Research Article
Rosemary Essential Oil and Lyophilized Extract as Natural Antioxidant Source to Prevent Lipid Oxidation in Pork Sausage

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Abstract: The aim of this study was to evaluate the effect of the use of Rosemary Essential Oil (REO) and Lyophilized Rosemary Extract (LRE) on the oxidative stability of pork sausages stored at -12°C. Potential antioxidants in sausages were determined by thiobarbituric acid reactive substances (TBARS) and expressed in mg of malonaldehyde MDA/kg. Measurements were taken one day each week starting from production day until 49 days after. The TBARS values increased over time, starting with a value of 0.45 and ending with 2.89 mg MDA/kg. The control treatment suffered more intense oxidation than those with Sodium Erythorbate (SE) and natural antioxidants. The lipid oxidation of sausage was significantly inhibited at 49 days of frozen storage in the presence of LRE when compared to the control (47.28%). The sausage containing LRE and REO showed a 75.67% and 56.41% acceptance rate, respectively. The LRE exhibited an excellent inhibitory effect on lipid oxidation of sausage and it has potential to use in meat products.

Keywords: Antioxidant activity, lipid oxidation, meat products, Rosmarinus officinalis, TBARS

INTRODUCTION

Currently, one of the biggest challenges for the meat products industry is to provide the consumer with fresh food, regarding taste, color and odor of the product. However, the process involving the preparation of meat products such as milling, mixing and cooking, results in lipid oxidation (Shah et al., 2014; Sáyago-Ayerdi et al., 2009; Osawa et al., 2005).

The lipid oxidation, denoted as one of the main degradation reactions, is responsible for the reduction of vitamins and unsaturated fatty acids, production of free radicals, creating undesirable flavors and decreasing the lifetime of the product (Okpala, 2016). It also leads to the formation of several other compounds which have negative effects on the quality of meat and meat products causing changes to the nutritional quality (Falowo et al., 2014; Shah et al., 2014). The use of antioxidants in foods is one method to minimize these problems (Das et al., 2012).

The synthetic antioxidants used in the meat industry, such as butylatedhydroxyanisole (BHA), Butylatedhydroxytoluene (BHT), Tert-Butylhydroquinone (TBHQ) and Propyl Gallate (PG) has been restricted by many countries due to possible risks to consumer health. The toxicological effects of these substances is growing the interest in studies of using natural substances with antioxidant characteristic in meat (Rocha et al., 2007; Lianhe et al., 2012; Sun-Waterhouse et al., 2011).

The demand for natural products with antioxidant potential has been an objective of scientific studies. Plants are a generous source of bioactive substances and are being evaluated as natural antioxidants to preserve and improve the overall quality of meat and meat products (Shah et al., 2014). Food seasonings are widely known to have bioactive compounds capable of decreasing lipid oxidation and the action of free radicals (Mariutti and Bragagnolo, 2007).

Rosmarinus officinalis, commonly known as rosemary (Mariutti and Bragagnolo 2007) has a high content of phenolic compounds, which includes the abundant compounds rosmarinic acid and rosmanol (Windisch et al., 2008) and it has been successfully applied to the oxidation inhibition in food (Sebranek et al., 2005; Keokamnerd et al., 2008). Rosemary Essential Oil (REO) contains many important bioactive compounds, such as monoterpenes hydrocarbons (camphene, verbene, α-pinene, β-pinene, limonene), oxygenated monoterpenes (1, 8-cineole, linalool,
camphor, borneol, verbenone) and sesquiterpenes (β-cariofillene) (Napoli et al., 2010). Essential oils and natural extracts of Rosmarinus officinalis L. are known to possess antimicrobial and antioxidant activities and some applications of these extracts in food active packaging systems were reported in literature (Realini and Marcos, 2014). It can be used as a natural preservative in order to inhibit lipid oxidation in sunflower oil (Chen et al., 2014), soybean oil (Casarotti and Jorge, 2012), pork-based products (Lara et al., 2011; Hać-Szymańczuk et al., 2011; Hernández-Hernández et al., 2009) and various types of foods, such as sausages, pastas, peanuts and butter. While rosemary essential oil has antioxidant potential, it can be used to inhibit lipid oxidation in sunflower oil, pork meat sausages (Jongberg et al., 2013) and other foods (Lara et al., 2011; Chen et al., 2014; Hać-Szymańczuk et al., 2011; Hernández-Hernández et al., 2009).

