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SYNERGISTIC EFFECTS OF NATURAL ANTIOXIDANTS FROM PLANT EXTRACTS ACEROLA, ROSEMARY, AND OLIVE IN BEEF PATTIES

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ABSTRACT

An increasing consumer trend towards “clean-label” foods (products with few easily understandable ingredients) is becoming more prevalent as consumers prioritize transparency, health, and natural ingredients in their food choices. “Clean-labels” may refer to the absence or reduction of a certain ingredient perceived as harmful (for example, sugar-free). In this context, the use of natural antioxidants as an alternative to food preservation has been of growing interest as consumers become more informed about the potential health hazards of synthetic additives, particularly in meat. The use of natural antioxidants could provide synergistic benefits for the overall quality and shelf-life of the meat.

Beef patties, a commonly consumed type of ground meat, undergo oxidative changes and develop rancidity more rapidly than an intact muscle. Sample beef patties were formed and used to test the blend of plant extracts namely, acerola, rosemary, and olive (ARO) at 0.4% concentration in comparison to negative (NC) and positive controls (PC) in determining the shelf-life and overall quality.

The Direct Analysis in Real Time – High-Resolution Mass Spectrometry (DART-HRMS) was used to identify the bioactive compounds in individual plant extracts. Results showed the presence of several detected compounds with antioxidative properties and few antimicrobial effects. Focusing on the antioxidant activity and its effect on shelf-life, lipid oxidation analyses, measuring the peroxide values (PV) and thiobarbituric acid reactive substances (TBARS) values, were conducted. Additionally, photo-oxidation through color assessment, and the pH measurement per time point were determined to evaluate shelf-life. Results showed that beef patties with ARO significantly delayed the oxidation rate, both lipid and photo-oxidation. The ARO blend exhibited the lowest pH and most prolonged the redness of patties throughout the period, attributed to the presence of organic compounds. Moreover, ARO was observed to delay

microbial growth due to its antimicrobial properties compared to the controls, suggesting its effectiveness in preventing microbial spoilage. Furthermore, sensory analysis was performed to test the overall acceptability of the ARO treated and untreated beef burgers. The ARO treated burgers were perceived as the most acceptable among the panel of judges. Therefore, the formulated blend of plant extracts could be a natural solution for preserving meat.

KEYWORDS: Synergistic effects, Natural antioxidants, Bioactive compounds, Oxidation, Shelf-life, Acerola, Rosemary, Olive, Beef burger

RIASSUNTO

Un crescente trend dei consumatori verso alimenti "clean-label" (prodotti con pochi ingredienti facilmente comprensibili) sta diventando sempre più diffuso, poiché i consumatori danno priorità alla trasparenza, alla salute e agli ingredienti naturali nelle loro scelte alimentari. Le “clean-label” possono riferirsi all’assenza o alla riduzione di un determinato ingrediente percepito come nocivo (ad esempio senza zuccheri). In questo contesto, l'uso di antiossidanti naturali come alternativa alla conservazione degli alimenti ha suscitato crescente interesse poiché i consumatori diventano più informati sui potenziali rischi per la salute degli additivi chimici di sintesi, in particolare nella carne. L'uso di antiossidanti naturali potrebbe fornire benefici sia in relazione alla qualità complessiva sia per la durata di conservazione della carne.

I burger di manzo, un tipo comune di carne macinata, subiscono cambiamenti ossidativi e sviluppano più rapidamente la rancidità rispetto alla carne intatta. In questo lavoro di tesi, sono stati ottenuti campioni di burger di manzo, utilizzati per testare una miscela di estratti vegetali, ovvero acerola, rosmarino e oliva (ARO), alla concentrazione dello 0,4% in confronto ai controlli negativi (NC) e positivi (PC) per determinare sia la shelf life che la qualità complessiva del prodotto ottenuto.

La- Spettrometria di massa ad alta risoluzione in tempo reale (DART-HRMS) è stata utilizzata per identificare i composti bioattivi nei singoli estratti vegetali. I risultati ottenuti hanno evidenziato che diversi composti rilevati hanno proprietà antiossidanti e alcuni proprietà antimicrobiche. Concentrandosi sull'attività antiossidante e sul suo effetto sulla shelf life, sono state condotte analisi dell'ossidazione lipidica misurando i valori dei perossidi (PV) e delle sostanze reattive dell'acido tiobarbiturico (TBARS). Inoltre, sono state determinate la foto-ossidazione attraverso la valutazione del colore e la misurazione del pH I risultati hanno mostrato che i burger di manzo con ARO hanno mostrato un tasso di ossidazione, sia lipidica

che foto-ossidativa, significativamente ritardato. La miscela ARO ha mostrato un pH più basso e una maggiore persistenza del colore rosso dei burger per tutto il periodo, effetto attribuibile alla presenza di composti organici. Inoltre, si è osservato che l'ARO ha ritardato la crescita microbica rispetto ai controlli, suggerendo la sua efficacia nella prevenzione dello deterioramento microbico. Inoltre, è stata effettuata un'analisi sensoriale per testare l'accettabilità complessiva dei burger di manzo e i burger con ARO sono stati percepiti come i più accettabili tra i membri del panel di valutazione. La miscela di estratti vegetali qui proposta potrebbe essere una soluzione naturale per la conservazione della carne.

INTRODUCTION

1.1 Consumer Trends Influencing Meat Consumption

Consumer preferences are undergoing significant changes in the meat industry. Some of the emerging trends shaping the meat sector, include a focus on “clean-labels” and the use of natural ingredients, convenience, health and nutrition, and flavor infusions.

1.1.1 Clean label trend and use of natural ingredients

More and more consumers are reading food labels before purchasing, and many prefer products with few easily understandable ingredients: “clean-labels”. “Clean labels” may refer to the absence or reduction of a certain ingredient perceived as harmful (sugar-free). There are many other claims related to the absence or low content of certain substances in foods. In addition to nutritional claims, there are other types of “clean-labels”: - palm oil-free, - glyphosate-free, - additives-free etc.

Consumers are increasingly demanding “clean-label” foods and the use of natural additives, as synthetic additives are linked to potential health hazards. The presence of synthetic additives in meat products such as nitrites, sulphites, butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), is a concern for consumer health. Therefore, substituting synthetic additives with natural alternatives, particularly those rich in polyphenols, offers a promising approach to preserving food quality, especially in meat products. This substitution provides consumers with healthier and safer meat alternatives (Munekata et al., 2020). Consequently, the meat industry must offer processed products that meet consumer demand for transparency and natural ingredients. These products should be labeled as "clean-label" or "free from" artificial additives and ingredients, while maintaining the same high standards for safety and quality (Sayas-Barberá et al., 2020).

Aligning with the “clean-label” trend is the use of natural antioxidants, which are gaining popularity as consumers seek healthier meat choices. These natural ingredients preserve meat quality, improve shelf life, and enhance flavor which gives similar effect when compared to artificial preservatives. However, it is important to consider the impact of using natural antioxidants as their combinations may lead to unfavorable sensory characteristics, adversely affecting the quality of the products.

Shahidi and Ambigaipalan (2015) stated that the safety concerns on the use of synthetic antioxidants have shifted the focus towards the use of natural antioxidants, which are primarily sourced from plant phenolics. These compounds can be found in various plant parts, including fruits, seeds, leaves, and roots. Notably, naturally occurring antioxidant compounds include flavonoids, phenolic acids, lignans, terpenes, tocopherols, phospholipids, and polyfunctional organic acids. These compounds have the potential to protect against various diseases such as cardiovascular diseases, cancer, type 2 diabetes, and neurological conditions. Given this, consumers would generally prefer natural antioxidants because these are associated with health benefits.

1.1.2 Foods with convenience

The increasing preference for convenience among consumers, particularly millennials and urban populations, has driven a surge in demand for ready-to-eat and ready-to-cook meat products. As a result, the market has witnessed a proliferation of flavored and pre-seasoned meat options (FHA-Food & Beverages, 2024). Additionally, convenience for consumers is associated with the price and value of the food product. According to a report by Nelson (2024), younger generations have distinct shopping values and are mindful of their grocery spending but will not compromise on quality or personal values.

Sayas-Barberá et al. (2020) indicated that numerous studies linked meat consumption to an increased risk of health issues like heart disease and certain cancers. Despite these findings and growing health consciousness, burgers remain a popular choice worldwide, especially among young people. Thus far, meat consumption fulfills the daily protein needs of half the global population (Straits Research as cited by FHA-Food & Beverages, 2024).

1.1.3 Health and nutrition focus

Another consumer trend is on the emphasis of healthy eating that led to an increase for high-quality meat products demand. Consumers are increasingly seeking ethically sourced options that offer nutritional benefits and are easy to prepare. Food manufacturers adhere to food safety regulations and clearly communicate a product's nutritional value to ensure consumers make informed choices (FHA-Food & Beverages, 2024). Additionally, the meat industry is creating value-added products in response to consumer demand for healthier options. These functional meats are enriched with vitamins, minerals, and beneficial compounds to cater to diverse dietary preferences and health goals (Etchem & Etchem, 2024).

The developed functional food products contain bioactive compounds that can provide additional health benefits (Hygreeva et al., 2014) beyond basic nutrition and can address specific health concerns of the consumers. Furthermore, the nutritional content of food products can be preserved, minimizing oxidative stress-related health risks, by using natural antioxidants (Petcu et al., 2023).

1.1.4 Flavor infusions

A new consumer trend is the demand for tastier meat. Consumer preferences are evolving, with a pronounced shift towards more flavorful meat products. While the desire for convenience remains, there's a growing demand for a wider range of meat options like salami, ham, sausages, and bacon. Global culinary influences have also contributed to this trend, with the incorporation of herbs, marinades, and spices to create distinctive tastes. As a result, meat producers are increasingly exploring techniques to enhance flavor and meet consumer expectations. This surge in flavor innovation is driving growth in meat preservation and product development (FHA-Food & Beverages, 2024).

1.2 Beef as A Consumer Demand

Beef contains proteins with high nutritional values, and it is widely consumed globally (Soares et al., 2021). Consumer demands on meat and its products regards low level of fat, cholesterol, sodium chloride and nitrite concentrations but an improved fatty acid profile composition. Beef is observed to be rich in saturated lipids. However, it also contains polyunsaturated fatty acids (PUFAs) such as omega -3 (n-3) fatty acids that are involved in brain and retinal development and prevention of human diseases. Additionally, it contains conjugated linoleic acid (CLA), a type of omega-6 (n-6) fatty acid, which has the potential to protect against cancer, heart disease, diabetes, and obesity, while also strengthening the immune system. Depending on several factors including the cow’s diet, the content of n-3 fatty acid varies. Increasing n-3 fatty acid levels in meat can be advantageous; however, it presents a challenge due to the heightened susceptibility of these products to lipid oxidation, which can lead to an easier product deterioration. Consequently, an increase in n-3 fatty acid content within beef has been associated with the development of undesirable off-flavors and rancidity (Realini et al., 2015).

