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Evaluation of natural extracts' antioxidant capacity for controlling fresh sausage oxidation

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Abstract

Lipid oxidation impacts the quality and shelf life of meat products. The use of natural antioxidants is an alternative to delay their oxidative deterioration. This study aimed to determine the antioxidant activity of natural extracts and evaluate the viability of replacing 2,3-tert-butyl-4-hydroxyanisole (BHA) in fresh sausages. Both tocopherols and grape seed extract showed similar antioxidant activity to BHA through the 2,2-diphenyl-1-picryl-hydrazyl-hydrate free radical (DPPH) and ferric reducing antioxidant power (FRAP) methods. The effectiveness of these antioxidants applied to fresh sausage was evaluated during 15 days of storage at 4.0°C under light incidence. The results indicated a pro-oxidant effect of grape seed extract (with 6.74% acidity and 2.74 mg MDA kg sample^{-1} thiobarbituric acid reactive substances (TBARS)) and showed the viability of replacing BHA with tocopherols. Tocopherols had a lower peroxide value (3.07 mE O_2 kg^{-1}) than the control (11.40 mE O_2 kg^{-1}), with no difference in TBARS or acidity content compared to BHA, and the lowest total mass loss (18.22%).

Keywords: rosemary; grape seeds; tocopherols; meat products; oxidative stability.

Practical application: Tocopherol mix can replace BHA as a preservative in meat products.

1 INTRODUCTION

Meat products are very susceptible to oxidation due to their chemical composition, richness in amino acids and lipids, and the mechanical and thermal processing to which the meat is subjected (Pereira et al., 2017). Lipid oxidation is recognized for reducing the quality of meat products and shortening their shelf life because of the formation of compounds that impact their taste, color, and odor (Kanner, 1994).

In light of this, antioxidants are an important resource to inhibit or delay the oxidative deterioration of foods in order to extend their shelf life (Oliveira et al., 2012). However, the safety of using synthetic antioxidants in the food industry, such as butyl hydroxyanisole (BHA) and butylhydroxytoluene (BHT), has been questioned. Thus, natural compounds, especially plant extracts, that exhibit this functionality have been studied (Del Ré & Jorge, 2012).

Among the plant extracts, rosemary is well known for its capability of controlling lipid oxidation. Carnosol and carnosic acid are primarily responsible for its antioxidant capacity (Fernández-López et al., 2005). Tocopherols exist naturally in many vegetable oils, and their antioxidant activity is due to their ability to interrupt the formation of hydroperoxides and the chain propagation of the reaction (Bertolin et al., 2011). Grape seed extract, obtained from by-products of wine or grape juice manufacturing processes, contains numerous phenolic compounds that can scavenge free radicals and act in synergy with other antioxidants (Yi et al., 2009).

Given the growing interest of consumers in products that deliver convenience, taste, texture, and healthiness, the food industry requires further research and innovation to meet consumer demand (Font-i-Furnols & Guerrero, 2014). The aim of this study was to determine the antioxidant potential of tocopherols mixture, rosemary, and grape seed extracts and find a natural alternative to replace the synthetic antioxidant BHA in fresh sausage.

2 MATERIALS AND METHODS

2.1 Samples

The Niagara grape seeds were supplied by a wine producer from Toledo city, Paraná, Brazil. The fermented seeds were separated from the peels and peduncles, washed, sun-dried, sieved, packed, and stored at room temperature. The grape seed extract was obtained according to Dalposso et al. (2021). The commercial rosemary extract (GNutra) was standardized to 3.5% of bioactive compounds; the commercial mixture of α, β, γ, and δ -tocopherols (BTSA) was standardized to 18% of bioactive compounds; and the BHA (Danisco) was standardized

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to 20% of bioactive molecules. The antioxidants, raw materials, and other ingredients were kindly provided by a meat processing company, located in Jundiaí, Brazil.

2.2 Reagents

The reagents used were methanol (Alphatec), acetone (Alphatec), petroleum ether (Anidrol), sodium acetate (Alphatec), Folin-Ciocalteu reagent (Dinâmica), sodium carbonate (Dinâmica), iso-octane (Dinâmica), phosphoric acid (Merck), ethyl alcohol (Alphatec), ether (Alphatec), potassium iodide (Neon), 2-thiobarbituric acid (TBA) (Êxodo científica), and trichloroacetic acid (Dinâmica). The standards 2,2-diphenyl-1-picrylhydrazyl (DPPH), (S)-6-methoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferrous sulfate, and gallic acid were purchased from Sigma-Aldrich.