The present study aims to evaluate the antioxidant capacity of essential oil and lyophilized rosemary extract when applied to pork sausage meat in order to prevent the lipid oxidation during frozen storage.

**MATERIALS AND METHODS**

**Sample:** Rosemary (Rosmarinus officinalis) sample was cultivated in Pato Branco, Paraná, Brazil. Was collected in usual flowering period, between January and May 2014. Rosemary leaves were dried in an oven with forced air circulation (Nova Ética 400/D, São Paulo, Brazil) at 40°C for 48 h and ground in an analytical mill (Quimis Q298A21, Diadema, Brazil) to a grain diameter of less than 0.5 mm.

**Chemicals:** Folin-Ciocalteu phenol reagents, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), β-carotene, linoleic acid, Trolox, DPPH (2,2-Diphenyl-1-picryl-hydrazyl), (2,2-azino-bis-(3-ethylbenzothiazolone-6-sulfonic acid)) (ABTS), 2, 4, 6-Tris(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbituric acid, acetonitrile, ferulic acid, gallicacid, catechin, chlorogenic acid, caffeic acid, p-coumaric acid, trans-cinnamic acid, syringic acid, kaempferol, myricetin, rutin, quercetin and resveratrol were obtained from Sigma-Aldrich (Sternheim, Germany). All reagents used were of analytical grade.

**Preparation of Lyophilized Rosemary Extract (LRE) and Rosemary Essential Oil (REO):** A sample containing 10 g of rosemary leaves was subjected to the extraction process with 100 mL of ethanol solution (800 mL/L) in a shaker (SL 222, São Paulo, Brazil) at 40°C for 60 min a 150 rpm stirring rate. After filtration of the extracts, the supernatants were evaporated in a rotary evaporator (Fisatom 802, São Paulo, Brazil) (vacuum pressure of 600 mm Hg and 40°C) until completely dry and lyophilized (Lirotop L101, São Carlos, Brazil). The lyophilized rosemary extract was named LRE.

Essential oil was extracted by hydrodistillation, the rosemary leaves (100 g) were homogenized with 1000 mL of distilled water and placed inside a Clevenger-type apparatus. The heating temperature for the system was controlled to 110°C, until a boil began in the sample flasks began and extraction was carried out for 4 h. The Rosemary Essential Oil (REO) was recovered after decantation and used for analysis.

**Total Phenolic Compounds (TPC):** TPC were quantified by the Folin-Ciocalteu method described by Singleton et al. (1999), using gallic acid as a standard. 500 μL of the samples was mixed with 2.5 mL of Folin-Ciocalteu and 2 mL of sodium carbonate 40 g/L (v/v). After two hours in darkness at room temperature, the absorbance of the extract was measured at 764 nm in spectrophotometer (UV-VIS Bel Photonics 2000, Piracicaba, Brazil). The results were expressed as mg GAE/g of sample (GAE: gallic acid equivalent, \( y = 0.021x-0.015, R^2 = 0.999 \)). All the assays were carried out in triplicate.

DPPH (2, 2 Diphenyl-1-picryl-hydrazyl) Radical Scavenging Assay: The measurement of DPPH free radical scavenging activity was measured as described by Brand-Williams et al. (1995). The reaction medium consisted of 0.5 mL of samples, 3.0 mL of ethanol and 0.3 mL of 0.5 mM DPPH• solution in ethanol. The mixture was incubated at room temperature in the dark for 45 min and the absorbance was read using a spectrophotometer (Bel Photonics 2000, Piracicaba, Brazil) at 517 nm. The \( EC_{50} \) (concentration required to obtain a 50% antioxidant effect) was measured and expressed in μg/mL and the mean percentage of antioxidant activity (%AA), according to the formula used by Carpes et al. (2013) (Eq. (1)). The assays were carried out in triplicate.

\[
\% AA = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \times 100
\]  

Ferric Reducing Antioxidant Power (FRAP) Assay: The FRAP assay was performed as described by Pulido et al. (2000). The absorbance was measured with a UV/VIS spectrophotometer (Bel Photonics 2000, Piracicaba, Brazil) at593 nm. Aqueous solutions of ferrous sulfate were used for calibration (\( y = 32.345x-0.0824, R^2 = 0.998 \)) and the results were expressed as mmol of Fe²⁺/g. All tests were carried out in triplicate.

**ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) Assay:** The ABTS method was performed as described by Re et al. (1999). The stock solutions included 7.4 mM ABTS⁺ and 2.6 mM potassium persulfate. The solution was prepared by mixing 1 mL ABTS⁺ solution with 60 mL ethanol to obtain an absorbance of 0.70 at 734 nm using the spectrophotometer (UV-VIS Bel Photonics 2000 Piracicaba, Brazil). The results were expressed in μmol/g of TEAC (Trolox-equivalent antioxidant
Coupled oxidation of β-carotene and linoleic acid assay: The β-carotene bleaching assays were conducted as previously described by Ahn et al. (2004). For emulsion stock, 40 mg of linoleic acid and 400 mg of Tween 40 were diluted in 3 mL of β-carotene chloroform solution (0.1 mg/mL). Chloroform was removed with nitrogen gas and then was added to oxygenated distilled water (100 mL) to form an emulsion. Absorbance of the samples at 470 nm were measured triplicate every 20 min until the color of β-carotene disappeared in the control reaction (t = 60 min). The antioxidant activity was determined by the inhibited percent in comparison to the control sample.

Preparation of the pork sausages: The preparation of the sausages was conducted according to procedures recommended by Brasil (2000). Fresh pork lean and fresh pork backfat were ground through a plate with 0.6 and 0.8 cm diameter holes, respectively and homogenized in a meat mixer with salt (1.8%), garlic, onion powder and dried parsley (all 0.1%). This basic formulation was divided into 4 lots. The first lot was designated as the control and no additional antioxidants were included. The second lot was designated as a positive control and was prepared by adding sodium erythorbate (0.2%), the third lot added LRE (0.2%) and the fourth lot added REO (0.2%). Separately, the meat lots were stuffed into natural pork casings, packed and stored at -15°C for 49 days. The sausages were analyzed for TBARS content and sensory analysis.

Oxidative Stability-Thiobarbituric Acid Reactive Substances (TBARS) assay: TBARS were measured by Raharjo et al. (1992). Measurements were made after processing and after 7, 14, 21, 28, 35, 42 and 49 days of frozen storage. Tetramethoxypropane (TMP) was removed with nitrogen gas and then was added to oxygenated distilled water (100 mL) to form an emulsion. Absorbance of the samples at 470 nm were measured triplicate every 20 min until the color of β-carotene disappeared in the control reaction (t = 60 min). The antioxidant activity was determined by the inhibited percent in comparison to the control sample.

Sensorial analysis: The stored frozen sausages were evaluated for sensory characteristics after 49 days. The color, odor, flavor, texture and overall acceptability were analyzed using a hedonic scale of 9 points, ranging from 1 (“I disliked very much”) to 9 (“I liked very much”) (Dutcosky, 2007). The samples randomly coded with three-digit numbers, were served in a monadic form in individual booths. Unsalted crackers and room temperature water were provided between samples to clean the palate. The percentages of agreement were analyzed of the grades assigned during the sensory analysis by testers. 115 panelists, including untrained students and staff members (75 females and 40 males), of Federal Technological University of Paraná (UTFPR) in Brazil participated in the test for the sensory evaluation. The members ranged in age from 19 to 45 years old.

Statistical analysis: The profiles were presented as the mean value±SD (standard deviation). The data was processed by one-way Analysis of Variance (ANOVA). The averages were compared by Tukey test and t test, considering the significance level of 95% (p<0.05), using the STATISTICA program 8.0 version (StatSoft, USA).