Like any meat, beef is subjected to chemical, physical, and microbiological changes. However, given its particularities, beef requires careful handling and storage as asserted by Soares et al. (2021).

Meat burger is a global food commodity, and it has intensified the need for effective shelf-life extension strategies, due to its susceptibility to spoilage (Incoronato et al., 2014). Transforming meat into products such as beef burgers typically maintains the original nutritional profile, but the processing imparts distinct sensory qualities like color, flavor, and aroma (Soares et al., 2021). A burger is typically ground meat molded into a patty, and according to Realini et al. (2015), ground meat is more susceptible to oxidation and rapid rancidity compared to an intact muscle. This increased susceptibility is due to the grinding process, which increases the surface area of the meat, thereby intensifying oxidation. Oxidative processes degrade lipids and proteins that eventually lowers the quality of the meat and nutritional content; hence, treatments with antioxidants such as vitamin E and vitamin C were observed to reduce pigment and lipid oxidation in meats. Lee et al. (2006) as cited by Realini et al. (2015) indicated that using a blend of antioxidants would be more effective in preserving n-3 fatty acids in meat products compared to a single antioxidant.

1.3 Lipid Oxidation

Lipid oxidation, or oxidative rancidity, is the deterioration of lipids through their reaction with oxygen, and this process deteriorates food quality. There are three main types of lipid oxidation: autoxidation, involving triplet oxygen ($^3O_2$); photo-oxidation, involving singlet oxygen ($^1O_2$); and enzymatic oxidation catalyzed by lipoxygenase (Kontogiorgos, 2021).

1.3.1 Autoxidation

Autoxidation is the primary mechanism by which lipids deteriorate in food (Abeyrathne et al., 2021). It is a chain reaction initiated by highly reactive molecules called free radicals. These

unstable molecules easily oxidize other compounds, causing oxidative damage. The process is divided into three stages: initiation, propagation, and termination (Kontogiorgos, 2021). In the initiation stage, free radicals removed susceptible hydrogen atoms from the methylene group of PUFAs. This initiates a molecular rearrangement (diene conjugation), thereby creating a more stable fatty acid radical. Pro-oxidants, commonly reactive oxygen species (ROS), accelerate lipid oxidation by either directly reacting with unsaturated fats to form lipid hydroperoxides or indirectly by stimulating the formation of free radicals. During the propagation stage, these conjugated dienes react with oxygen forming highly reactive peroxyl radicals. Afterwards, these peroxyl radicals react with additional fatty acids, generating hydroperoxides and new alkyl radicals, which starts a new oxidation cycle. Hydroperoxides break down into various compounds, including aldehydes, ketones, hydrocarbons, furans, and acids, which adversely impact food quality. These compounds contribute to off-flavors in oils, altered melting points of triglycerides, color changes, the formation of toxic substances, and loss of nutritional value. The cycle will continue until termination stage when the unstable peroxyl radicals become unreactive molecules (Abeyrathne et al., 2021 & Kontogiorgos, 2021). A summary of autoxidation mechanisms is shown in Figure 1 (Abeyrathne et al., 2021).

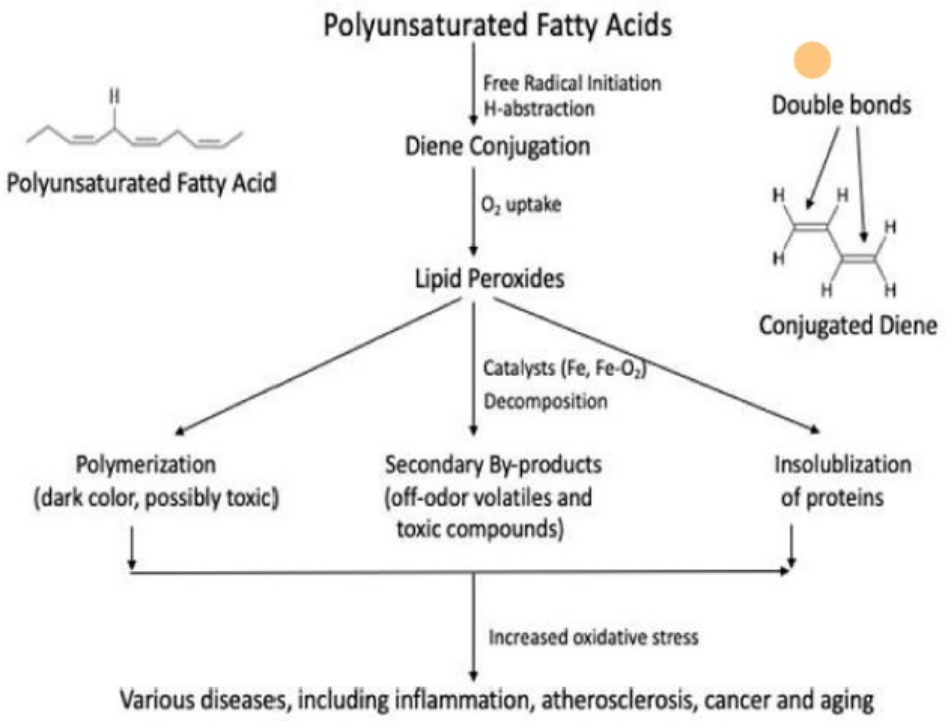


Figure 1. A schematic diagram of autoxidation and its produced products (from (Abeyrathne et al., 2021).

1.3.2 Photo-oxidation

Photo-oxidation involves a singlet oxygen for oxidation (as shown in Figure 2) and it is more reactive than triplet oxygen. It requires light and photosensitizers, which are compounds that absorb light and then convert $^3O_2$ to $^1O_2$. This reactive oxygen directly attacks the unsaturated bonds in fatty acids, leading to the formation of hydroperoxides. Photosensitizers like chlorophyll and riboflavin are naturally present in many raw foods and are difficult to remove (Kontogiorgos, 2021).

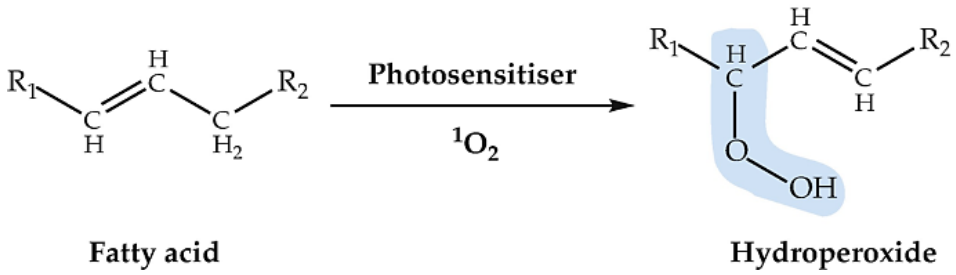


Figure 2. A schematic diagram of photo-oxidation.

Autoxidation and photo-oxidation differ significantly. Unlike autoxidation, photo-oxidation does not involve free radicals and an initial induction period. Additionally, it is less influenced by factors such as temperature, fatty acid composition, and oxygen levels. The hydroperoxides produced by these two processes differ, leading to distinct breakdown products. This distinction aids in identifying the primary oxidation mechanism. While antioxidants are effective against autoxidation, they offer limited protection against photo-oxidation. Strategies to mitigate photo-oxidation include light-blocking packaging, vacuum packaging, removal of photosensitizers, and the addition of singlet oxygen quenchers (e.g., ascorbic acid, tocopherol, or carotenoids). Foods like soybean oil and milk are particularly susceptible to photo-oxidation and should be stored away from light (Kontogiorgos, 2021).

1.3.3 Impact of oxidation in foods

Lipids have an essential role in food quality, and their degradation is a key factor in determining shelf-life (Abeyrathne et al., 2021). Lipids, particularly PUFAs, are primary targets of oxidation and this poses a significant challenge to the quality of both fresh and processed foods. Lipid oxidation in food results in undesirable changes to taste, smell, texture, and color. These alterations not only diminish the sensory and nutritional quality of food products but also pose safety concerns due to the formation of potentially toxic compounds such as polyoxygenated compounds from cholesterol of animal meat (Gutiérrez-Del-Río et al., 2021). Most meat and

meat-based products, especially those rich in polyunsaturated fats, their shelf-life is determined based on the level of oxidation. The resulting rancid flavor of the meat would significantly diminish the overall sensory quality of the final product (Abeyrathne et al., 2021). Therefore, preventing food oxidation is a critical challenge for the food industry as it directly impacts consumer acceptance (Gutiérrez-Del-Río et al., 2021). Moreover, the aim for food scientists is to extend product shelf-life by delaying oxidation as stated by Kontogiorgos (2021).

1.4 Natural Antioxidants in Meat Applications

Antioxidants, when added to foods, effectively inhibit rancidity, prevent the formation of harmful oxidation byproducts, preserve nutritional value, and extend product shelf life (Shahidi and Ambigaipalan, 2015). Ideally, as suggested by Gutiérrez-Del-Río et al. (2021), an antioxidant should be cost-effective, harmless, potent at low doses, stable, resistant to food processing, and undetectable in terms of sensory characteristics such as taste, odor, or color.

The addition of natural antioxidants is more favored from a consumer point of view. Natural antioxidants are mainly sourced from phenolic compounds found abundantly in plants. Phenolic compounds are dietary staples renowned for their antioxidant properties and potential health benefits, reducing the risk of certain diseases. Additionally, these compounds, derived from a main class of secondary metabolites in plants, act as natural food preservatives by inhibiting oxidative rancidity at low concentrations. Phenolic antioxidants function by neutralizing free radicals and, in some cases, binding to metal ions, thus interfering with the oxidation process (Shahidi and Ambigaipalan, 2015).

Phenolic antioxidants can be sourced from a wide range of plant-based foods, including cereals, legumes, fruits, vegetables, essential oils, nuts, seeds, and even beverages.

1.4.1 Rosemary extract

Herbs are Generally Recognized as Safe (GRAS) and have an increasing application in meat due to their antioxidant properties. These have been regarded as substitutes of synthetic antioxidants to prevent oxidative reactions and extend the shelf-life of meat. Among herbs, rosemary (*Rosmarinus officinalis* L.) is considered among the safest and most efficient because its use as a food additive does not give rise to safety concerns including genotoxicity (Hoelscher et al., 2023). It is an effective oxidative stabilizer of various meat and poultry products, fish oils, and vegetables. Regulations governing ingredients in meat, poultry, and egg products specifically permit the use of rosemary extract as an antioxidant (Oswell et al., 2018).