2.3 Antioxidant activity

Considering the different mechanisms that lead to lipid oxidation in meat, the antioxidant ability of the natural extracts was analyzed using three methodologies: DPPH radical scavenging activity, total phenolic compounds, and ferric-reducing properties, which indicate their capacity for interrupting lipid peroxidation and chelating free iron.

2.3.1 DPPH radical scavenging activity

The DPPH radical scavenging capacity was determined using the methodology described by Bondet et al. (1997), with modifications proposed by Boroski et al. (2012).

2.3.2 Total phenolic compounds by Folin-Ciocalteau method

The method described by Singleton and Rossi Jr. (1965) to determine the concentration of total polyphenols was employed with some modifications. Initially, methanolic solutions of grape seeds (2.5 mg mL^{-1}) and rosemary (25 mg mL^{-1}) extracts, tocopherol mixture (2.5 mg mL^{-1}), and BHA (1.5 mg mL^{-1}) were prepared. The final solutions were prepared by adding 250 µL of the extracts, 250 µL of Folin-Ciocalteu reagent (diluted to 1:1 in Milli-Q water), 500 µL of sodium carbonate saturated solution, and 4.0 mL of Milli-Q water. The solutions were kept in the dark at room temperature for 25 min. Afterward, they were centrifuged for 10 min at 3000 rpm, and their absorbance was read at 725 nm using a UV-VIS spectrophotometer (PG Instruments Ltd., Model T 80+, Coventry, United Kingdom).

2.3.3 Ferric-reducing antioxidant properties

The reduction power was determined using the FRAP method, following the procedure described by Benzie and Strain (1996) and adapted by Dalposso (2018).

2.4 Fresh sausage manufacturing

The raw materials were stored at a refrigerated temperature of 4.0°C until they were processed. Pork ham and fat were ground and blended with a brine previously prepared with water, salt, sugar, seasonings, additives, and the antioxidants BHA, grape seed extract, and tocopherol mix, at the concentrations of 0.01% (w/w), 0.027% (w/w), and 0.024% (w/w), respectively. The antioxidants' dosage levels were defined according to the total phenolic compound results and the Brazilian regulations. The amount of each ingredient in the formulation was measured in an analytical scale. A control sample, without any antioxidants, was also prepared. The batches were homogenized for 2 min in a paddle mixer and stuffed in natural casings. The fresh sausages were packaged in low-density polyethylene plastic bags, manually sealed, and stored at -12°C. All samples were defrosted before further analysis.

2.5 Fresh sausage centesimal composition and pH

The moisture, protein, lipids, and ash contents were determined according to the methods described by AOAC (2019), ISO 1442 (1997), ISO 1871 (2009), and ISO 936 (1998), respectively. The value of pH was determined by the pH meter manual.

2.6 Oxidative stability during refrigerated storage

Lipid oxidation during the products' shelf life was measured by peroxide value, which demonstrates the initial stages of oxidation, and acidity and thiobarbituric acid reactive substances (TBARS), which evaluate the levels of the final products of lipid oxidation. Additionally, thermogravimetry, an accelerated method, was employed to analyze oxidative stability.

2.6.1 Peroxide value

Samples were analyzed using the methodology described by Instituto Adolfo Lutz (2008).

2.6.2 Acidity value

Acidity levels were measured using the methodology described by Instituto Adolfo Lutz (2008).

2.6.3 Thiobarbituric acid reactive substances

The determination of TBARS was performed using the procedures described by Raharjo et al. (1992) and modified by Wang et al. (2002). The TBARS were measured in a UV-VIS spectrophotometer (PG Instruments Ltd., Model T 80+) at 531 nm.

2.6.4 Thermogravimetric analysis

Thermogravimetric curves of fresh sausage samples were obtained using a simultaneous thermal analyzer (STA) (PerkinElmer 6000 Series, PerkinElmer Inc., São Paulo, Brazil). The non-isothermal tests were performed using samples of approximately 10 mg in an alumina crucible with an oxygen atmosphere, a flow rate of 20 ml min^{-1}, and a heating rate of 10 °C min^{-1}, ranging from 50 to 550°C.

