such differences can be explained by peculiar properties of fatty acids metabolism in ruminants. The C4:0 – C14:0 fatty acids are synthesized *de novo* in mammary gland, the synthesis of C16:0 occurs both *de novo* and from fatty acids of feed in the process of digestion in cows' small intestine, and the content of long chain acids depends on their content in feed (Mitani, 2016; Palmquist, 2006).

In our study significantly differences ($P < 0.00001$) of the C16:0 content reflected the share of green grass and hay in the cattle diet. The diet based only on fresh grass and hay feeding leads to the energy feed deficiency and increasing the role of fat reserve and decreasing *de novo* synthesis. Correspondingly, this may reflected in the decreasing of the C16:0 content (Roca Fernandez, 2012). Thus, the C16:0 content can be considered as a marker for determination of the share of green grass and hay in cows' diet.

Our studies have shown that the total PUFA content was higher by 30.8% and 15.4% in outdoor and indoor periods respectively in milk from O2 farms than in milk from O1 farm. In particular, the average annual content of ω3-fatty acids was higher by 89.4% and ω6-fatty acids was lower by 25.64% in milk from O2 farms. The average annual ratio of ω3- and ω6-fatty acids was higher by 51.0% in milk from O2 farms than in milk from O1 farms.

The intensity of transformation processes of long chain fatty acids in cow body directly depends on intensity of feed biohydrogenation in rumen and amount of long chain fatty acids, especially PUFA, in livestock feed (Jenkins, T. C., 1993).

In mammary gland stearic acid (C18:0), which is the final product of biohydrogenation of feed PUFA, is the substrate for C18:1*trans*-11 *de novo* synthesis and its following transformation to C18:2*cis*-9,12 and CLA with the help of Δ9-desaturase. From 64% to 97% of CLA content in milk fat comes from C18:1*trans*-acids (Roca Fernandez et al., 2012; Palmquist, 2006; Kemp & Lander, 1984; Kemp et al., 1984). Thus, the large share of feed such as fresh grass on pasture or fresh-cut grass with high level of PUFA leads to increasing of vaccenic acid (C18:1*trans*-11) and conjugates of linoleic acid content in milk fat (Palmquist, 2006).

Table 2. Fatty acid composition of milk fat from cows with different diets, g/100g of fat

Fatty acid	Organic farms with silage-haylage diet (O1)						Organic farms with grass-hay diet (O2)						P-value
	Outdoor period		Indoor period		Average annual		Outdoor period		Indoor period		Average annual		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
C 4:0	4.000	0.228	3.947	0.419	3.961	0.321	3.779	0.401	4.123	0.398	3.988	0.426	>0.05
C 6:0	2.508	0.148	2.410	0.215	2.465	0.184	1.972	0.287	2.237	0.254	2.134	0.293	<0.00001
C 8:0	1.480	0.104	1.446	0.171	1.456	0.137	1.099	0.207	1.257	0.208	1.195	0.218	<0.00001
C 10:0	3.219	0.262	3.142	0.209	3.165	0.245	2.338	0.570	2.266	0.421	2.294	0.474	<0.00001
C 10:1	0.312	0.053	0.332	0.051	0.316	0.052	0.260	0.061	0.253	0.054	0.256	0.056	<0.001
C 11:0	0.057	0.029	0.048	0.027	0.053	0.028	0.038	0.024	0.035	0.011	0.037	0.017	<0.05
C 12: 0	3.542	0.303	3.521	0.198	3.521	0.260	2.451	0.535	2.443	0.454	2.446	0.475	<0.00001
C 13:0	0.189	0.038	0.199	0.020	0.194	0.032	0.218	0.039	0.245	0.051	0.234	0.047	<0.001
C 14:0	10.477	0.867	11.00	0.615	10.68	0.794	8.868	1.671	8.703	1.488	8.768	1.527	<0.00001
Σ *trans* C14:1	0.583	0.057	0.622	0.036	0.602	0.053	1.121	0.245	0.927	0.271	1.003	0.273	<0.00001
C 14:1 *cis*	0.985	0.095	0.932	0.057	0.959	0.086	0.819	0.300	0.845	0.169	0.835	0.223	<0.01
C 15:0	1.062	0.087	1.193	0.060	1.120	0.101	1.280	0.184	1.097	0.245	1.169	0.237	>0.05
C 15:1	0.366	0.046	0.397	0.036	0.379	0.044	0.448	0.054	0.436	0.105	0.441	0.087	<0.01
C 16:0	28.059	1.166	29.31	1.656	28.51	1.473	21.40	3.586	23.00	2.799	22.381	3.153	<0.00001
Σ *trans* C16:1	0.377	0.113	0.336	0.055	0.357	0.084	0.069	0.024	0.067	0.030	0.046	0.027	<0.00001
Σ *cis* C16:1	2.178	0.343	1.824	0.729	2.001	0.536	2.025	0.750	2.010	0.392	2.018	0.571	<0.01
C 17:0	0.697		0.740	0.054	0.716	0.081	0.779	0.102	0.743	0.236	0.757	0.193	>0.05
C 17:1 *cis*	0.240	0.093	0.233	0.021	0.237	0.019	0.224	0.076	0.254	0.054	0.242	0.064	>0.05
C 18:0	9.807	0.018	9.515	1.178	9.783	1.163	12.21	2.947	13.64	2.278	13.082	2.595	<0.00001
C 18:1n (1-9) t	0.531	1.187	0.479	0.128	0.511	0.114	0.612	0.211	0.638	0.182	0.627	0.189	<0.01
C 18:1n (10-13) t	1.394	0.102	1.369	0.334	1.394	0.324	3.255	0.701	1.941	0.633	2.455	0.919	<0.00001
C 18:1n9 *cis*	21.791	0.326	20.89	1.209	21.45	1.227	22.70	2.934	25.71	3.387	24.532	3.488	<0.0001
C 18:2n6 *trans*	0.351	1.162	0.276	0.077	0.324	0.113	0.610	0.184	0.321	0.136	0.434	0.210	<0.05
C 18:2n6 *cis*	2.502	0.129	2.251	0.226	2.403	0.250	2.194	1.485	2.132	0.459	2.156	0.963	>0.05
C 18:3n6	0.208	0.225	0.203	0.018	0.207	0.028	0.241	0.048	0.380	0.148	0.326	0.137	<0.0001

