Production of Functional Cooked Sausage by *Mentha piperita* Essential Oil as a Natural Antioxidant and Antimicrobial Material

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Abstract

Background: Natural antioxidants with plant origin are incorporated to foods in the forms of essential oils or extracts. They can retard lipid oxidation and control spoilage bacteria in foodstuffs (e.g. meat products).

Objective: This work was aimed to evaluate the effect of nitrite partial replacement with *Mentha piperita* essential oil (MPEO) on oxidative, microbial and sensory properties of cooked sausage.

Methods: GC/MS was used for the identification of essential oil components. Nitrite content (120 ppm) was reduced and replaced with 20, 40 and 60 ppm of MPEO. The effect of MPEO on product rancidity was assessed by peroxide and TBARS values in sausage samples. Antimicrobial properties of the essential oil were evaluated by MICs and MBCs determination against *Escherichia coli* and *Clostridium perfringens* by microdilution method. Also, the effect of this replacement on the product color stability was evaluated by determination of L*, a* and b* values, Hue angle, and $\Delta E_{(2-30)}$.

Result: Results indicated that peroxide and TBARS values of sample with 20 ppm of MPEO were significantly lower than samples with 40 and 60 ppm of MPEO and control, at the end of storage period. With respect to color parameters, hue angle of the sample with 60 ppm of MPEO was significantly higher than samples with lower essential oil levels and control after 30 days of storage. Moreover, total color difference of sample with 60 ppm of MPEO was lower than other samples and control ($p<0.05$). Antimicrobial activity of MPEO against *Escherichia coli* determined as MICs and MBCs were 0.226 and 0.453 mg/ml, respectively. These values turned out as 0.453 and 0.906 mg/ml against *Clostridium perfringens*.

Conclusion: all samples with different essential oil levels were acceptable after 30 days of storage according to PV and TBARS thresholds determined in literature. Replacement of 50 % of nitrite with MPEO is a reasonable approach in order to put down harmful effects of nitrite in sausage and to enhance functionality of the product.

Keywords: Cooked sausage, *Mentha piperita*, Essential oil, Antioxidant, Nitrite

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Introduction

Lipid oxidation is defined as the main process which affects nutritional and sensory characteristics of muscle foods and leads to fatty acid degradation and generation of products like malondialdehyde (MDA). Moreover, lipid oxidation may cause gene mutations, leading to carcinogenesis [1]. Prooxidant substances generated by lipid peroxidation, may react with oxymyoglobin (OMb) and produce metmyoglobin (MMb) which affects color of meat products [2]. Moreover, protein oxidation which occurs through free radical chain reaction and pro-oxidative activity of heme proteins like myoglobin (Mb) and hemoglobin, leads to a significant decrease in meat nutritional value in terms of availability of essential amino acids and digestibility of oxidized proteins. Strategies applied to control oxidation in muscle foods include addition of antioxidants directly to the foods.

Nitrite has been used in meat products in order to inhibit lipid oxidation and microbial spoilage while influencing color and flavor of the product. Despite all these benefits, serious concern exists about its use as nitrite is responsible for *N*-nitrosamine formation which has strong carcinogenic effect [3]. So there is an essential need in finding safe compounds to replace nitrite. Plant derived antioxidants are incorporated to foods in the forms of essential oils or extracts and can retard lipid oxidation in meat products. Microbial deterioration in heated meat products may occur due to microorganisms that survive heat treatment. *Clostridium perfringens* is a contaminant of meat with anaerobic and spore forming properties which make it an important bacteria in heat treated meat products. Plant essential oils have active components with a large extent of antimicrobial activity to control spoilage bacteria in processed foods including meat products [4].

Antimicrobial activities of *Mentha piperita* essential oil have been studied previously and showed that the essential oil could inhibit the growth of different gram positive and gram negative bacteria [5]. Radical scavenging and antioxidant activities of *Mentha piperita* essential oil and extract have been observed in different systems [6]. In addition, herbal compounds have anti-inflammatory, antimutagenic and anticancer activities which increase functionality of foods against diseases [7]. So, partial replacement of nitrite with *Mentha piperita* essential oil not only reduces nitrosamine formation but also increases the product's functionality.