2.7 Statistical analysis

The analyses were performed in triplicate, and the results were expressed as mean±standard deviation. The results were

submitted to the Kolmogorov-Smirnov and Liliefors normality test, followed by a comparison of the averages using Tukey's and Kruskal-Wallis tests (5% of probability) performed using Statistica (Statsoft, 10th version).

3 RESULTS AND DISCUSSION

3.1 Antioxidant activity

The results for the antioxidant activity of the plant extracts and synthetic compound BHA are presented in Table 1.

It is observed that the antioxidant capacity of rosemary extract, tocopherol mixture, grape seed extract, and BHA was significantly different in all methodologies applied, except for one of the tocopherols mix and grape seed extract in the DPPH radical scavenging technique. The differences in the antioxidant activities of the extracts are related to the concentration of total phenolic compound and can also be explained by different types of phenols present in the extracts, since the antioxidant activity depends on both the amount and position of hydroxyls in the molecule (Fukumoto & Mazza, 2000).

The results obtained showed that, after the synthetic standard BHA, the mixture of tocopherols presented the highest content of phenolic compounds and the best results for the analysis of DPPH and FRAP, followed by the grape seed extract. One factor that may have led to the better performance of the mixture of tocopherols and grape seed extract is that both are crude extracts, i.e., they have not undergone purification steps. Estévez (2021) emphasized that plant materials and their crude extracts have a more expressive and consistent antioxidant action than processed extracts. The author explained that these raw materials consist of a complex mixture of naturally occurring bioactive compounds that act through various mechanisms, resulting in a highly efficient biological protective effect on the meat matrix.

Pereira et al. (2017) evaluated the antioxidant activity of commercial α-tocopherol, revealing a value of 61.22±0.06 μg mL^{-1} for the DPPH EC_{50} method and 1328.80±22.09 μmol Fe^{+2} g^{-1} for FRAP. These results are similar to those obtained for the tocopherol mix analyzed in this study. Variations may be related to the composition of commercial ingredients, such as the concentration and types of tocopherols present in the extract.

In contrast, the results obtained for the grape seed extract differ from those obtained by Dalposso et al. (2021): phenolic compounds of 179.97 mg EAG g $extract^{-1}$, FRAP 3449.33 μmol $EFeSO_4$ g $extract^{-1}$, and DPPH 1144.00 μmol TE g $extract^{-1}$; Casarotto (2013): phenolic compounds of 9670.1 mg EAG L^{-1} extract and 83.3% inhibition of DPPH; and Shirahigue et al. (2010): phenolic compounds of 430.55 mg EAG 100 g^{-1} extract. The differences between the results obtained in these studies can be explained by the various grape varieties analyzed (Bordô, Merlot, Isabel, and Niagara), as well as different wine fermentation processes adopted by the producers. In addition, the method adopted to obtain the extracts can impact the efficiency of extracting the active compounds (Casarotto, 2013; Dalposso et al., 2021). Additionally, storage of the samples may lead to the degradation of antioxidant compounds in grape seeds, resulting in a reduction of antioxidant activity (Carvalho, 2020).

Finally, rosemary extract was the natural antioxidant that showed the lowest capacity for capturing the DPPH radical (33.36 μmol ET g $sample^{-1}$) and the ability to reduce iron (125.82 μmol $EFeSO_4$ g $sample^{-1}$). These results can be explained by the low content of total phenolic compounds (4.10 mg EAG g $sample^{-1}$), which are the substances responsible for the antioxidant action verified in this sample. The rosemary extract analyzed presented different results from those achieved by Martínez et al. (2019), who reported 35.95 mg EAG g $sample^{-1}$ for the content of total phenolic compounds, 88.76% of DPPH radical chelating activity, and 73.43 mg EAG g $sample^{-1}$ for FRAP in rosemary extract samples with lecithin as an emulsifier. Saini et al. (2020) reported 136.66±7.41 mg EAG g $sample^{-1}$ for the content of phenolic compounds, 32.17±1.12 mg EAG g $sample^{-1}$ for FRAP, and 40.76±2.81 μg mL^{-1} for the capacity of DPPH radical capture when evaluating the crude rosemary extract. This extract was produced by the processes of grinding the leaves, extraction with solvent, and concentration of the extract. This variation in the results can be explained by different concentrations of rosmarinic acid and diterpenes (carnosic acid and carnosol) in the extracts that were analyzed, which might be due to the soil and climatic conditions where rosemary was cultivated, as well as post-harvest conditions and methods of extracting active compounds from the plant (Del Ré & Jorge, 2012). In fact, a large variability is observed among the results of total phenolic compounds content in published articles (Martínez et al., 2019; Pereira et al., 2017; Saini et al., 2020). Moreover, commercial rosemary extract undergoes purification steps to remove flavoring compounds such as monoterpenes, which can impact the sensory profile of the products (Amaral et al., 2018; Damodaran et al., 2010). As a result, there could be a loss of some bioactive compounds when compared to crude extracts. Moreover, for commercialization, vegetable oil is used as a carrier for the rosemary extract. Then, it is estimated that higher application dosages might be necessary for better results. Because the rosemary extract showed the lowest antioxidant capacity when compared to the other antioxidants, it was not used in the further studies.