RESULTS AND DISCUSSION

Total Phenolic (TPC) Compounds: The mean value of total phenolic compounds in LRE and REO was 45.67±1.08 mg GAE/g and 0.21±0.08 mg GAE/g, respectively. Afonso et al. (2013) reported 16.67±0.40 mg GAE/g aqueous rosemary extract and Erkan et al. (2008) found 162 mg GAE/g for the methanol rosemary extract, higher than what was found in aqueous and ethanol extract. This study demonstrates that; for the extraction of phenolic compounds present in rosemary, a less polar solvent, such as methanol, is the most efficient. However, other studies show that the mixture of methanol and water are highly efficient in extracting rosenyphenolic compounds (Lapornik et al., 2005; Švarc-Gajić et al., 2013). Olmedo et al. (2015) found 8.3 mg GAE/g for the essential oil of rosemary, much higher than found in this study (0.21±0.08 mg GAE/g). This difference can be attributed to differences in the conditions of extraction and the season in which the harvest was taken.

In previous studies, gallic acid was found in rosemary extracts from Czech Republic (Dvorackova et al., 2014), Murcia (Herrero et al., 2010) and Turkey (Hossain et al., 2011). Maldini et al. (2016) studied methanol rosemary extracts of different parts of Sarnidia and detected the presence of kaempferol, quercetin and rutin but the most abundant was the kaempferol (1.68±0.12 mg/g). Vallverdu-Queralt et al. (2014) also found ferulic acid in hydroalcoholic extract of rosemary, although to a lesser extent (1.99±0.08 µg/g) than the present study. Mulinacci et al. (2011) identified thirty phenolic compounds, including rosmarinic acid, caffeic acid, carnosol, carnosic acid and cirsimaritin and assigns these compounds antioxidant activity exhibited by the extract.

Antioxidant capacities: The antioxidant activity was determined by four distinct in vitro methods due to the complexity of constituents of LRE and REO. It was found that there is a significant difference (p<0.05) between the results for the extract and oil (Table 1). The concentration of the extract required to obtain a 50% antioxidant effect against DPPH radical is
Table 1: Antioxidant activity by different methods in LRE, REO and commercial antioxidants

<table>
<thead>
<tr>
<th>Samples</th>
<th>EC₅₀ (µg mL⁻¹)</th>
<th>FRAP (µmol de Fe²⁺ g⁻¹)</th>
<th>ABTS (µmol Trolox g⁻¹)</th>
<th>β-caroteno (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRE</td>
<td>127.33±0.04ab</td>
<td>112.14±2.80bc</td>
<td>198.07±0.99ab</td>
<td>92.31±0.17ab</td>
</tr>
<tr>
<td>REO</td>
<td>79.63±0.03bc</td>
<td>14.61±0.48c</td>
<td>6.42±0.00e</td>
<td>89.10±0.11bc</td>
</tr>
<tr>
<td>BHT</td>
<td>114.66±0.07cd</td>
<td>2310.11±70.59bc</td>
<td>4277.33±33.53cd</td>
<td>63.68±0.18cd</td>
</tr>
<tr>
<td>BHA</td>
<td>72.25±0.07cd</td>
<td>2717.79±71.46cd</td>
<td>4743.04±51.37cd</td>
<td>68.06±0.22cd</td>
</tr>
<tr>
<td>Alfa-tocopherol</td>
<td>61.22±0.06de</td>
<td>1328.80±22.09de</td>
<td>5270.03±57.27de</td>
<td>70.29±0.17ec</td>
</tr>
</tbody>
</table>

LRE: Lyophilized Rosemary Extract; REO: Rosemary Essential Oil; EC₅₀: Concentration required obtaining a 50% antioxidant effect; Values are presented as mean±SD (n = 3); Different lower-case letter in the same column indicate significant difference (p<0.05) by the Tukey test; Different capital letters in the same row indicate significant difference (p<0.05) by the Tukey test; BHT: Butylated hydroxytoluene; BHA: Butylated Hydroxyanisole

Fig. 1: Effect of LRE (Lyophilized Rosemary Extract), REO (Rosemary Essential Oil) and sodium erythorbate in oxidative stability (TBARS) of sausage during 49 days of frozen storage. Different lowercase letter indicate significant differences between treatments (p<0.05) by the Tukey test. Different capital letters indicate significant differences between the storage time (p<0.05) by the Tukey test.