This work concerns evaluation of MPEO inhibitory activity against two meat spoilage bacteria (*Escherichia coli* and *Clostridium perfringens*) and investigation of antioxidant activity of MPEO in cooked sausage as well as its effects on the product color changes during storage.

Material and Methods

Methods

GC/MS analysis of MPEO

GC/MS analysis was carried out on a HP-6890 gas chromatograph equipped with a HP-5 capillary column (30 m × 0.25 mm; 0.25 µm film thickness). The oven temperature was held at 40 ^{o}C for 5 min, and then raised to 240 ^{o}C with the rate of 3 ^{o}C/min. The temperature of injection port and detector was 290 ^{o}C. Helium gas was used as carrier with a flow rate of 0.8 ml/min while split method was

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used with the ratio of 1:10. The gas chromatograph was coupled with HP-5973 mass spectrometer with the same temperature programming and mass spectra were taken at 70 eV. The percentage of MPEO components was obtained from FID peak areas and identified by comparison of Kovats Index (KI) with those already published [8].

In vitro antibacterial screening

One gram-negative and one gram-positive bacteria which cause poisoning and spoilage in meat were studied. Stock cultures of *E. coli* and *C. perfringens* were prepared in Mueller-Hinton Broth (Merck, Germany) and Fluid Thioglycollate Media (Gibco, Scotland), respectively, and kept at refrigerated temperature (4 ^{o}C).

Antimicrobial effect of MPEO was evaluated by determining Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Broth dilution method was used for MIC and MBC determination according to Gachkar *et al.* with some modifications [9]. *E. coli* suspension was prepared with turbidity equivalent to 0.5 McFarland standard and microbial count of 1.5×10^{8} cfu/ml. 100 µl of MPEO dispersion in Tween 20 was added to 100 µl Mueller-Hinton Broth (MHB) medium in microtiter plate wells and inoculated with 10 µl *E. coli* suspension. Positive and negative controls were applied and incubation was done at 37 ^{o}C for 24 hours. MIC and MBC values were obtained by secondary culture in MacConkey agar medium (Merck, Germany).

The same method was used to determine MIC and MBC values of the MPEO against *C. perfringens* by using Fluid Thioglycollate medium (FTG). To maintain anaerobic conditions in test tubes, 0.5 ml of liquid paraffin was added on top of the culture medium.

Sausage production

Four sausage samples were prepared according to the following formula: MPEO:nitrite ratios of 0:120 (control), 20:100 (P20), 40:80 (P40), and 60:60 (P60) ppm. The formulation contained 70% meat (35% chicken meat and 35% beef meat). Other ingredients were 1.53 kg ice, 0.72 kg soybean oil, 0.27 kg starch, 0.027 kg $Na_5P_3O_{10}$, 0.144 kg NaCl and 0.0045 kg ascorbic acid for 9 kg paste. The components were mixed in a cutter (Seydelmann, Germany) and the paste was divided into separate batches and mixed manually with sodium nitrite and essential oil at different levels. The batches were filled in polyamide casings separately and cooked at 75 ^{o}C for 1 hour. Then, the product was cooled under cold water flow and stored at 4 ^{o}C for 30 days.

Peroxide value (PV) measurement

At first, sausage oil was extracted according to Folch method [10]. PV of samples was determined after 2, 9, 16, 23 and 30 days of storage according to AOAC method [11].

Thiobarbituric acid reactive substances (TBARS)

The TBARS of samples was measured after 2, 9, 16, 23 and 30 days of storage [12].