Table 1. Results obtained for the antioxidant activity of natural extracts and BHA*.

Analysis	Rosemary extract	Tocopherol mixture	Grape seed extract	BHA
DPPH (μmol ET g $sample^{-1}$)	33.36±0.10[a]	472.89±10.42[b]	275.71±2.03[b]	1271.00±183.58[c]
Phenolic compounds (mg EAG g $sample^{-1}$)	4.10±0.19[a]	30.48±0.13[b]	27.40±0.25[c]	72.68±1.14[d]
FRAP (μmol $EFeSO_4$ g $sample^{-1}$)	125.82±3.15[a]	1065.25±11.01[b]	665.87±6.92[c]	1446.11±41.68[d]

*Means±standard deviation, followed by the same letters in the same line, do not differ significantly (p>0.05) from each other by the Tukey's test. Analyses were performed in triplicates.

3.2 Characterization of fresh sausage

The results for centesimal composition (moisture, proteins, lipids, and ash) and pH of fresh sausage samples are presented in Table 2.

The Brazilian regulation establishes that fresh sausages must contain a minimum protein content of 12% and maximum levels of moisture and fat of 70 and 30%, respectively (Brasil, 2000). Therefore, the sausage samples prepared for the evaluation of oxidative stability are in accordance with current legislation. The addition of natural extracts and BHA did not interfere with the main parameters of the composition of fresh sausages since there was no statistical difference between the results of the samples for moisture, fat, and proteins. Similarly, the pH values of the samples produced with different antioxidants showed no significant difference, indicating that the antioxidants did not impact the pH of fresh sausages. The pH values of around 6.20 are similar to the pH of the meat and to the values mentioned by Pires (2014), which varied from 5.97 to 6.03.

3.3 Oxidative stability monitoring

Fresh sausage samples were analyzed at 0, 4, 7, 11, and 15 days of cold storage (4°C) under light incidence. The peroxide values measured during fresh sausages' shelf life are shown in Table 3.

The higher peroxide values at the beginning of the study indicate that the oxidation reactions had possibly started already in the stages of obtaining the raw material and manufacturing the sausage. The formation of hydroperoxides occurs through a complex process in which unsaturated fatty acids react with molecular oxygen initiators, resulting in the formation of free radicals. In the propagation step, a series of chain reactions happen through lipid-lipid interactions, increasing the formation of radicals. The peroxy radical extracts hydrogen from an adjacent lipid, forming a hydroperoxide (Amaral et al., 2018). These hydroperoxides are highly unstable and decompose into other compounds such as aldehydes, ketones, and alcohols, which are responsible for changes in taste and odor (Cheng, 2016).

Between the 4th and 11th day of storage, there was certain stability in the results of the peroxide index, indicating a balance between the formation and decomposition rates of peroxides, except for the sample containing grape seed extract, which showed representative variations between days 4 and 7. Casarotto (2013) explained that the increase in the peroxide index is probably due to a rate of peroxide formation higher than degradation. The drop in peroxide levels is related to the decomposition of hydroperoxides into other compounds (Feiner, 2006). During the period of evaluation (0–15 days), a significant reduction of about 80% was observed in the peroxide index for the samples with BHA and tocopherols. The main contributors to the breakdown of lipid hydroperoxides in foods are heme and non-heme iron (Erickson, 2002). Heme iron is present in fresh sausages and is released during the meat cutting and grinding process. It can therefore act as an accelerator for the decomposition of hydroperoxides.