C 18:3n3	0.715	0.034	0.782	0.142	0.745	0.155	1.468	0.236	1.354	0.343	1.398	0.305	<0.00001
C20:0	0.155	0.162	0.155	0.021	0.155	0.019	0.178	0.015	0.240	0.030	0.209	0.023	<0.00001
Σ CLA	0.387	0.011	0.400	0.050	0.394	0.044	1.871	0.523	0.822	0.372	1.347	0.448	<0.00001
C 20:1	0.270	0.038	0.280	0.045	0.275	0.051	0.551	0.056	0.553	0.095	0.552	0.076	<0.05
C 22:0	0.037	0.056	0.041	0.021	0.039	0.018	0.036	0.021	0.049	0.022	0.043	0.022	>0.05
C20:3n6	0.021	0.014	0.033	0.018	0.027	0.017	0.021	0.015	0.037	0.011	0.029	0.013	>0.05
C22:1n9	0.051	0.015	0.074	0.026	0.063	0.024	0.048	0.036	0.066	0.035	0.057	0.036	>0.05
C23:0	0019	0.022	0.024	0.012	0.022	0.012	0.056	0.031	0.054	0.029	0.055	0.030	>0.05
C20:4n6	0.032	0.011	0.038	0.017	0.035	0.013	0.074	0.036	0.088	0.044	0.081	0.040	<0.05
C20:5n3	0.018	0.009	0.025	0.011	0.022	0.011	0.072	0.056	0.059	0.032	0.066	0.044	>0.05
C22:6n3	0.041	0.011	0.032	0.012	0.037	0.017	0.061	0.032	0.056	0.026	0.059	0.029	>0.05
Σ SFA	65.307		66.70		65.85		56.71		60.14		58.799		<0.00001
Σ UFA	32.769		31.19		32.17		37.62		38.02		37.862		<0.00001
Σ MUFA	29.077		27.77		28.58		32.13		33.70		33.084		<0.00001
Σ PUFA	4.275		4.041		4.193		6.611		5.249		5.781		<0.01
Σ n/i*	1.924		2.108		1.971		5.669		1.835		3.338		
Σ omega-3	0.774		0.839		0.804		1.601		1.469		1.523		<0.00001
Σ omega -6	3.501		3.202		3.389		5.010		3.780		4.258		<0.001
Omega 3/6	0.221		0.262		0.237		0.320		0.388		0.358		<0.00001

Σ SFA - the sum of saturated fatty acid; Σ UFA - the sum of unsaturated fatty acid; Σ MUFA - the sum of monounsaturated fatty acid;
Σ PUFA - the sum of polyunsaturated fatty acid; Σ n/i – the sum of non-identified peaks.