Color stability evaluation

Samples stored at 4 ^{o}C were subjected to color measurements after 2, 9, 16, 23 and 30 days using the Hunter-Lab Colourflex Colorimeter (Hunter Associated Lab, Inc.,

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Reston, Virginia, USA). CIE Lab coordinates were reported as lightness (L*), redness (a*), and yellowness (b*) and hue angle and total color difference (ΔE) of samples between 2 and 30 days of storage ($\Delta E_{(2-30)}$) were calculated using the following equations: H^o= arctg b*/a*; $\Delta E_{(2-30)} = [(L_{30} - L_2)^2 + (a_{30} - a_2)^2 + (b_{30} - b_2)^2)]^{1/2}$.

Spore inoculation and microbiological analysis

C. perfringens strain (RITCC 2752) was used to test the antimicrobial activity of MPEO in sausage. The stock culture of the bacteria was prepared and sporulation was performed in Duncan and Strong medium. Spore suspension was transferred to microtubes and the medium separated by centrifugation. The deposited spores were re-suspended in sterile 0.1% peptone water, heat-shocked (20 min at 75 ^oC) and mixed manually with meat paste [13]. The product was cooked in water bath set at 75 ^oC for 1 hour and then cooled with cold water.

C. perfringens growth in samples selected from oxidation analysis was evaluated during 30 days of storage at 4 ^oC. For this purpose, 10 g of sample was homogenized in 90 ml of sterile salt solution (NaCl, 0.85%) using a sterile blender (Sanyo, Japan). Bacterial enumeration was performed using an anaerobic culture in Sulfite Polymyxin Sulfadiazine agar.

Sensory evaluation

The odor and taste of samples selected from oxidation analysis were evaluated by 30 trained panelists using hedonic test according to Fattahi-far *et al.* at five scales (100: excellent, 75: good, 50: fair, 25: poor, and 0: very poor) [14].

Materials

M. piperita essential oil was obtained from Institute of Medicinal Plants (ACECR).

Escherichia coli strain (ATCC 25922) was supplied by the Bacteriology Department of Tarbiat Modares University (Tehran, Iran) and *Clostridium perfringens* strain (RITCC 2752) was purchased from Razi Institute (Karaj, Iran). Mueller-Hinton Broth, MacConkey agar medium and Fluid Thioglycollate Media were purchased from (Merck, Germany) and (Gibco, Scotland), respectively.

Chloroform, methanol, acetic acid, sodium chloride, sodium thiosulphate, trichloroacetic acid, thiobarbituric acid, starch and Tween 20 were obtained from Fluka Chemical Co. (Buchs, Switzerland) with highest purity available without further purification.

Statistical analysis

All experiments were done in triplicate and mean values with standard deviations were reported. The data obtained from experiments were analyzed by SPSS 15.0.1 software to assess the statistical significance. Analysis of variance (ANOVA) was conducted and means were compared using LSD test. Differences were considered significant at $p \leq 0.01$.

Results

The major constituents of MPEO were identified by GC/MS method (Table 1). They include menthol (24.40 %), neo-isomenthol (23.36 %) and 1,8-cineole (8.73 %) which appear consistent with Ka *et al.* report on

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major constituents of *M. piperita* essential oil: menthol (18 mg/g) and neo-menthol (0.72 mg/g) [6], as well as menthol (28-42 %), menthone (19-27 %), and 1,8-cineole (4-5 %) reported by others [5].

MIC and MBC values of MPEO against *E. coli* and *C. perfringens* were determined (Table 2).

The antioxidant effect of different essential oil levels on sausage samples are determined by PV (Fig. 1). An overall increase in PV was observed during storage. As can be seen, the sample with higher essential oil level showed lower PV at the beginning of storage period (day 2). After 30 days of storage, PV of P40 and P60 samples were higher than control sample ($p< 0.05$) and there was no significant difference between PV of P20 and control samples. Also, we found an increase of MPEO antioxidant activity at higher levels toward day 16 of storage. This was consistent with Goli *et al.* finding where peroxide content of soybean oil decreased with increasing of pistachio hull extract level [15]. It is noteworthy that PVs of all samples were below 25 meq O_2/kg oil which is mentioned as acceptability limit for PV of fatty foodstuffs [16].