defined as EC₅₀. It is important to note that high values of EC₅₀ are negatively related to the antioxidant activity, in other words, EC₅₀ value is inversely proportional to the antioxidant capacity of the compounds (Almeida et al., 2017; Kontogianni et al., 2013; Mishra et al., 2012; Vicente et al., 2013). Given all these considerations, the LRE had lower antioxidant activities by DPPH method EC₅₀ = 127.33 µg/mL than the REO by EC₅₀ = 79.63 µg/mL (Table 1). Similar to LRE, EC₅₀ value was found by Rocha et al. (2007) (125.0 µg/mL) for ethanolic extracts of rosemary and Maldini et al. (2016) (29.86 µg/mL) in methanolic extract. Zaouali et al. (2010) reported the antioxidant capacity of six essential oils of the species *Rosmarinus officinalis*, obtained DPPH values ranging from 6 to 28.5 µL/mL and Yosr et al. (2013) obtained values ranging from 7.73 to 12.8 µL/mL.

Different than what is observed in the EC₅₀ FRAP and ABTS the LRE and REO had lower antioxidant activity when compared to the commercial antioxidants by FRAP and ABTS methods (Table 1). The FRAP values found for the REO and LRE were 14.61 and 112.14 µmol Fe²⁺/g respectively. Yosr et al. (2013) evaluated the essential rosemary oil and obtained FRAP levels between 13.28 and 17.75 mM Fe²⁺/g, similar to present study results (Table 1). The antioxidant activity by ABTS’ method for REO and LRE were 6.42 and 198.07 µmol Trolox/g of the sample, respectively (Table 1). These results are similar to Aouadi et al. (2014) who studied the effect of dietary supplementation in lambs containing essential oil of rosemary (*Rosmarinus officinalis*) and found ABTS value of 12.29 µmol Trolox/mL of the sample.

In the β-carotene/linoleic acid test, the free radicals attacked the highly unsaturated β-carotene molecules and as a result of this, the β-carotene was oxidized, smaller molecules were broken and the system lost its color. Therefore, the antioxidants inhibit the formation of peroxyl radicals, thereby inhibiting the oxidation of β-carotene (Seneviratne et al., 2016).

The antioxidant activity of LRE by different methods was higher than REO (Table 1). These results showed that LRE and REO contain compounds which are responsible for capturing free radicals and therefore can be used to stabilize and slow down lipid oxidation. According to Pérez-Fons et al. (2010) and Rašković et al. (2014), the antioxidant activity of ethanolic rosemary extracts is mainly due to phenolic compounds (phenolic acids and flavonoids).

*Thiobarbituric Acid-Reactive Substances (TBARS):*

The results were obtained by TBARS to assess lipid oxidation in the pork sausages frozen for 7 weeks in storage (Fig. 1). During the storage the control
Values are presented as mean±SD (n = 3); Different lower-case letter in the same column indicate significant difference (p<0.05) by the Tukey test.

### Table 2: Percent decrease in TBARS values in sausage treated with antioxidants (LRE and REO) during storage

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Days of storage</th>
<th>Percent decrease in TBARS compared to control (T1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage</td>
<td>Treatment</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td>8.33</td>
<td>30.23</td>
</tr>
<tr>
<td>T3</td>
<td>10.42</td>
<td>43.02</td>
</tr>
<tr>
<td>T4</td>
<td>10.42</td>
<td>33.72</td>
</tr>
</tbody>
</table>

4 Data not statistically analysed, but percentages were computed from the statistical data in Table 3; T1: Control -without antioxidant; T2: synthetic antioxidant-sodium erythorbate; T3: Lyophilized Rosemary Extract (LRE); T4: Rosemary Essential Oil (REO)