Bertolin et al. (2011) measured the peroxide index for beef jerky samples added with natural antioxidants and also observed a subsequent reduction of peroxides, evidencing their degradation in secondary oxidation products such as aldehydes, ketones, and alcohols. Analogously, Casarotto (2013) observed an increase followed by a decrease in the peroxide index of sausages during the evaluated period. The author observed that natural antioxidants had lower peroxide levels only in specific periods of shelf life, as observed in this study. Specifically, at 4, 7, and 15 days, the sample with mixed tocopherols showed significantly lower peroxide levels ($p<0.05$) than the sample containing BHA.

The acidity levels verified in the samples during 15 days of refrigerated storage are shown in Table 4.

The results of the acidity index at the end of the evaluated period varied between 4.85 and 6.74% for the fresh sausage samples. Kaipers (2017) found similar acidity values after 15 days

Table 2. Mean centesimal composition and pH of fresh sausages*.

Samples	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	pH
Control	69.57±0.3^{a}	10.43±0.25^{a}	17.24±0.42^{a}	2.84±0.12^{ab}	6.19^{a}
BHA	69.20±0.36^{a}	10.00±0.61^{a}	17.44±0.43^{a}	2.93±0.19^{b}	6.19^{a}
Tocopherol mixture	69.63±1.01^{a}	10.30±0.82^{a}	17.80±0.87^{a}	2.91±0.18^{b}	6.22^{a}
Grape seed extract	70.00±0.06^{a}	9.53±0.35^{a}	17.50±0.32^{a}	2.49±0.12^{a}	6.21^{a}

*Means of proximate composition results±standard deviation and pH. The same letters in the same column indicate that there is no significant difference at $p>0.05$ assessed by the Tukey's test.

Table 3. Average peroxide levels during the shelf life study*.

Sample	Storage time (days) at 4°C				
	0	4	7	11	15
Control	12.10^{aA}±0.17	1.75^{aB}±0.15	2.50^{bAB}±0.00	2.00^{aAB}±0.40	11.40^{dAB}±0.00
BHA	29.90^{bA}±3.40	11.50^{cAB}±0.00	7.90^{dAB}±0.00	8.70^{cAB}±1.70	5.40^{bB}±0.00
Tocopherol mixture	29.10^{bA}±2.60	6.05^{bAB}±0.05	5.10^{cAB}±0.00	9.47^{cAB}±0.50	3.07^{aB}±0.06
Grape seed extract	6.85^{aA}±0.75	13.10^{cAB}±2.20	2.03^{aAC}±0.06	5.80^{bA}±0.00	7.43^{cA}±0.02

*Means±standard deviation, followed by the same letters in the same column, do not differ significantly from each other by the Tukey's test. Different capital letters in the same line indicate a statistical difference by the Kruskal-Wallis test ($p<0.05$). Analyses were performed in triplicates.

of storage of colonial sausages added with rosemary extract and sodium erythorbate, with acidity ranging from 3.5 to 6%.

There was an increase in the acidity value between time 0 and the 15th day of the shelf life for samples containing the mixture of tocopherols and grape seed extract. Similarly, Singh et al. (2018) found that the storage period resulted in a significant increase in free fatty acids while comparing the initial time to 12 days of storage in samples of buffalo veal slices treated with grape seed extract (addition of 0.1–0.2%).

This increase in the acidity index is explained by the advance in hydrolytic rancidity reactions, which increase the content of free fatty acids in food. Hydrolytic rancidity is the result of the hydrolysis of the ester bond by lipase enzymes or chemical agents (acids/bases) that, in the presence of moisture, break the ester bond of lipids, releasing fatty acids (FIB, 2014). Moreover, the increase in the acidity content of foods might be related to the production of acids, which are secondary products of lipid oxidation. It is known that the presence of free fatty acids from triacylglycerols accelerates lipid oxidation reactions (Erickson, 2002).

The synthetic antioxidant BHA was able to control these factors that lead to an increase in the acidity content of foods since the acidity index of this sample did not show a significant difference over the evaluated period, ranging from 4.08 to 4.92%. In contrast, the natural extracts were not as efficient as BHA in reducing the speed of these reactions, and a pro-oxidant effect of grape seed extract was observed, which had the highest acidity content among all samples (6.74%) at the last point of analysis.