Table 1- Chemical composition of *Mentha piperita* essential oil identified by GC/MS

Components	R.T. (min) [a]	K.I. [b]	Amount (%)
α-Pinene	8.36	939	1.56
β-Pinene	10.33	980	3.30
1,8-Cineole	13.16	1033	8.73
cis-Sabinene hydrate	14.90	1071	3.05
Menthol	19.79	1173	24.40
Terpin-4-ol	20.03	1177	8.67
Isomenthol	20.18	1179	1.49
Neo-isomenthol	21.18	1188	23.36
Carvone	23.08	1242	4.40
p-Menthon-3,8-diol	25.90	1301	4.40
Linalool butyrate	31.10	1422	2.77
Germacrene D	33.65	1480	2.57
Others (<1.00)	-	-	9.85
Total			98.55

a: Retention Time; b: Kovat`s index

Table 2- Antimicrobial activity results of the MPEO (MIC and MBC, mg ml^{-1})

Microorganism	MIC	MBC
Escherichia coli	0.226	0.453
Clostridium perfringens	0.453	0.906

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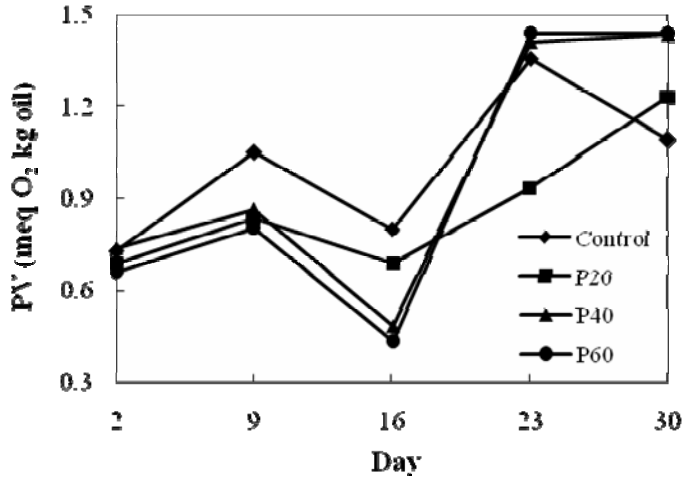

Fig 1- Peroxide values of the sausage samples during 30 days of storage at 4°C (Control: blank sample with 120 ppm sodium nitrite and without essential oil (120:0); P20, P40 and P60: samples containing 100:20, 80:40 and 60:60 ppm of nitrite: MPEO, respectively)

There was no significant difference between TBARS values of P40, P60 and control samples at day 2 of storage (Fig. 2). At day 30, the P20 sample had significantly lower TBARS value than control sample while other samples had no significant difference or significantly higher values. These results are inconsistent with that reported by Ka *et al.* (2005) who observed no inhibitory activity of peppermint extract on MDA level [6]. The TBARS values of all samples had a decreasing trend from day 9 to 23 which is in agreement with results observed by Georgantelis *et al.* (2007) who reported the highest amount of MDA in pork sausage after 15 days of storage at 4 °C and a decrease afterwards [17]. The acceptable limit for TBARS value in meat products is 1 mg MDA/kg sample [18]. In our experiments, it was observed that all MPEO containing samples had lower TBARS quantities than the above limit, thus, they were considered acceptable.

Color changes in sausage samples were expressed by changes in hue angle (H^o) and total color difference (ΔE). There was no significant difference between H^o values of samples at day 2 of storage (Fig. 3). But after 30 days, the value of P60 sample was significantly higher than other samples while no significant difference was observed between P20, P40 and control samples.

Total color difference (ΔE) of samples between days 2 and 30 of storage were probed (Fig. 4). The results showed that P60 sample had the least ΔE and its value was significantly different from that of P20, P40, and control samples. Results of microbiological analysis showed that no growth of *C. perfringens* occurred during 30 days of storage at 4 °C in sausage samples with reduced nitrite content.