### Table 3: Sensory evaluation of sausage in different treatments at 49 days frozen storage

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Color</th>
<th>Flavor</th>
<th>Texture</th>
<th>Taste</th>
<th>Global impression</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>6.67±1.38b</td>
<td>6.09±1.59c</td>
<td>6.76±1.54b</td>
<td>6.95±1.38c</td>
<td>6.78±1.42b</td>
</tr>
<tr>
<td>T2</td>
<td>7.03±1.14a</td>
<td>6.58±1.50b</td>
<td>7.21±1.20a</td>
<td>7.38±1.26a</td>
<td>7.29±1.21a</td>
</tr>
<tr>
<td>T3</td>
<td>6.04±1.82d</td>
<td>6.01±1.66d</td>
<td>6.19±1.79d</td>
<td>5.92±2.10d</td>
<td>6.04±1.83d</td>
</tr>
<tr>
<td>T4</td>
<td>6.59±1.54a</td>
<td>6.65±1.81a</td>
<td>6.68±1.69a</td>
<td>5.27±2.61a</td>
<td>5.68±2.28a</td>
</tr>
<tr>
<td>Average</td>
<td>6.58±0.40</td>
<td>6.33±0.33</td>
<td>6.71±0.42</td>
<td>6.38±0.96</td>
<td>6.45±0.42</td>
</tr>
</tbody>
</table>

T1: Control -without antioxidant; T2: commercial antioxidant-sodium erythorbate; T3: Lyophilized rosemary extract; T4: Rosemary essential oil; Values are presented as mean±SD (n = 3); Different lower-case letter in the same column indicate significant difference (p<0.05) by the Tukey test.

Some authors have reported that the addition of REO and LRE are efficient in lipid oxidation in meat (Jongberg et al., 2013; Sebranek et al., 2005; Han and Rhee, 2005). Naveena et al. (2013) evaluated the effect of carnosic acid dried rosemary added to chicken patties during refrigerated storage and observed reduction of TBARS (37% to 87%) in comparison with the control sample. Teruel et al. (2015) showed the effectiveness of the rosemary extract as antioxidants, after 9 months of storage of chicken nuggets. Sebranek et al. (2005), showed the rosemary extract was more effective than BHA/BHT for preventing increased TBARS values in precooked-frozen sausage and showed similar results as that of BHA/BHT in refrigerated sausage.

**Sensory evaluation:** Mean sensory scores for odor, taste, flavor, textural attributes and global impression for the control sausage (T1) and sodium erythorbate (T2) and natural antioxidants (T3 and T4) are shown in Table 3.

Among the sausage containing natural antioxidant, the most liked sausage was that containing the LRE (75.67%) and the least acceptable was the sausage containing REO (56.41%). T3 and T4 differ from other treatments in all attributes. The sample T2 obtained higher average values close to 7.0 ("like regular") for attributes such as color, texture, taste and global impression (Table 3).

According to the panelists, the treatment containing the lyophilized extract showed green color change and a bitter taste. Similar results were found by Savadkoohi et al. (2014) in his study with beef sausage containing tomato pomace. Color intensity scales for beef sausages containing tomato residue were lower than control, resulting in an increased shade of orange in the sample.

According to the grades obtained, it was observed that the two samples of sausage containing natural antioxidant (REO and LRE) were accepted. However, the averages of sausages with synthetic antioxidants and control had higher values. Therefore, the panelists indicated that the pork sausage containing the synthetic

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antioxidants and control got better sensory acceptance than the sausages containing REO and LRE.

In study by Venturini et al. (2011) consumer acceptability for meat products is related to their color, odor, appearance, flavor and juiciness. Regarding the acceptability rate of the product, it was observed that treatment 3, with an addition of 0.2% rosemary essential oil, showed the lowest acceptance rate in the global impression of the product.

CONCLUSION

The LRE was more effective in the lipid oxidation inhibition in pork sausage than REO. However, this study confirms that the use of LRE and REO as a natural antioxidant, combined with storage under low temperatures, can be considered an effective method to retard lipid oxidation in pork sausage. This protection may be due to high antioxidant activity and the presence of phenolic compounds in LRE and REO. The sausage containing LRE obtained greater acceptance (75.67%) among the tasters than the sausage containing REO (56.41%). Considering the interest in finding natural antioxidants that have similar performance to synthetic antioxidants in meat products, rosemary showed promising results. The sausage with rosemary extract can be an alternative substitution for synthetic antioxidants, showing the possibility of using in meat products resulting with satisfactory acceptance.

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REFERENCES