Table 3. Ratio of carbon stable isotopes in organic milk fat phase from cows with different diets

Parameter	Values of $\delta^{13}C$ in milk fat phase, ‰												
	Outdoor period				Indoor period				Average annual				P-value
	Mean	Min	Max	SD	Mean	Min	Max	SD	Mean	Min	Max	SD	
Milk fat from cows with silage-haylage diet (O1)	-24,53	-28,00	-22,30	1,76	-24,85	-26,70	-22,90	1,32	-24,70	-28,00	-22,30	1,57	<0,00001
Milk fat from cows with grass-hay diet (O2)	-31,58	-32,90	-29,80	1,08	-29,31	-29,80	-28,60	0,33	-30,20	-32,90	-28,60	1,33	

The average content of ΣC18:1*trans*-acids was 1.6 times higher in O2 milk compared to O1 milk. The differences between O2 and O1 milk in outdoor and indoor periods were 2.0 times and 1.4 times, respectively.

According to our results the average annual CLA content in O2 milk was 3.4 times higher as compared to O1 milk. The difference in CLA content between O1 and O2 milk during the indoor period was equal to 2.0 times, while during the outdoor period – 4.8 times.

The annual content of C18:2*cis*-9,12 was lower by 10.3% in O2 milk than in O1 milk; during indoor and outdoor period – by 5.3% and 12.3% respectively.

However, despite the high statistically significant difference between individual fatty acids of milk from different farms, the analysis of the absolute values ranges of the most of fatty acids does not allow to identify the milk origin. For example, the annual values of CLA content ranged from 0.327 g / 100 g to 0.506 g / 100 g of fat in milk from O1 farms and from 0.478 g / 100 g to 2.523 g / 100 g of fat in milk from O2 farms. During the indoor period the characteristic range of CLA for milk from O1 farms was 0.329 – 0.506 g / 100 g of fat, and for milk from O2 farms is 0.478 – 1.535 g / 100 g of fat. The most significant variations were observed during the outdoor period: for O1 milk the values ranged from 0.327 g / 100 g to 0.441 g / 100 g of fat, and for O2 milk – from 1.035 g / 100 g of fat to 2.523 g / 100 g of fat. It is possible that the CLA content of more than 1.000 g / 100 g of fat is distinctive only for organic milk from cows with the grass-fed diet.

Overlapping of the annual values for ΣC14:1*trans* has been detected: for O1 milk it was 0.489–0.670 g / 100 g of fat, and for O2 milk it was 0.663 – 1.650 g / 100 g of fat. During indoor period the values were 0.557 – 0.670 g / 100 g for O1 and 0.663 – 1.631 g / 100 g of fat for O2. However, during outdoor period they are clearly distinguished – 0.489 – 0.670 g / 100 g of fat for O1 milk and 0.811 – 1.650 g / 100 g of fat for O2 milk.

Annual ranges of the total content of C16:1*trans*-acids were not overlapped: in O1 and O2 milk were ranged 0.157 – 0.585 g / 100 g and 0.017 – 0.112 g / 100 g of fat respectively. It is also possible to suggest a hypothesis that the total C16:1*trans*-acids content, which is not higher than 0.134 g / 100 g of fat, may be characteristic only for organic milk from farms with the grass-fed diet.

The ranges of annual values of the C18:3*cis*-6,9,12 content were overlapped: for O1 milk was 0.163 – 0.268 g / 100 g of fat and O2 milk was 0.174 – 0.857 g / 100 g of fat. However, during the indoor period the possibility for samples discriminate ion was higher: the ranges were 0.174 – 0.231 g / 100 g for O1 and 0.195 – 0.857 g / 100 g of fat O2 milk.

The similar situation was observed for the C6:0 during the outdoor period. Its values ranged from 2.251 to 2.870 g / 100 g for O1 milk and from 1.365 to 2.393 g / 100 g of fat for O2 milk. Also, the C8:0 content ranged from 1.171 to 1.733 g / 100 g in O1 milk and from 0.693 to 1.461 g / 100 g of fat O2 milk during the outdoor period.