P20 and P40 samples were selected from the results of oxidation tests and were evaluated in terms of sensory characters. As can be seen, there were no significant difference between sensory scores of P20, P40, and control samples (Table 3). In agreement with our findings, Kanatt *et al.* (2008) notified that there were no significant differences

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between odor and taste scores of salami samples containing mint extract and control sample [19].

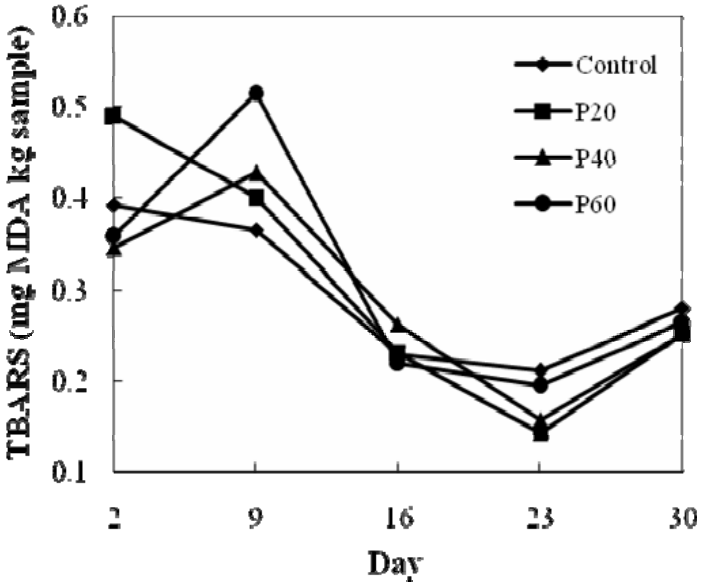

Fig 2- TBARS values (mg MAD/kg oil) of the sausage samples during 30 days of storage at 4°C (Control: blank sample with 120 ppm sodium nitrite and without essential oil (120:0); P20, P40 and P60: samples containing 100:20, 80:40 and 60:60 ppm of nitrite: MPEO, respectively)

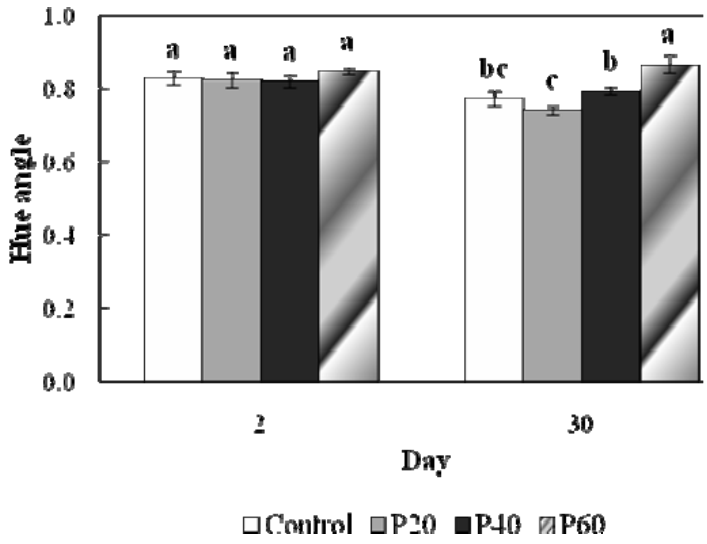

Fig 3- Hue angle values of sausage samples after 2 and 30 days of storage at 4°C (Control: blank sample with 120 ppm sodium nitrite and without essential oil (120:0); P20, P40 and P60: samples containing 100:20, 80:40 and 60:60 ppm of nitrite: MPEO, respectively). Different letters represent significant differences at p<0.05