Rosemary's application in beef products can prevent the formation of potential carcinogenic substances such as heterocyclic amines (HCA) due to the inhibitory effect of its compound, oleoresin (Lee et al., 2020). Numerous studies have demonstrated its potential in inhibiting oxidation and growth of microorganisms in food products. The concentration of the rosemary extract varies depending on the type of meat, but at least 150 ppm is enough to decrease lipid and protein oxidation. Rosemary extract comprises of phenolic diterpenes including carnosol and carnosic acid that act as hydrogenic donors in the chain reaction of free radicals, and its addition provided lower values of TBARS, decreasing lipid oxidation in both raw and cooked meat (Bellucci et al., 2022). Additionally, the combination of rosemary extract with pomegranate extract is more effective in preventing oxidation compared to synthetic antioxidants when applied in meat (Fruet et al., 2019). However, not limiting onto this blend, rosemary can be combined with other natural extracts as some possesses both antioxidant and antimicrobial properties that can enhance product shelf-life and safety.

Rosemary extract has several benefits on meat particularly on liver pâtés, wiener sausages, beef burgers, and chicken nuggets. With liver pâtés, its usage allowed to decrease the amount of

sodium nitrate and effectively retarded oxidation. For wiener sausages, it generally retarded oxidation. In raw pork sausages, rosemary extract proved to be effective in reducing the TBARS value and retained redness. Also, it was similarly effective as BHA/BHT in lowering the lipid oxidation of pre-cooked-frozen sausage. As for beef burgers, its effect together with oregano extract decreased mono- and poly-unsaturated fatty acids and slightly increased the content saturated fatty acids. While for chicken nuggets, rosemary extract improved the oxidative stability in frozen conditions (Aminzare et al., 2019).

1.4.2 Acerola extract

Among fruits, acerola (*Malpighia emarginata*) is an abundant source of vitamin C (ascorbic acid) with a level of at least 10 times higher than citrus fruits. Other bioactive compounds present are phytochemical components such as anthocyanins, phenolic, and carotenoids which are compounds that can prevent the effects of chronic diseases such as oxidation, hyperglycemic, inflammation, and obesity. The unripe fruits are commonly used to extract bioactive compounds particularly antioxidant compounds that are found to be richest at this stage, while the ripen fruits are suitable for food processing as this maturity stage gives high organoleptic quality. Additionally, acerola is a functional ingredient to improve nutrition and shelf-life of food products (Hoang et al., 2022). Martínez et al. (2020) found that the acerola extract contained 0.4-0.6g ascorbic acid per 100g extract. In a study conducted by Li et al. (2022), acerola extracts were found to effectively reduce the formation of heterocyclic aromatic amines (HAAs) from a commercial fish, the large yellow croaker. This study suggested that fruit extracts can inhibit the formation of HAAs in various meat systems to produce healthier meat products. In addition, the antioxidative activity of acerola exhibited a stronger ability to prevent thiol loss (sulfhydryl groups) in an oxidized lipid (OXLip) system compared to an oxidized hydrolysate (OXHydro) system; however, it becomes prooxidative when given a high

concentration of ascorbate in the extract, thereby increasing radical signal intensity in the OXLip system (Martínez et al., 2020).

In a study on water buffalo steaks, besides its antioxidant activity, acerola extract at concentrations of 0.0063% and 0.0125%, showed promising antimicrobial efficiency as it reduced the growth of *Brochothrix thermosphacta* and *Pseudomonas* spp. Hence, the extract's potent antimicrobial properties may be attributed to its high concentration of phenolic compounds (Šojić et al., 2022).

1.4.3 Olive extract

Olive (*Olea europaea*) leaves are a promising, abundant, and affordable source of bio-phenols. The specific composition of these bio-phenols in olive leaves is influenced by various factors, including geographical origin, leaf maturity, and extraction methods. Oleuropein is the prevalent phenolic compound, constituting a significant portion of the leaf's dry weight. Other notable phenolic compounds in olive leaves include luteolin, apigenin, and their respective glycosides, as well as phenolic acids like caffeic, p-coumaric, chlorogenic, vanillic, and homovanillic acids. Additionally, flavonoids such as diosmetin, rutin, quercetin, hesperidin, and various forms of luteolin and apigenin have been identified. Besides, olive tree extracts and their isolated polyphenols offer a promising alternative to synthetic preservatives in the meat industry. These compounds, particularly oleuropein, hydroxytyrosol, and tyrosol, have been shown to enhance the oxidative stability of fresh meat by improving the redox state of meat-producing animals. Furthermore, these extracts and their polyphenols can extend the shelf life of meat and meat products by inhibiting microbial growth and preventing oxidative deterioration, both as direct additives and as components of protective packaging (Munekata et al., 2020).

Both olive leaf extract (OLE) and fruit extract (OFE) contain oleuropeosides, flavones and phenolic acids. The OLE demonstrated a dose-dependent and strong antioxidant effect due to the synergism of its phenolic compounds. In contrast, OFE showed weaker antioxidant and reducing properties; however, it was more effective at neutralizing superoxide radicals. In addition, the active compounds from olive leaves prove to be beneficial for human health due to their antioxidant activities (Xie et al., 2015).

In a recent study by Difonzo et al. (2022), the oxidative stability test revealed that increasing amounts of OLE prolonged the initiation phase of lipid oxidation (known as “induction period”). In a separate study, olive leaf extract was employed to enhance the nutritional profile of table olives, specifically increasing their levels of tocopherols, oleuropein, and hydroxytyrosol.

Numerous investigations have explored the use of olive leaves to prevent foods from oxidative damage (Aouidi et al., 2017). Besides, olive oil was incorporated into dry fermented sausages and it delayed lipid oxidation while enhancing the ratio of monounsaturated to saturated fatty acids (Petcu et al., 2023).

1.5 European Union (EU) Regulations on Antioxidants

The European (EU) Regulation 1129/2011 categorizes certain antioxidants using E-numbers and includes them under the category of "other food additives". This regulation identifies rosemary extract (E392), tocopherols (E306-E309), and ascorbic acid (E300) as natural antioxidants permitted for use in foods within the European Union (Hassanpour & Doroudi, 2023). However, only ascorbic acid has a clear maximum permitted level of *quantum satis*, which means that the amount should be just enough to achieve the desired effect.

The EU regulation 1333/2008, as amended by 601/2014, strictly defines the use of antioxidants in meat products. It has a limited application wherein antioxidants can only be used in

prepacked preparations of fresh minced meat and meat preparations that contain ingredients other than additives or salt. Additionally, the carry-over principle may be applied to meat preparations as amended in Article 18. Therefore, the presence of an unauthorized food additive is regarded legal when the additive is approved for one of the ingredients used in the preparation (Cenci-Goga et al., 2020). For a better knowledge, the carry-over principle means that if an ingredient is allowed to contain a particular additive, the additive is automatically allowed in the final product. An example is a spice blend containing preservatives legally approved for use in spices. The preservatives are then allowable in the application of meat.

1.6 Aims of the Project

The main objective of this research was to propose a natural solution with antioxidative properties and that gives flavor to meat.

Specific objectives were:

- Identify bioactive compounds of acerola, rosemary, and olive extracts possessing antioxidative properties.
- Evaluate the synergistic effects of the natural antioxidants when incorporated in beef patties particularly on photo-oxidation and lipid oxidation. The combination of acerola, rosemary, and olive extracts is coded as ARO.
- Compare the antioxidant effect of the natural solution (ARO) with a standard of sodium citrate (E331), considered the positive control.

MATERIALS AND METHODS

2.1 Sample Beef Patty Preparation

The sample patties were obtained using the state-of-the-art equipment inside one of a globally partnered food and nutrition industry. These patties were set into three batches: negative control (NC) without additives; positive control (PC) with sodium citrate; and the blend comprising of acerola, rosemary, and olive (ARO). In forming the beef patties as shown in Figure 3, meat with a lean-to-fat ratio of 80:20 was used, then ground, mixed with the respective ingredients (Table 1), and molded into patties (Hamburger maker: la Minerva Hamburgatrice, Model C/E 653, Serial no. P21 20621). A total of 120 patties were made as these were accounted for every analysis per batch and each weighed around 135 ± 5 grams. These beef patties were individually sealed and stored inside food-grade plastic containers and were placed inside the chiller (+4°C) until usage (Figure 4).

A

B

Figure 3. Formation of beef patties. **A**: selecting, grinding, mixing**. B**: molding into patty.

Figure 4. Sealed packaging and storage of beef patties at 4°C.

Table 1. Beef patty composition defined per batch.

Batch	Ingredients	Composition (%)	Quantity (g)
NC	Meat 80:20	97.05	5823
	Water	1.95	117
	Food-grade rock salt	1	60
	Total	**100**	**6000**
ARO	Meat 80:20	97.05	5823
	Water	1.55	93
	Food-grade rock salt	1	60
	Blend of plant extracts*	0.4	24
	Total	**100**	**6000**
PC	Meat 80:20	97.05	5823
	Water	1.85	111
	Food-grade rock salt	1	60
	Sodium citrate (E331) *	0.1	6
	Total	**100**	**6000**

*Additives mixed into the baseline ingredients. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate.

The burger patties followed 80:20 lean-to-fat ratio. This is considered an optimal ratio for burger patties because this proportion provides sufficient fat content to enhance flavor and juiciness without compromising texture or leading to excessive grease or flare-ups during cooking (Moontide & Moontide, 2022).

2.2 Timeline of the Analyses

The analyses were conducted for 15 days with seven time points (T0-T6) and were mostly taken at an interval of 3 days (Table 2) except at the initial stages T0 and T1. Analyses were aimed to obtain preliminary data and continuously evaluate the progression of oxidation, in particular lipid oxidation and photo-oxidation of the beef patties.

Table 2. Time points of the analyses.

Timepoint	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Days	0	1	3	6	9	12	15

At the beginning (T0), some of the patties were transferred to an external laboratory for lipid oxidation analyses. Once received, they placed these patties inside a chiller (+4°C) until usage.