3.3. $\delta^{13}C$ analysis and chemometric data processing

For the complete discrimination of organic milk it was proposed the integrated approach based on the application of PCA to obtained multidimensional data of fatty acid composition and stable isotopes ratios $^{13}C/^{12}C$ ($\delta^{13}C$) in milk fat. Such an integrated chemometric approach has allowed to discriminate the milk obtained from organic farms with silage/haylage and grass/hay feeding diets (Figure 1).

The analysis of $\delta^{13}C$ values in the fatty phase of milk has revealed statistically

significant differences between the samples from the farms of both types (Table 3).

PCA has been carried out in several stages and in different variants. Thus, Figure 1a shows the data distribution plot by the first two principal components when analysing only absolute values of fatty acid composition of the milk samples. Such an analysis didn't allow to reliably distinguish milk from O1 and O2 farms. It was shown that the largest contribution into the first two principal components (which explained ≈92% of data variations) was ensured by the content of palmitic (C16:0) (0.69 in the loadings of PC1), oleic (C18:1*cis*-9) (-0.44 in the loadings of PC1), stearic (C18:0) (-0.45 in the loadings of PC1) and myristic (C14:0) (0.26 in the loadings score of PC1) acids. It should be noted that the influence of these parameters could not be considered as characteristic. This is explained by positively correlation of their relatively high content in milk fat and contribution significance PC1 and PC2. Contingently, they can be attributed to macroparameters of fatty acid composition.

The introduction of absolute $\delta^{13}C$ values into the data matrix (Figure 1b) greatly improved the samples discrimination – the zones overlapping on the plot become minimal. The loadings analysis has shown that this parameter has the largest contribution to PC1 (0.43) and PC2 (0.64). Unlike the previous distribution the significance of C16:0 (-0.64 in the loadings of PC1), C18:0 (0.40 in the loadings of PC1) and C14:0 (-0.23 in the loadings of PC1) content decreased, but C18:1*cis*-9 content increased (0.37 in the loadings of PC1).

At the second stage the data of fatty acid composition and $\delta^{13}C$ values in fatty phase were normalized (by the maximum value for each parameter).

Such an approach allowed to minimize the main problem of PCA – the scale, i. e., to equalize the impact significance of macroindicators and minor components on the results of chemometric analysis. As a result of such actions the distribution picture has been improved, but not completely (Figure 1c). Also, the loadings analysis has shown another contribution of parameters to the principal components structure. While PC1 explained only ≈40% of data variations, PC2 and PC3 had the equal value of 10%.

It should be noted that using just this approach the significance of such acids as C16:1*trans*-9 (-0.42 in the loadings of PC1), C18:1*trans*-11 (0.28 in the loadings of PC1), C18:3*cis*-9,12,15 (0.30 in the loadings of PC1), C18:2*trans*-9,12 (0.20 in the loadings of PC1) and CLA (0.31 in the loadings of PC1) were established. These acids are characteristic for milk fat composition and especially for milk from cows with grass and hay diet (Elgersma, 2006). The effect of other UFA content on the possibility of distinguishing the milk samples from different farms was also quite high. At the same time the significance of $\delta^{13}C$ influence decreased (0.14 in the loadings of PC1).

Taking into account the results of chemometric analysis of normalized data we have introduced the additional derivative parameters describing the milk samples composition. Such parameters are the total *cis*-isomers and *trans*-isomers of UFA content, total UFA content, total MUFA content, total PUFA content, total dienes and trienes content, C18:0 content, total C18 UFA content, the ratio of ΣC18 UFA and Σ*trans*-isomers, the ratio of ΣC18 UFA and Σtrienes; the ratio of ΣC18 UFA and CLA content.