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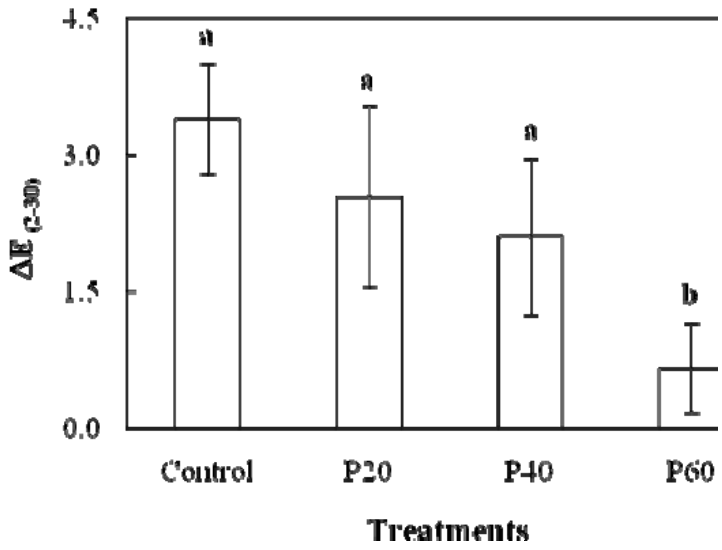

Fig 4 - Total color difference (ΔE) of sausage samples at 2 and 30 days of storage at 4°C (Control: blank sample with 120 ppm sodium nitrite and without essential oil (120:0); P20, P40 and P60: samples containing 100:20, 80:40 and 60:60 ppm of nitrite: MPEO, respectively). Different letters represent significant differences at p<0.05

Table 3- Sensory scores of sausage samples*

Samples	Sensory attributes	
	Odor	Taste
Control	48.33±22.68[a]	54.17±25.50[a]
20 ppm MPEO	48.33±20.69[a]	51.67±26.21[a]
40 ppm MPEO	50.83±24.99[a]	48.33±28.57[a]

* Means with different letters in the columns represent significant differences at p<0.05

Discussion

Upon plant growth, ketone compounds like menthone are reduced and alcoholic components like menthol increases. This explains higher amounts of menthol and neo-isomenthol in our study. *M. piperita* plant at a growth stage with high number of basal leaves yields an essential oil with high concentration of menthol and low content of menthone. Another reason for composition differences is that spring planted crops show significantly higher menthol content and lower concentration of terpinen-4-ol compared to autumn planted crops. Also, mineral fertilization seems to increase the menthol content of the essential oil and treated crop shows lower menthone content.

In previous studies, MIC value of MPEO was found to be 1.25-2.5 mg/ml against *E. coli*, whereas the value in present study was 0.226 mg/ml which shows higher antibacterial activity of the investigated essential oil [5]. This difference in MIC and MBC values may be due to different seasons and conditions of planting and different harvest times resulting in dissimilar chemical composition. MBC

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values of MPEO were reported to be 0.7 and 2 % previously which indicated lower activity against *E. coli* than the studied essential oil [20].Menthol as a major constituent in MPEO was shown to be an active component against *C. sporogenes* [21]. Menthol seems to be the major constituent responsible for bioactivity of the essential oil and other terpenes contribute to the antimicrobial activity through synergistic effects [5]. *Mentha arvensis* var. *piperacens* has shown an effective antimicrobial inhibition against *C. perfringens* determined by diameter of inhibition zone [22]. The MIC value of *Satureja montana* L. essential oil against *C. perfringens* was reported to be 1.56 % which was higher than the value obtained for MPEO (0.05 %) and showed lower antibacterial activity [23]. MBC values of other essential oils (*Erodium ciconium*, *E. cicutarium* and *E. absinthoides*) against *C. perfringens* were reported previously as 2.5-5 mg/ml. As can be seen, MPEO indicated more bactericidal activity on *C. perfringens* than *Erodium* species [24]. Further comparisons were not made as there was no report about MIC and MBC values of MPEO against *C. perfringens*.

Terpenes are hydrocarbon compounds present in essential oils and menthol is a monoterpene constituent of MPEO. Because of hydrophobic character, hydrocarbons cause toxicity in cell membrane. Accumulation of these compounds in the lipid biolayer expands the membrane and alters its structure and function. These compounds can decrease the cytochrome C oxidase activity in cell membrane and increase proton permeability causing cell toxicity [25].