2.3 Oxidation Analyses

The lipid oxidation tests were performed at Mérieux NutriSciences – CHELAB S.R.L, Italy Laboratory. The primary indicators used to assess the extent of lipid oxidation in food are peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) assay (Rahman et al., 2015) that were measured as described below:

2.3.1 Lipid oxidation: Measurement of Peroxide Value

In determining PV, the fat of the beef patties was extracted first. Fifty grams (50 g) of each burger sample was transferred into separate glass beakers and submerged with an acetone and hexane (1:1, v: v) solution. Then, the mixture was agitated and sonicated for 10 minutes. Subsequently, 10 mL of the solution were filtered and collected into a vacuum filtration flask. The extraction process was repeated twice, and the extracts were dried using a rotary evaporator.

To assess fat oxidation, 1 g of the extracted fat was dissolved in a solution containing 15 mL glacial acetic acid, 10 mL chloroform, and 500 uL of saturated aqueous potassium iodide (KI). The mixture was kept in the dark for 10 minutes. Afterwards, 50 mL distilled water and 2mL of 1% starch solution were added, and the resulting solution was titrated with sodium thiosulfate solution, 0.01N.

2.3.2 Lipid oxidation: Thiobarbituric Acid (TBA) Test

This test is recognized as one of the most widely used methods for assessing lipid oxidation products in meat and meat products. Also, this method is valued for its simplicity and speed. More specifically, 2-thiobarbituric acid (TBA) is a compound employed in the determination of autoxidative spoilage of fats (Tata, 2024). Its reaction process with malondialdehyde is shown in Figure 5.

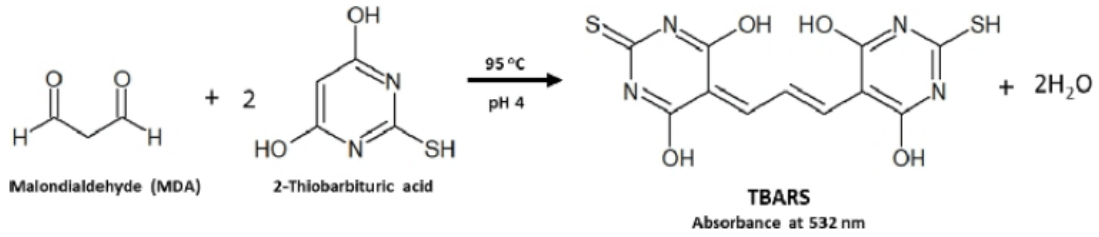


Figure 5. Reaction process of MDA with TBA in a sample, then products are measured using spectrophotometry.

The analytical method is described as follows. A 0.081g quantity of 2-thiobarbituric acid was dissolved in 100 mL of 0.01 N NaOH. Subsequently, 1 g of fat was placed inside a 30 mL glass test tube. This fat was then dissolved with 10 mL of 2-thiobarbituric acid solution and 5 mL of 0.7 N HCl. The mixture was heated in a controlled temperature bath at 60 ± 5 °C for 30 minutes. After cooling the solution in water, 5 mL of 20% trichloroacetic acid were added. The resulting solution was filtered and transferred into a cuvette with an optical path of 1 cm and analyzed using colorimetry at 532 nm.

2.3.3 Photo-oxidation

Color assessment of the beef patties was achieved using a digital single-lens reflex (DSLR) camera with a standardized manual focusing: aperture F5.0, 800, ISO 800, and zoom range 90mm, and using Photoshop CS6 software to obtain the color values expressed as: i) L*, indicating lightness, ii) a*, the red/green coordinate, iii) b*, the yellow/blue coordinate, iv) C*, the chroma, and v) H, the hue angle. Also, a lightbox was used to provide a uniform and controlled lighting environment for photographing the samples. The intensity of lighting was set at the maximum level.

In identifying color differences, the L*a*b* color space, defined by the International Commission on Illumination (CIE), is based on the color-opponent theory, which suggests that colors cannot simultaneously be both red and green or yellow and blue as shown in Figure 6. Deltas for L* (ΔL*), a* (Δa*) and b* (Δb*) may be positive (+) or negative (-); however, the overall difference, Delta E (ΔE*), is always positive as this is calculated using the common equation:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

One beef patty per batch was taken, photographed, and its photo was run in the software to obtain the color values per time point. As these patties were heterogenous in color, three sections of the photo were randomly selected, and the color values were recorded and averaged.

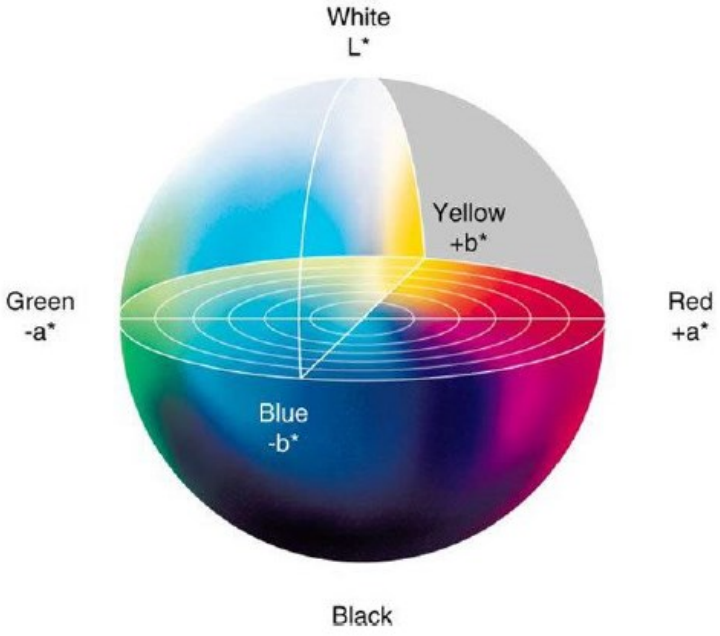


Figure 6. CIELAB color space coordinates.

To compare the color differences among the batches per time point, the negative control (NC) was compared against the blended plant extracts (ARO) and the positive control (PC), and PC was compared as well to ARO labeled as ΔE*[NC-ARO], ΔE*[NC-PC], ΔE*[ARO-PC] respectively.

2.4 pH Analyses

A pH meter (Figure 7) was used to determine the pH levels of raw beef patties per batch per time point. Three different areas of the patty were measured and then averaged.

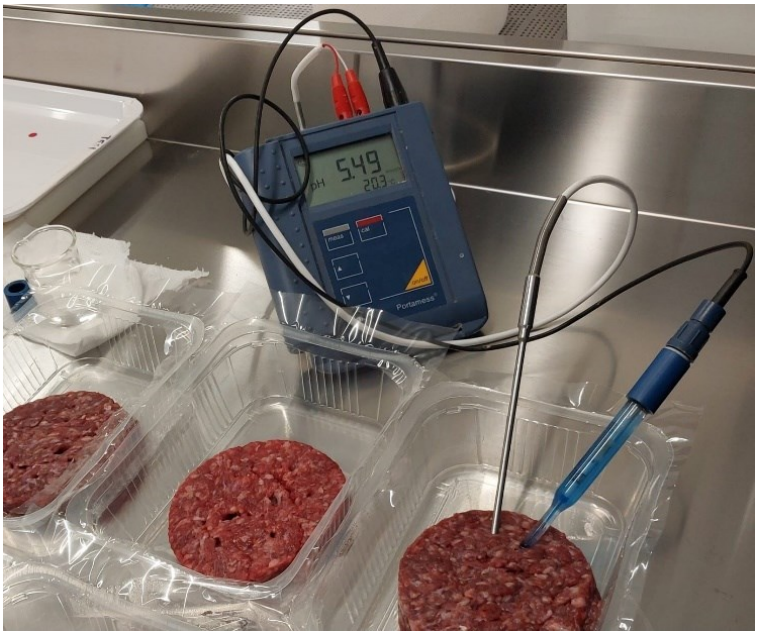


Figure 7. pH meter used for food samples.

2.5 Sensory Analysis

Before sensory analyses, beef burgers were cooked using a heavy skillet that was preheated over medium-high heat. Then, a small amount of oil was added to the hot pan. Afterwards, patties were placed inside and cooked until these were “well-done”, reaching at least an internal temperature of 71°C (160°F). This was to ensure food safety as well. After cooking, the burgers were allowed to rest for a few minutes before serving.

A descriptive test with a 9-point scale was used to describe the cooked beef burgers based on the sensory qualities perceived by trained panels. The perceived intensities on appearance, odor, texture, and flavor were graded. At T1, highly trained panels (7 judges) received pieces of the cooked burger labeled with the 3-digit sample codes (as shown in Figure 8) and handed to them in a different order of appearance. Alongside tasting, the panelist received water to cleanse.

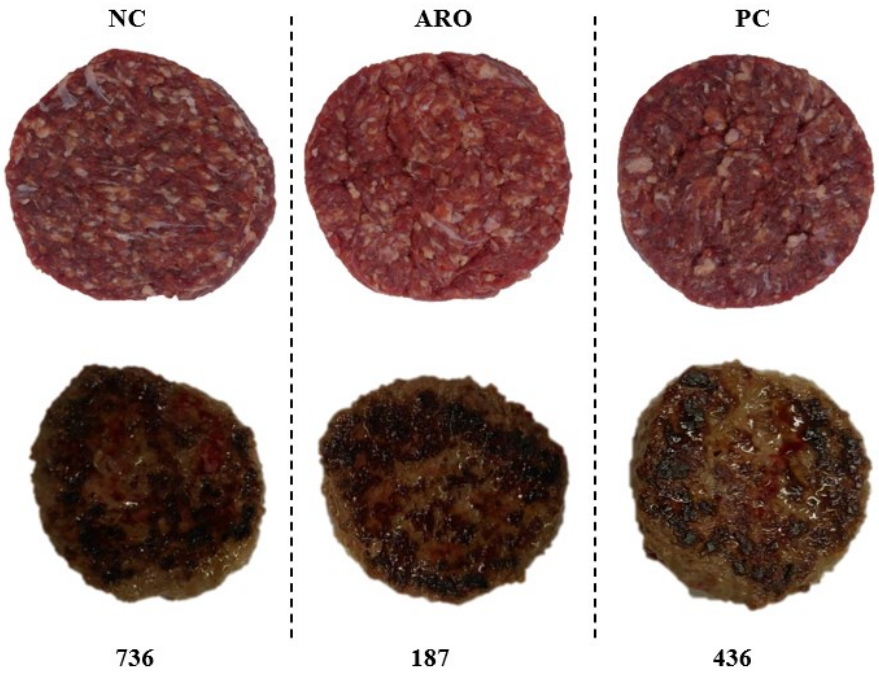


Figure 8. Comparison of raw beef patties and cooked burgers.