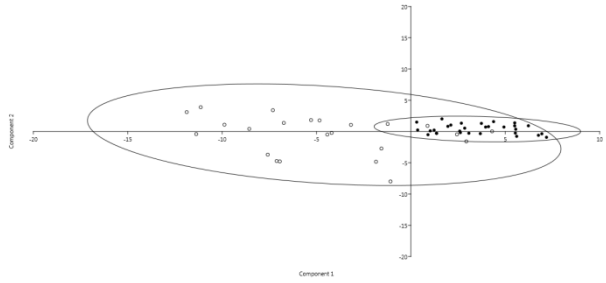

Figure 1a. PC1 and PC2, fatty acids composition, absolute values

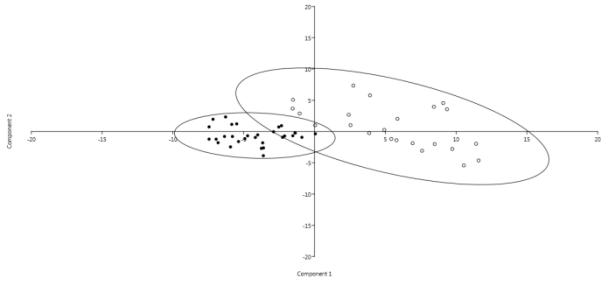

Figure 1b. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute values

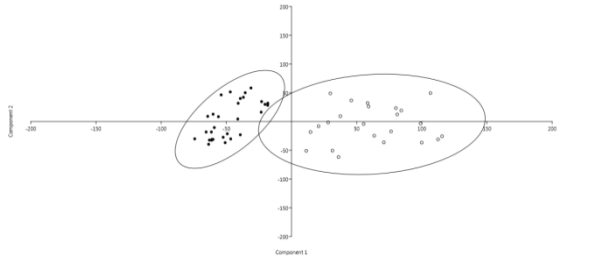

Figure 1c. PC1 and PC2, fatty acids composition and $\delta^{13}C$, absolute normalized values

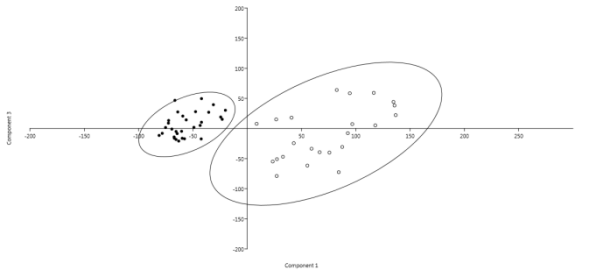

Figure 1d. PC1 and PC3, fatty acids composition and $\delta^{13}C$, absolute and derivative indicators, normalized values

Figure 1. The effect of the integrated approach on discrimination of organic milk from farms with different cows' diets by PCA:
○ – farms with silage-haylage diet; ● – farms with grass- hay diet.

At the third stage the calculated data set of derived parameters has been added to the matrix of absolute values of fatty acid composition and $\delta^{13}C$. Then the matrix normalization has been done (in the same way as at the previous stage) followed by its processing by PCA. This analysis of such complex database completely divided two data groups on the plot (Figure 1d). While PC1 explains only ≈45% of variations, and PC2 and PC3 still have an equal value of 10%.

It should be noted that it is rather problematic to allocate the parameters with the greatest contribution to the principal components in the loadings matrix. The most of UFA have high contributions, however, the contribution of C16:1*trans*-9 (-0.35 in the loadings of PC1), vaccenic (C18:1*trans*-11) (0.25 in the loadings of PC1), linolenic (C18:3*cis*-9,12,15) (0.27 in the loadings of PC1), linolalidine (C18:2*trans*-9,12) (0.17 in the loadings of PC1) acids and CLA(0.28 in the loadings of PC1) remained maximal.

Among calculated derivative parameters the contribution into the structure of the first, second and third components is maximal for such parameters as the total *trans*-isomers content (0.18 in the loadings of PC1), total trienes content (0.23 in the loadings of PC1), total unsaturated C18 UFA (0.11 in the loadings of PC1), the ratio of ΣC18 UFA/CLA content (-0.29 in the loadings of PC1) and far exceeds the contribution significance of $\delta^{13}C$ values (0.12 in the loadings of PC1).

4. Conclusions

The method of milk examination, including the organic one, to detect the presence of grass share in cows' diet has been proposed. This method is based on the integrated approach, which includes the analysis of fatty acid composition, the ratios of carbon stable isotopes in milk fat phase followed by the calculation of the set of derivative parameters, the normalization of the absolute values of these parameters (by the maximum value) and the processing of obtained data matrix by PCA. Among the calculated derivative parameters the contribution into the structure of the first, second and third components is maximal for such parameters as the total *trans*-isomers content, total trienes content, total unsaturated C18 UFA, the ratio of ΣC18 UFA and CLA content. The proposed approach has allowed to distinguish organic milk from farms with different percentage of green grass and hay in cattle diet during indoor and outdoor periods. It could be a potential tool for confirming organic milk authenticity, especially labelled as "grass fed".

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