The reduction in inhibitory effect of MPEO against product oxidation might be the result of prooxidant effect of the essential oil at higher concentrations. In consistence with the results, Genot *et al.* (1991) reported that some natural antioxidant compounds show antioxidant activity at lower concentrations but act as a prooxidant at higher quantities [2]. Du and Li (2008) reported a PV decrease with an initial increase in the essential oil level. Further increase of the latter causes a sudden increase of the former [1].

Moreover, a sudden decrease in PV of samples was observed at day 16 of storage after which it increased toward day 30. This result is in agreement with that reported by Georgantelis *et al.* (2007) who observed the same decrease in PV of beef burger and stated that the decrease might be the result of higher hydroperoxides breakup rate than the rate at which they are formed [26].

The reduction of TBARS is attributable to the decomposition of MDA by some microorganisms, further oxidation of MDA to other products like alcohols and acids which can not react with thiobarbituric acid or MDA reactions with proteins and sugars. In consistence with our results, Dorman *et al.* (2003) have previously reported that *Mentha* species extracts were capable of preventing TBARS formation by scavenging hydroxyl radicals generated in the presence of iron ions and ascorbic acid in a dose dependent manner and therefore, were able to prevent the propagation of lipid peroxidation process in complex matrices like foodstuffs. They reported *M. piperita* as a good source of natural antioxidants and it contains the highest levels of total phenols [27]. In addition, Mimica-Dukić *et al.* (2003) reported that pure MPEO shows higher hydroxyl radical scavenging capacity than 0.1 M BHT solution and they identified 1,8-cineole as the most powerful scavenging compounds with regards

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to synergistic effects of other terpenic constituents [28].

Myoglobin and oxymyoglobin oxidation to brown metmyoglobin reduces meat red color and increases H^o value. Similar to our observed results, Kamdem *et al.* (2007) had found that H^o of sausage samples with reduced nitrite or without any nitrite addition will increase during storage [29]. This difference may be because of prooxidant activity of higher level of MPEO (60 ppm) which causes myoglobin oxidation, red color reduction and H^o increase.

In another investigation, Zarringhalami *et al.* reported that nitrite content could be reduced up to 60% with significantly higher *a** value as a positive parameter of sausage color [30]. This result which is in agreement with our observations, shows that nitrite reduction not only is ineffective on sausage color attenuation but also causes lower ΔE in samples. Meat red color is related to the reduced form of iron ion (Fe^{2+}) of myoglobin and antioxidants would protect the reduced form and red color. Polyphenol oxidases (PPOs) which are present in plants can oxidize plant polyphenols and form darkened compounds. Therefore, natural antioxidants containing PPOs can contribute to browning of the product.

In agreement with the results of ΔE, Estévez *et al.* (2005) reported that higher levels of rosemary essential oil added to frankfurter (300 and 600 ppm) caused significant reduction of total color difference compared to sample with lower essential oil level (150 ppm) [31]. Color changes measured instrumentally are considered as observable changes when ΔE value is more than 2. In this study, we perceived that ΔE value of the P60 sample is below 2, thus, this level of essential oil could prevent color changes in sausage during shelf life.

Conclusion

In conclusion, *M. piperita* essential oil showed higher *in vitro* antibacterial activity against *E. coli* and *C. perfringens* compared to previous studies. With respect to results of antioxidant activity, it can be deduced that despite better results observed from lower levels of the essential oil, all samples with different essential oil levels were acceptable after 30 days of storage according to PV and TBARS thresholds determined in literature. Also, it was found that sample with 60 ppm of the essential oil had lower total color difference during 30 days of storage compared to other samples, that shows little effect of nitrite in sausage color development. Therefore, replacement of 50 % of nitrite with MPEO is a reasonable approach to put down harmful effects of nitrite in sausage and to enhance functionality of the product.

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