Sample codes:736=NC, 187=ARO, 436=PC

2.6 Analysis of the Bioactive Compounds from the Plant Extracts

The metabolic profiling of the plant extracts, acerola, rosemary and olive, was conducted at the Istituto Zooprofilattico Sperimentale delle Venezie (IZVe), Laboratorio Tecnologie Alimentari c/o Sezione Territoriale di Vicenza. The analysis was performed using DART-HRMS, and the process of metabolomic fingerprinting is summarized in Figure 9. Detailed methodology results, and data interpretation were provided by the external analysts.

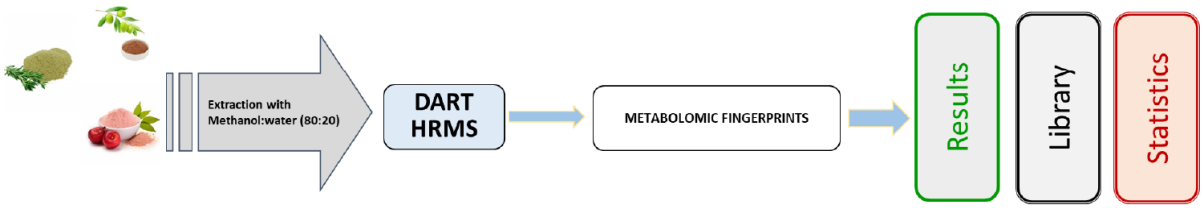


Figure 9. Metabolomics workflow using DART-HRMS: from sample extraction to statistical analysis.

During metabolic fingerprinting using DART-HRMS, analysts initially performed manual mixing of each plant extract. Subsequently, one gram (1 g) portion of each sample was solubilized with a methanol water solution (80:20, v: v). Five milliliter (5 mL) of methanol (Sigma Aldrich, St. Louis, MI, USA) was used for extraction, and samples were vortexed for 1 minute. The resulting sample extract was centrifuged at 15000×g for 5 minutes, and 1 mL of the supernatant was transferred to a new plastic tube. The extraction process is summarized below (Figure 10).

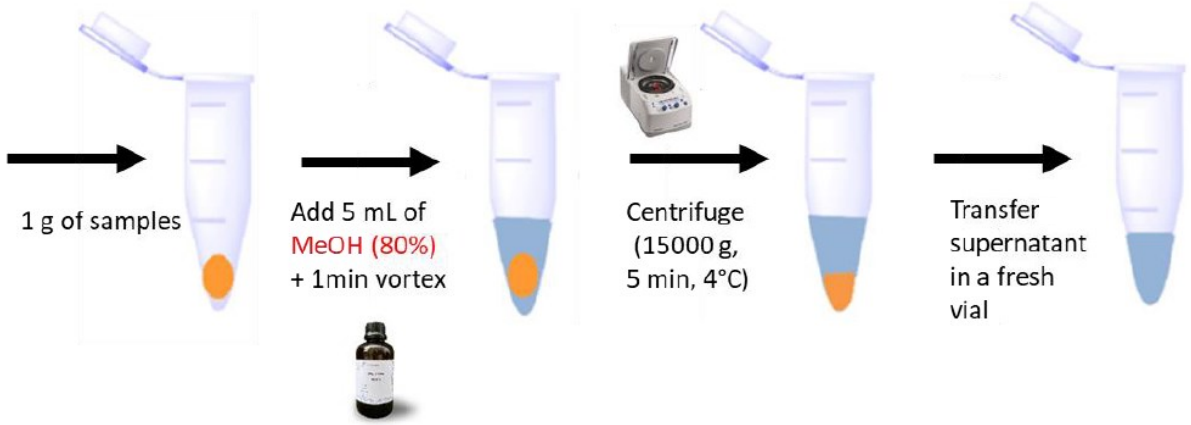


Figure 10. Extraction of the plant extracts for bioactive compounds analyses.

Untargeted metabolomic analysis of the extracts was performed using a DART SVP 100 source (IonSense, Saugus, USA) coupled to an Exactive Plus Orbitrap Mass Spectrometer (Thermo

Fisher Scientific, Waltham, USA). Five microliter (5 µL) volume of each extract was spotted onto melting point rod tubes inserted into a custom-made holder of the Dip-it® autosampler (IonSense, Saugus, MA, USA) as shown in Figure 12. The air-dried extracts on the melting point tubes were then automatically moved by the autosampler at a constant speed of 0.3 mm/s through the DART gun exit and ceramic tube of the Vapur interface, positioned in front of the Orbitrap mass spectrometer. The resolution was set to 70,000 full widths at half maximum, and the mass range was set to 75-1125 Da in both positive and negative ion modes.

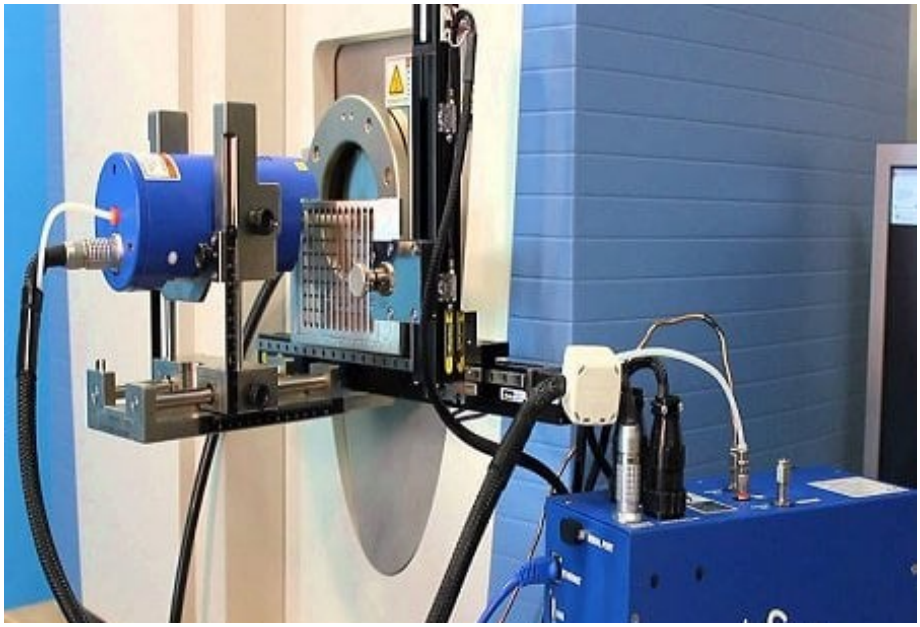


Figure 11. Sample image of the DART-HRMS equipment.

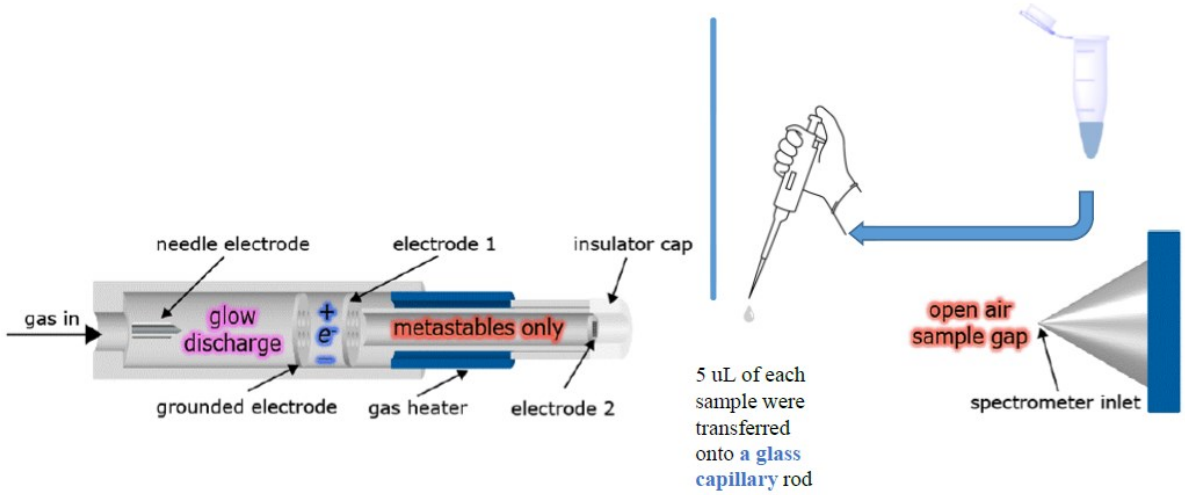


Figure 12. A schematic diagram of the DART-HRMS system.

2.6.1 Statistical analysis

The DART-HRMS data were subjected to statistical analysis using principal component analysis (PCA) and non-parametric analysis of variance (ANOVA) with false discovery rate (FDR) correction. The PCA, an unsupervised technique for data visualization and dimensionality reduction, was employed. The calculation of principal components facilitated this analysis. This approach allowed for the identification of patterns, outliers, or relationships among the three plant extracts through graphical representations.

The explained data variance was expressed by each component which contained the most significant molecular features (m/z values) for differentiating the three study groups. The loading values provided information on the significance of these features within the components. A biplot, calculated by overlaying a PCA score plot with a PCA loading plot, was used. This allowed for the graphical display of information on both samples and variables. Samples were represented as points, while variables were depicted as vectors, with vector length corresponding to variable significance.

Non-parametric ANOVA with FDR correction was employed to assess statistically significant differences between the mean intensity values of the molecular features (m/z values) of the three plant extracts. This approach served to control false discoveries of significant features during multiple hypothesis testing.

RESULTS AND DISCUSSION

As the analyses were conducted over 15 days, the temperature varies only at timepoint zero (T0) as shown in Table 3. A subset of the beef patties was transported to an external analyst, which may have resulted in a sudden temperature fluctuation. These patties were likely exposed to room temperature (+25°C) before being returned to the chiller (+4°C) for storage. All patties were maintained at +4°C throughout the storage period. The period of transfer may have caused temperature change at T0 and so might have an adverse effect on the quality of the product. The beef patties could be prone to oxidative stress regardless of the packaging. But is clear that this stage, this temperature shift could simulate a "situation" of patties transfer from the retailer to the house of the consumers.

Table 3. Timepoints and temperatures.