Carvalho (2020) also identified a pro-oxidant effect of Bordô grape extract in oils, increasing the acidity value during storage. The author attributed the increase in oil acidity to extract composition and dosage. Palade and Chedea (2016) stated that grape seed extracts may have pro-oxidant activity depending on their structural characteristics, the concentration used, pH, and interactions with other components of the food matrix such as metals and the enzyme lipoxygenase. Estévez (2021), Palade and Chedea (2016), and Schaich (2017) indicated that high doses and contact time can provoke a pro-oxidant action in natural and synthetic antioxidants. The authors also reported that there is a relationship between the concentrations of metals and matrix chelators with the pro or antioxidant effect. Also, the pro-oxidant effect evidenced in the sample containing the grape seed extract may be related to the increase in the solubility of metal ions in lipids, which is caused by the reduction of their polarity when they bind to antioxidants (Schaich, 2017).

The results for TBARS are presented in Table 5.

The results for TBARS analysis showed an increase in the amounts of malonaldehyde (MDA) per kg of sample in all treatments until the 11th day of cold storage. Cava (2007) also verified an increase in TBARS levels in pork sausages during shelf-life studies. Malonaldehydes are secondary oxidation products and the main products of hydroperoxides of polyunsaturated fatty acid decomposition (Osawa et al., 2005). Therefore, they are not present in the initial evaluation stages, and their quantity increases with reaction time.

Even though TBARS levels increased in all samples, they were more expressive in the control and in the sample with the addition of grape seed extract. From the second point of analysis (4 days), there was a significant difference between the results of the sample containing the grape seed extract and the others. Grape seed extract showed a pro-oxidant effect for half of the shelf life (7 days). These results reinforce those obtained in the acidity analysis and suggest that at a concentration of 0.027%, the grape seed extract presents pro-oxidant activity for fresh sausages.

Between the 11st and 15th days of the study, there was a decrease in the TBARS values in the samples containing BHA and the mixture of tocopherols. This is due to the fact that malonaldehydes can undergo reactions and be oxidized to other molecules, which do not react with thiobarbituric acid.

Table 4. Means of acidity index results (%) in fresh sausage samples*.

Sample	Storage time (days) at 4°C				
	0	4	7	11	15
Control	5.06^{bA}±0.11	4.00^{aAB}±1.01	4.83^{cA}±0.00	4.49^{aB}±0.86	4.85^{aA}±0.12
BHA	4.08^{abA}±0.88	4.76^{aA}±0.63	4.62^{bA}±0.12	4.47^{aA}±0.44	4.92^{aA}±0.12
Tocopherol mixture	4.31^{aA}±0.59	4.44^{aAC}±0.32	5.47^{dBD}±0.00	4.72^{aC}±0.27	5.34^{bD}±0.01
Grape seed extract	4.56^{abA}±0.22	4.25^{aA}±0.35	3.97^{aB}±0.00	4.06^{aB}±0.12	6.74^{cC}±0.12

*Means±standard deviation, followed by the same lowercase letters in the same column, do not differ significantly from each other by the Tukey's test ($p<0.05$). Different capital letters on the same line indicate statistical differences by the Tukey's test ($p<0.05$). Analyses were performed in triplicates.

Table 5. Average amounts of malonaldehyde (mg per kg of the sample) TBARS*.

Sample	Storage time (days) at 4°C				
	0	4	7	11	15
Control	0.000^{a}±0.000	0.112^{a}±0.005	0.361^{b}±0.004	0.767^{b}±0.001	1.319^{b}±0.001
BHA	0.039^{b}±0.002	0.147^{a}±0.004	0.242^{a}±0.003	0.473^{a}±0.004	0.053^{a}±0.006
Tocopherol mixture	0.000^{a}±0.000	0.259^{c}±0.006	0.277^{a}±0.001	0.441^{a}±0.001	0.256^{a}±0.002
Grape seed extract	0.000^{a}±0.000	0.203^{b}±0.003	0.708^{c}±0.002	1.598^{c}±0.005	2.740^{c}±0.046

*Means±standard deviation, followed by the same letters in the same column, do not differ significantly from each other by the Tukey's test. Analyses were performed in triplicates.