Timepoint	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Days	0	1	3	6	9	12	15
Temperature	varies	+4°C	+4°C	+4°C	+4°C	+4°C	+4°C

3.1 Determination of Shelf-life based on Oxidation

3.1.1 Lipid oxidation

Meat packaged with oxygen (O_2) has a relatively short shelf-life of at most 16 days. The presence of O_2 inside a packaging can extend the redness of the meat as the oxygen maintains the myoglobin in its oxygenated form (Del Nobile et al., 2009). However, its presence supports lipid oxidation and the growth of spoilage bacteria. The shelf-life of raw meats is mostly determined by the activity of microorganisms which causes off-odors, off-flavors, textural changes and slime formation resulting in meat spoilage. Microbial analysis is often prioritized

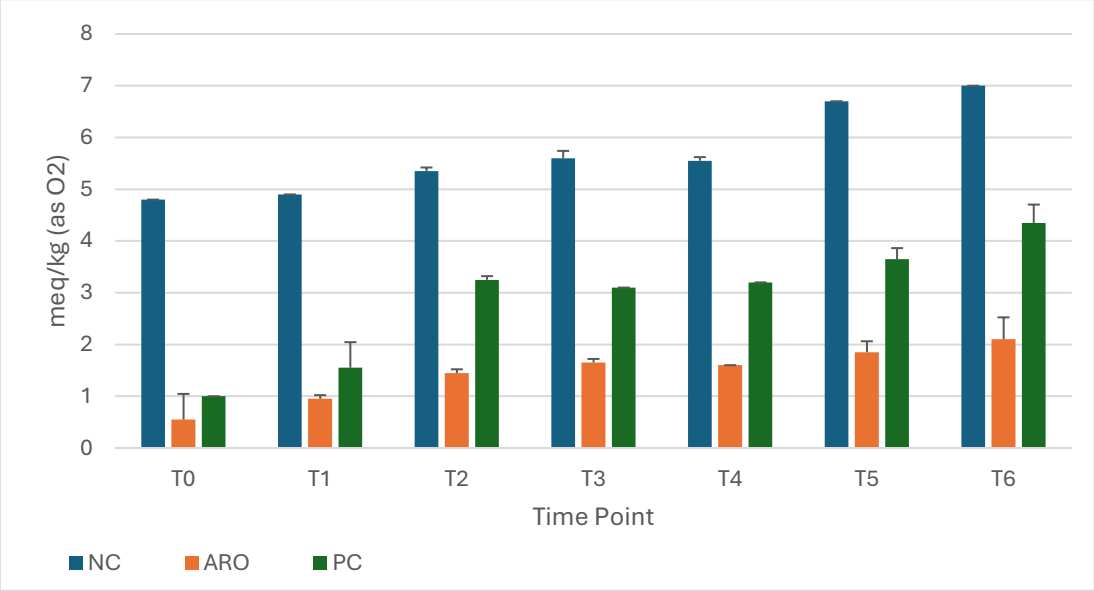
in shelf-life studies as microbial growth directly affects food safety. While microbial contamination is the primary cause of meat spoilage, chemical modifications can precede microbial growth, altering the meat's appearance and signaling the onset of spoilage. Biochemical factors such as lipid oxidation cause undesirable flavors, surface discoloration, rancidity and other quality deteriorations in meat which are important indicators for shelf-life (Sun & Holley, 2012).

Among other experiments, in this thesis the shelf-life of beef patties was evaluated by detecting lipid oxidation products, which are categorized into primary and secondary products. The primary oxidation compound that was measured was peroxide using titration method. This method as summarized by Ruffato (2023) is designed to determine the peroxide value (PV) in animal fats and vegetable oils. The PV is expressed in milliequivalents of active oxygen per kilogram (meq O_2/kg), that oxidize potassium iodide (KI) under the specified test conditions. The secondary oxidation compound that was measured was a by-product known as malondialdehyde (MDA), which is an aldehyde released during the breakdown of unsaturated fatty acid. The thiobarbituric reactive substances (TBARS) assay was used to determine MDA as described in Abeyrathne et al., 2021. In this test, the TBA value is expressed in milligrams of malondialdehyde per kilogram of sample (mg/kg) and serves as an indicator of the extent of oxidative decomposition in polyunsaturated fats. The determination is facilitated by the unique reactivity of TBA with the C=O group of MDA, and this reactivity arises due to the lability of the methylene group at the C-5 position on the MDA molecule. Moreover, the TBA reagent undergoes an additional reaction with MDA (Tata, 2024).

The results of both methods (Figures 13 and 14) demonstrated that beef patties with the blend of plant extracts, acerola, rosemary, and olive (ARO) had the lowest values of oxidized products compared to the negative and positive controls throughout the evaluated period. The blend’s peroxide values started (T0) with an average of 0.55 meq O_2/kg (SD = 0.49) and

gradually increased to an average of 2.1 meq O_2/kg (SD = 0.42) until the end of the period (T6). While for TBARS values, it had an initial average value of 0.65 mg MDA/kg (SD = 0.64), which was higher than its average value at the end of the period, 0.49 mg MDA/kg (SD = 0.11). This data indicates that the use of the blend, ARO, in beef patties was significantly effective in inhibiting oxidation.

A



B

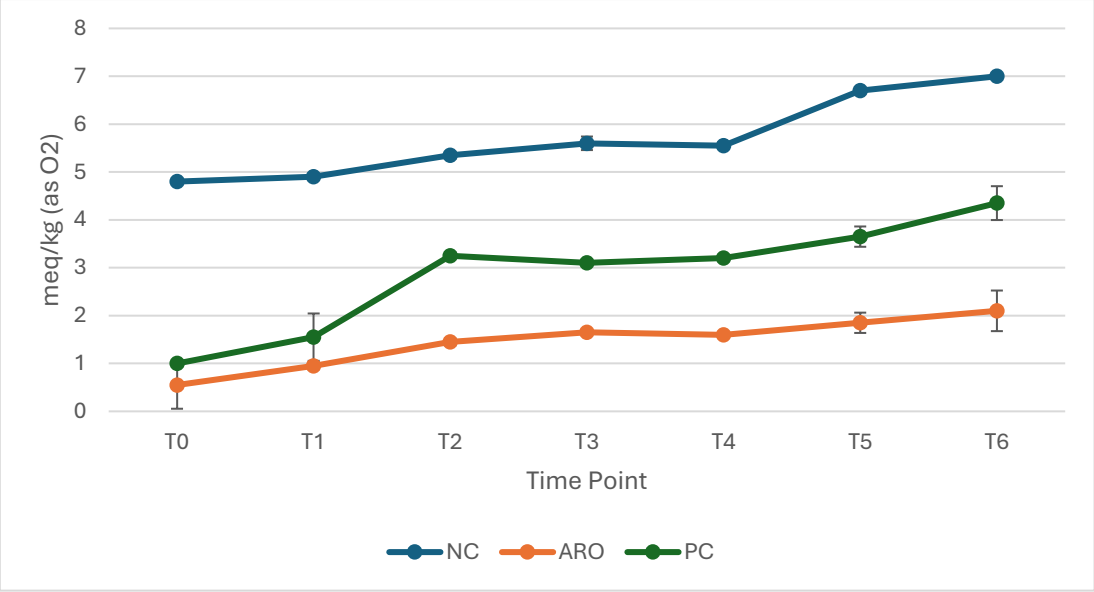
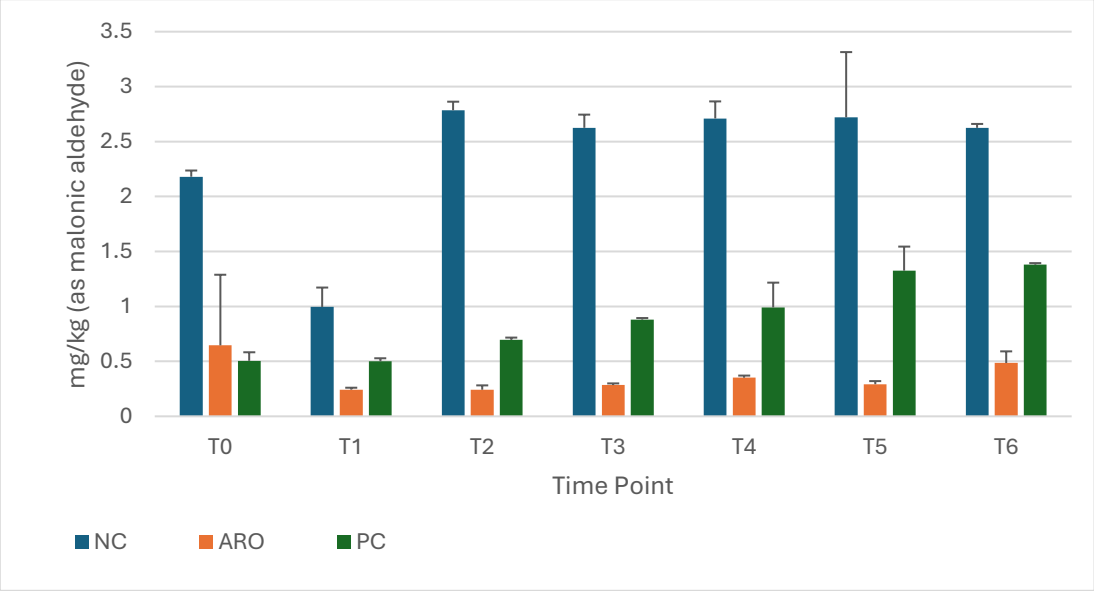


Figure 13. Peroxide values among the samples throughout time. **A**: Comparison among batches. **B**: Trend per batch. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate. Error bars represent standard deviation.

A



B

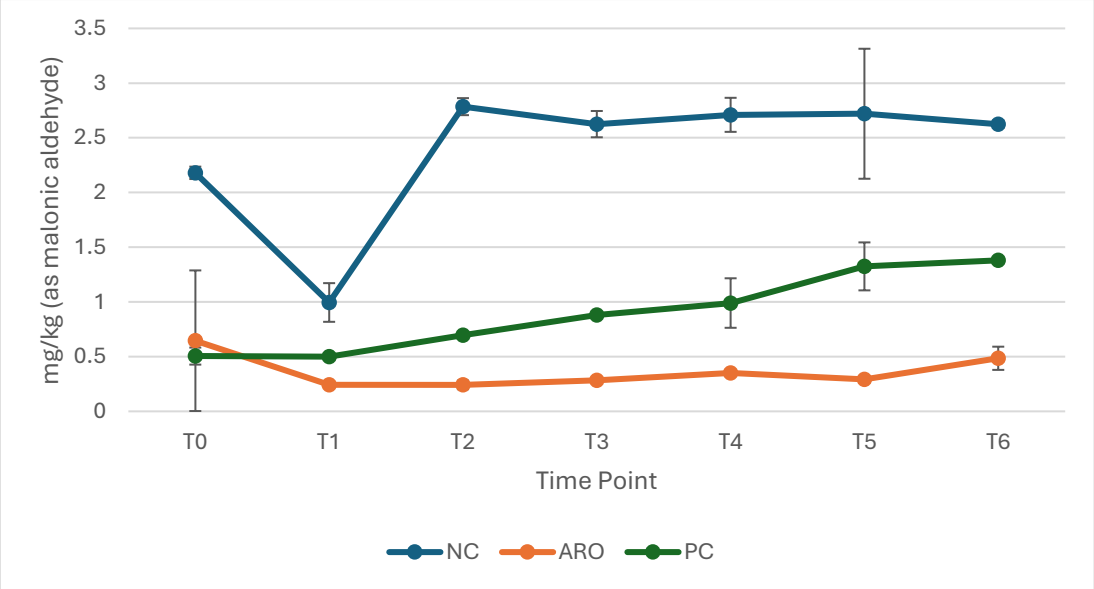


Figure 14. TBARs value among the samples throughout time. **A**: Comparison among batches. **B**: Trend per batch. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate. Error bars represent standard deviation.

Both ARO and PC contained additives wherein one was a combination of natural extracts at a 0.4% concentration and the other was containing sodium citrate (E331), which is a standard chemical compound used to preserve meat, added at *quantum satis* (0.1%), respectively. The blend's effect resulted in at least half of the oxidized products compared to the positive control containing chemicals. Regardless of the differences in concentration, the blend had a significant effect in delaying oxidation rate because of the combined antioxidative properties of the plant extracts. Moreover, considering that the use of natural ingredients is among the top consumer trends currently, these results are very interesting and could pave the way for future products.

All batches of beef patties showed a comparable trend in oxidation. In measuring the peroxide values (Figure 13), all had a gradual increase of oxidation throughout time. Even when the negative control (NC) started with the highest average value of 4.8 meq O_2/kg (SD = 0), it still had a slow increase until the end, reaching an average value of 7 meq O_2/kg (SD = 0). While in measuring the TBARS value (Figure 14), all batches had a decrease from T0 to T1; NC samples showed the most drastic drop of mean values from 2.2 mg/kg (SD = 0.057) to 1 mg/kg (SD = 0.18), followed by ARO from 0.65 mg/kg (SD = 0.64) to 0.24 mg/kg (SD = 0.018), and PC with the smallest decrease from 0.505 mg/kg (SD = 0.078) to 0.5 mg/kg (SD = 0.028). After this time point, oxidation started to increase gradually. The sudden drop in oxidation products at T1 suggests that temperature change might have influenced the TBARS value, decelerating oxidation once stored back at +4°C. Another reason could indicate that most of the available oxidizable substrates have been consumed.

The edibility threshold for peroxide values (PV) should not exceed 30 meq O_2/kg fat (Gotoh & Wada, 2006). However, from a study cited by Rahman et al. (2015), beef and beef products become unacceptable when peroxide values exceed 25 meq O_2/kg. Thus, freezing beef can slow down the formation of peroxides compared to refrigeration. If PV exceeds, it will indicate excessive lipid oxidation and rancidity in fats and therefore leads to off-flavors, odors, and

overall quality deterioration in food products, making food products unfit for consumption. Peroxide values as high as 100 meq O_2/kg fat have been linked to cases of food poisoning (Gotoh & Wada, 2006). Based on the results, in this study all batches have peroxide values below 10 meq O_2/kg fat; hence, this implies that all beef patties were edible and safe throughout the period (15 days).

Whereas for TBARS values, it does not have a standardized MDA threshold in food because TBARS measure the extent of lipid oxidation and not directly on safety consumption. Indeed, this test is used to monitor lipid oxidation of food products such as meat and correlate the values with sensory quality and shelf-life. Some studies have suggested that acceptable limits of TBARS values range from 1 to 2 mg MDA/kg. The negative control, since the beginning, already exceeded the acceptable limit with a mean of 2.2 mg MDA/kg (SD = 0.057). Only at T1, it had lower mean TBARS value of 1 mg MDA/kg (SD = 0.18). As for the blend and the positive control, both remained at an acceptable limit throughout the shelf-life with averages of 0.49 mg MDA/kg (SD = 0.11) and 1.38 mg MDA/kg (SD = 0.014), respectively (Figure 14). Hence, these treated batches of beef patties would have a better overall sensory quality as these are correlated with lower TBARS values.

3.1.2 Photo-oxidation

One of the categories of lipid oxidation is photo-oxidation, which involves interaction of light i.e., ultraviolet and visible light, with molecular O_2 leading to the production of reactive oxygen species (ROS) that can initiate and propagate oxidative reactions in a food product. It is a distinct process from lipid oxidation; however, it also affects the sensory quality and shelf-life of food products.

In assessing photo-oxidation, colorimetry is the main technique to quantify the color values: lightness (L*), chromaticity (a*, b*), chroma (C*), and hue (H). It is an indirect method to

measure photo-oxidation wherein changes in color indicate a chemical reaction. Beef patties or red meat may undergo browning due to oxidative reactions throughout time.

Instead of colorimetry, a different approach was used in this work. A lightbox and a camera with a standardized setting were used to take photos of the beef patties per time point as shown in Figure 15. Afterwards, photoshop software was applied to obtain the color values per batch of beef patties. These color values were averages of three different areas of the burgers as their colors were heterogeneous (Figures 16-18).

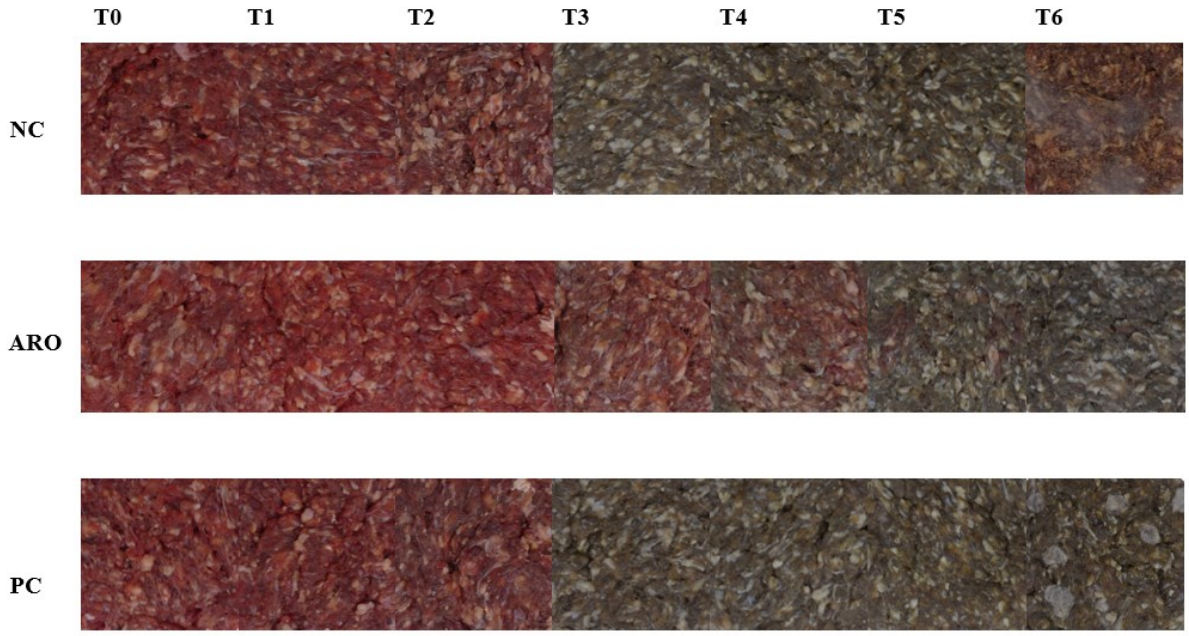


Figure 15. Progression of photo-oxidation of the meat throughout the period. Manual camera setting: F 5.0, 800, ISO 800, 90mm. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate.

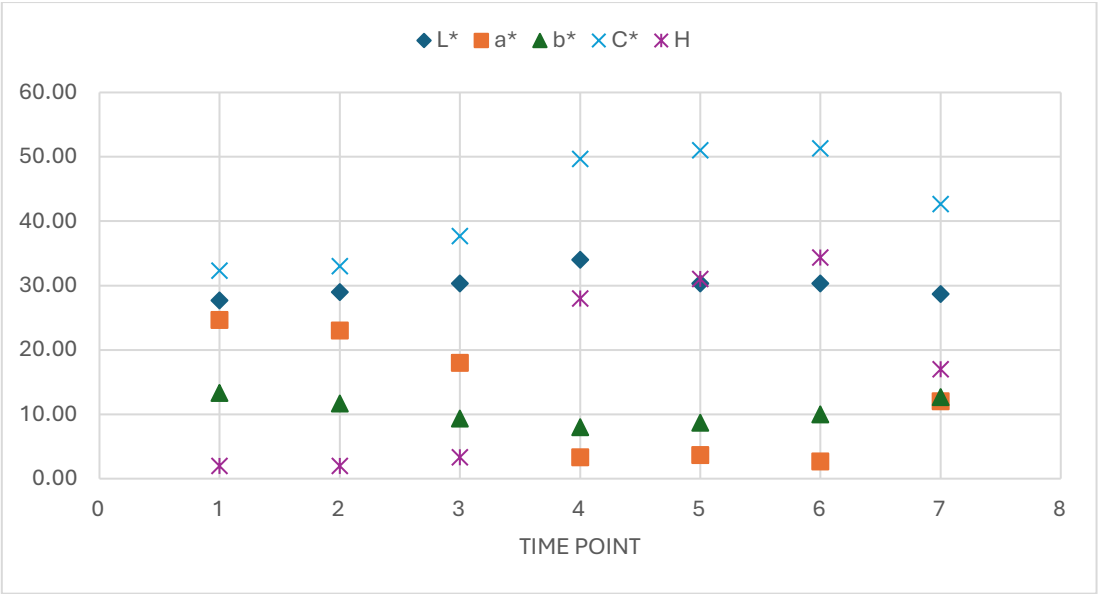


Figure 16. Measurements of Lightness (L*), Chromaticity (a*, b*), Chroma (C*), and Hue (H) in the negative control (NC) batch of beef patties.