Bragagnolo et al. (2005), Liu et al. (2010), Osawa et al. (2005), and Pereira et al. (2017) also explained that malonaldehydes can bind to meat proteins, producing stable compounds, and as a result, they are not identified by the method.

Moreover, there was no significant difference between the TBARS results for samples treated with BHA and a tocopherol mixture on the 7th day of the shelf life of fresh sausages. Thus, it can be concluded that this natural antioxidant had comparable efficiency to that of BHA in the control of lipid oxidation. Similarly, Resurreccion and Reynolds (1990) concluded that tocopherols (addition of 0.1% in relation to fat content) were as effective as BHA/BHT (addition of 0.02% in relation to fat content) in preventing oxidation in frankfurter sausages through TBARS analysis.

The thermogravimetric curves of the samples are shown in Figure 1.

Table 6 illustrates the temperature ranges at which mass losses occur in fresh sausages and their respective percentages of mass loss.

The peaks obtained in the analysis are referenced by numbers 1 to 7, and the residual mass by RM.

From the analysis of the thermogravimetric curves and the percentages of mass loss in each stage, it is possible to infer that the thermal decomposition of the fresh sausage samples took place in three main stages: dehydration, fat degradation, and carbonization of the residual mass. Chen et al. (2017) and Lazzarotto et al. (2014) also identified these steps when evaluating residues from oilseed plants and pine resin using thermogravimetry.

The mass loss that occurred in the first steps correlates with the moisture content of each sample. Discreet variations may be related to the heterogeneity of the sausage segments, intrinsic to the manufacturing process, and the release of some volatile compounds of lower molecular weight already in the initial stages of the analysis.

In the second stage, the degradation of free fatty acids and triacylglycerols occurs. In terms of total mass loss in the fat degradation stages, all samples added with antioxidants

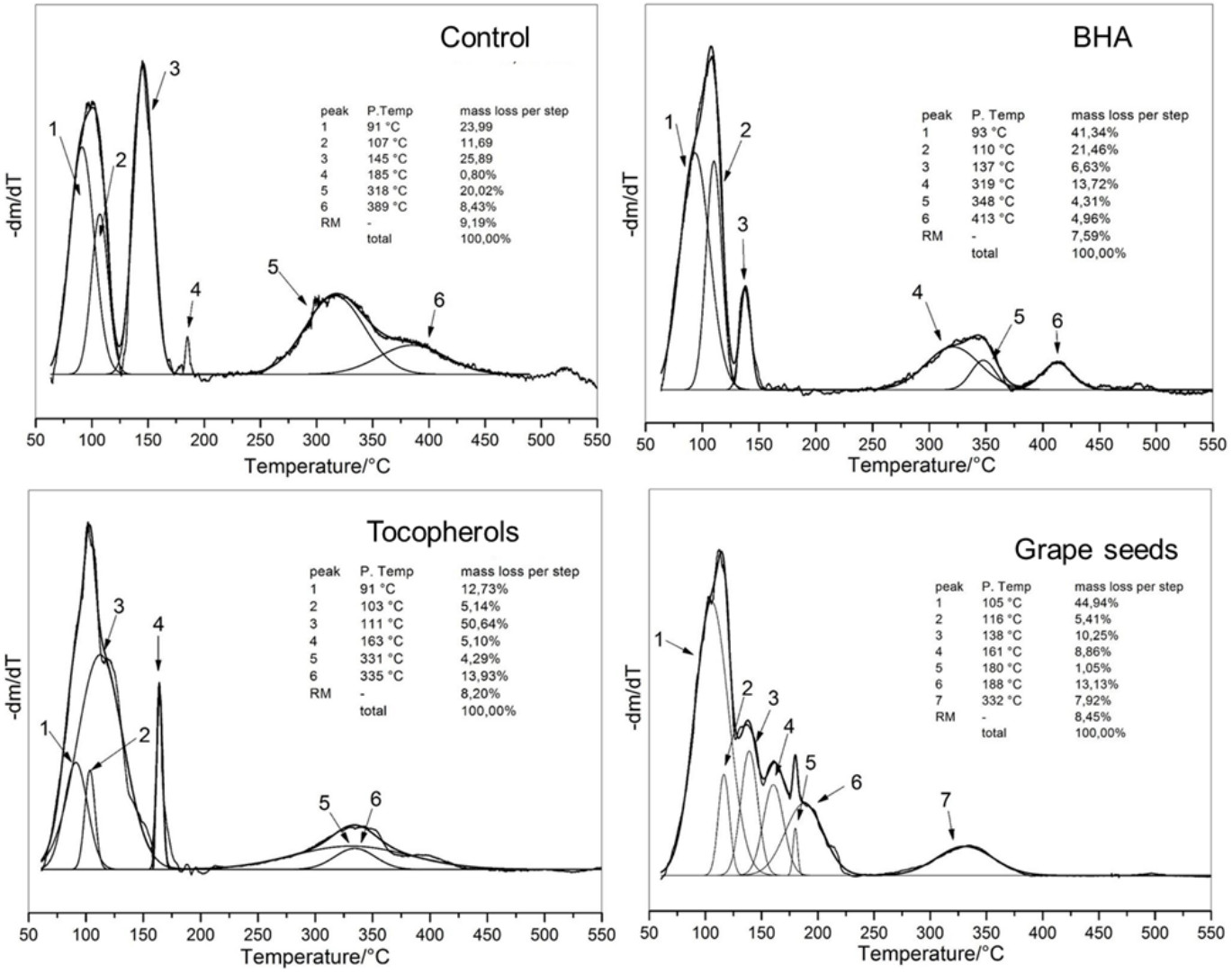

Figure 1. Thermogravimetric analysis of fresh sausage samples with antioxidants.

Table 6. Thermal events identified in the thermogravimetric analysis of fresh sausages.

Sample	Steps	Temperature (°C)	Mass loss (%)
Control	1	91 – 107	23.99
	2	107 – 145	11.69
	3	145 – 185	25.89
	4	185 – 318	0.80
	5	318– 389	20.02
	6	389 – 550	8.43
	RM	-	9.19
BHA	1	93 – 110	41.34
	2	110 – 137	21.46
	3	137– 319	6.63
	4	319 – 348	13.72
	5	348 – 413	4.31
	6	413– 550	4.96
	RM	-	7.59
Tocopherol mixture	1	91 – 103	12.73
	2	103 – 111	5.14
	3	111 – 163	50.64
	4	163– 331	5.10
	5	331 – 335	4.29
	6	335 – 550	13.93
	RM	-	8.20
Grape seeds extract	1	105 – 116	44.94
	2	116 – 138	5.41
	3	138 – 161	10.25
	4	161– 180	8.86
	5	180 – 188	1.05
	6	188 – 332	13.13
	7	332– 550	7.92
	RM	-	8.45

performed better than the control, showing total loss percentages of 22.99, 18.22, and 21.05% for BHA, mixed tocopherols, and grape seed extract, respectively. In addition, Gennaro et al. (1998) and Marinho (2012) explained that the higher the temperatures at the onset of thermal decomposition, the longer the predicted lifetimes of the samples. The curves obtained in the thermogravimetric analyses allow the identification of the initial temperatures of degradation of the fats, which were 238, 250, 285, and 250 °C for the control samples, with the addition of BHA, the mixture of tocopherols and grape seed extract, respectively. Therefore, all antioxidants demonstrated the ability to increase the presumed shelf life of fresh sausage compared to the control sample.

4 CONCLUSION

Rosemary extract was the natural antioxidant that showed the lowest antioxidant capacity in the phenolic compounds, DPPH, and FRAP methods among all samples studied. Considering that it is the natural antioxidant mostly used by the meat industry, this study showed that it is important to carefully validate the dosage levels applied in terms of functionality and also that there are already other more effective alternatives on the market to replace synthetic antioxidants and keep products' quality during shelf life.

Grape seed extract, despite its prominent antioxidant activity, demonstrated a pro-oxidant action on fresh sausage, accelerating the production of substances reactive to thiobarbituric acid and increasing the acidity index of the samples.

The tocopherol mixture showed significantly lower peroxide levels than the sample containing BHA at 4, 7, and 15 days, presented similar TBARS values, and had the lowest total mass loss among the samples, indicating that this natural antioxidant has comparable efficiency to the synthetic one in controlling oxidation lipid.

The results showed the effectiveness of the tocopherol mixture in increasing the oxidative stability of fresh sausage. Therefore, it can be used as an alternative to replace synthetic BHA, maximize the product's shelf life, and deliver more healthy and clean labelled products.

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