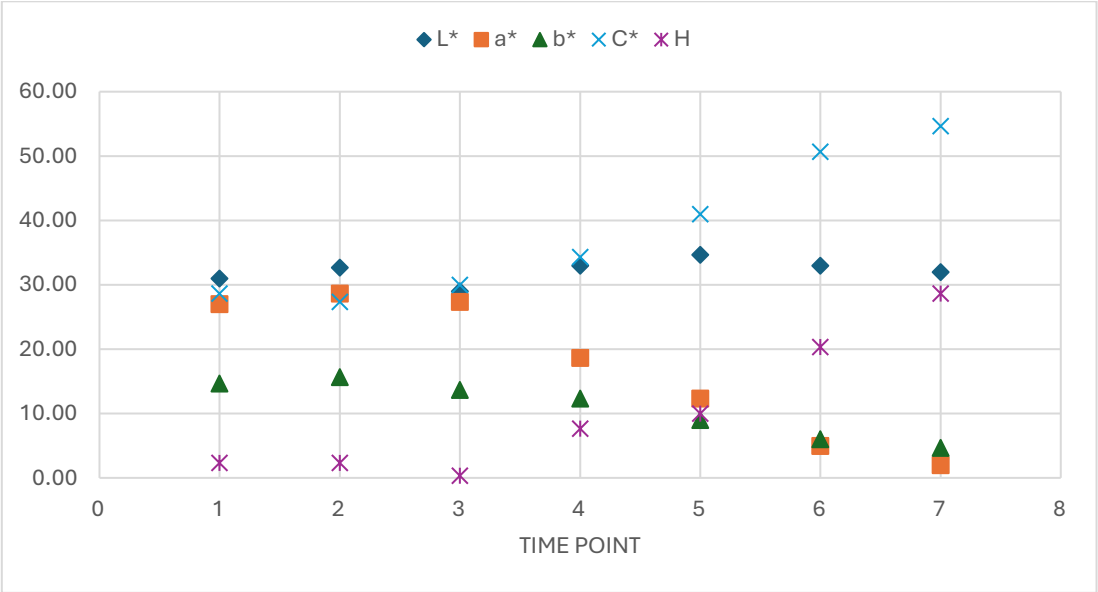


Figure 17. Measurements of Lightness (L*), Chromaticity (a*, b*), Chroma (C*), and Hue (H) in the batch of beef patties with the blend of plant extracts (ARO).

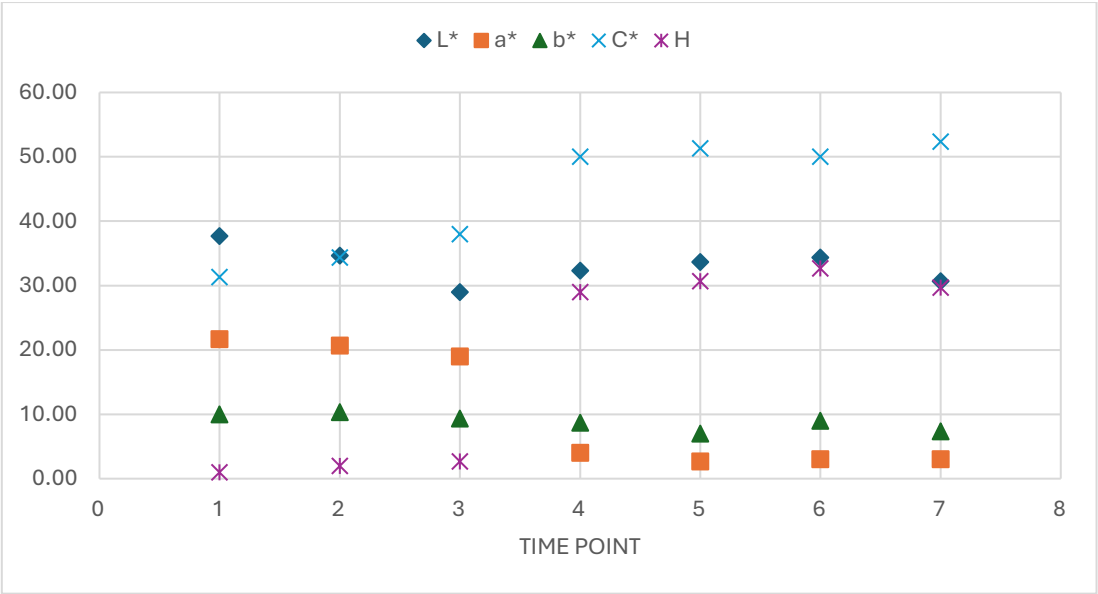


Figure 18. Measurements of Lightness (L*), Chromaticity (a*, b*), Chroma (C*), and Hue (H) in the positive control (PC) batch of beef patties.

In color assessment, the a* axis is relative to the green–red opponent colors, with negative values toward green and positive values toward red. The b* axis represents the blue–yellow opponents, with negative numbers toward blue and positive toward yellow. Since red meats are initially red and indicate freshness, accounting for the chromaticity +a* was enough to measure the changes, from red to brown (Figure 19). Based on the results, the blend, ARO, had the highest a* value until T5, indicating that it mostly retained the redness of the beef patties throughout the period. However, at T6, NC obtained the highest value but this change in color was influenced by the growth of spoilage microorganisms.

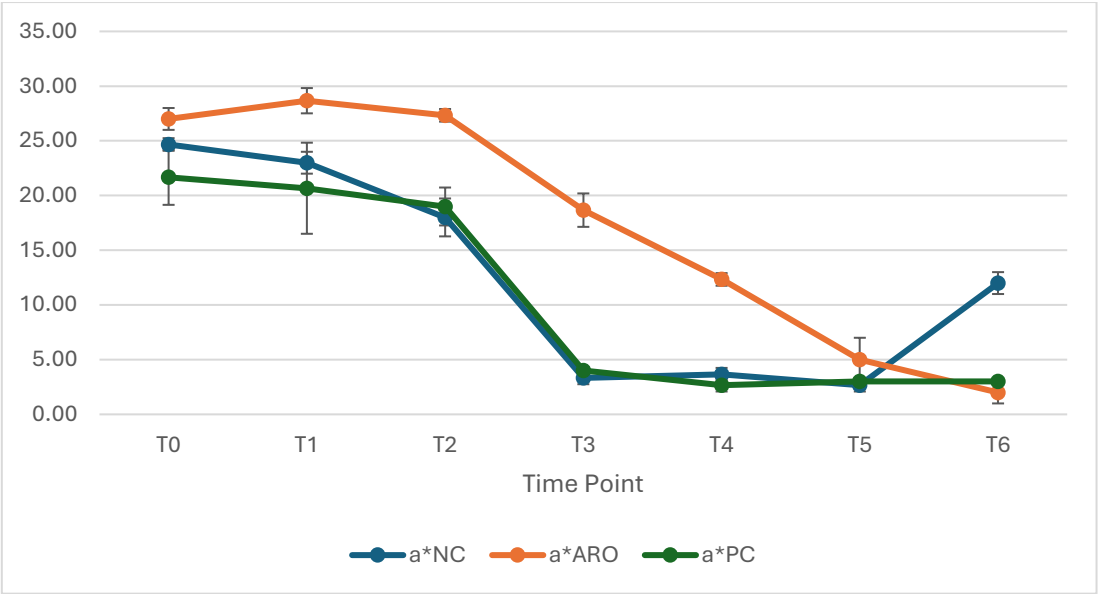


Figure 19. Measurements of a* Chromaticity in the samples. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate. Error bars represent standard deviation.

The differences of color values ΔE* between batches are summarized in Figure 20. The blend's color differences against the controls were significantly different for most of the time points. At T3, a color difference of 15 was quantified when ARO was compared with the controls. This difference was indicative that the beef patties from the controls turned brown while the one with the blend retained redness; thus, ARO delayed photo-oxidation due to the combined antioxidants, deactivating the photosensitizers.

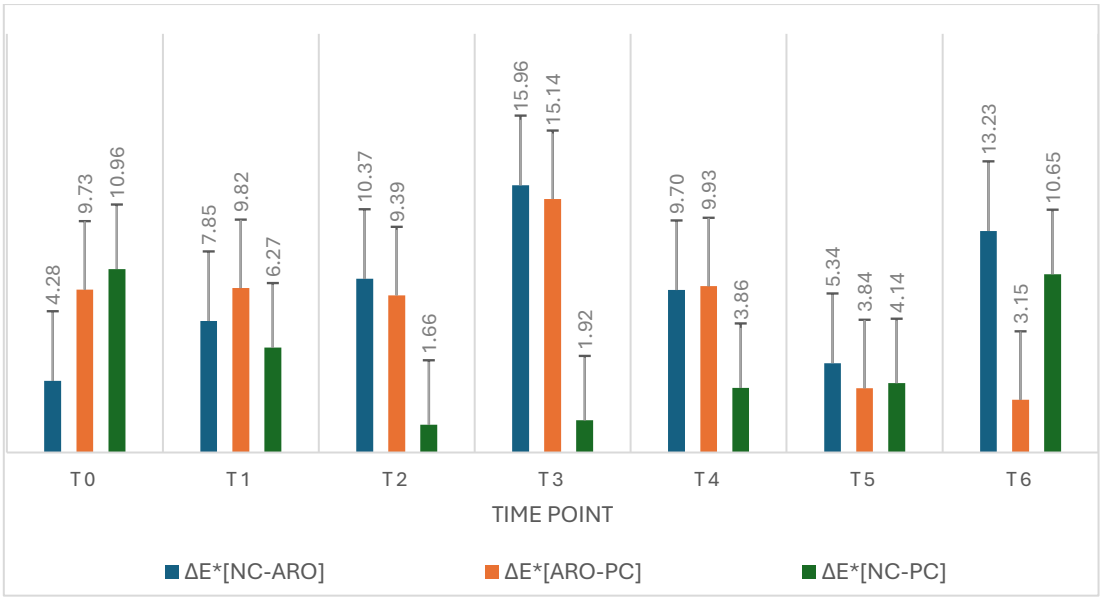


Figure 20. Differences of color values ΔE* between batches per time point. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate. Error bars represent standard deviation.

3.2 pH

Another indicator for shelf-life is the pH, that also influences the color of the meat. The standard range of raw beef patties is from pH 5.4 to 5.6 (Huff-Lonergan and Lonergan, 2005 as cited by Van Buren et al., 2023), and within this range, patties have a better water-holding capacity and quality. However, it was mentioned by Serdaroğlu et al. (2018) that the range is specifically from pH 5.67 to 5.71 based on their study. Initially, the beef patties have a pH around 5.6; NC =5.66, ARO = 5.65, and PC=5.58. In Figure 21, it was observed that the pH of the patties decreased in all batches until T3, with ARO having the lowest mean pH 5.26 (SD = 0.018). This indicates that the patties became slightly acidic. According to Hajlaoui et al. (2019), a lower pH suggests that there has been bacterial growth, and they are producing acids that lead to spoilage and deterioration of meat quality. While a higher pH is associated with fresher meat that has not yet undergone significant microbial degradation. Additionally,

hydroperoxides, which are formed during primary oxidation, are highly unstable and quickly decompose into secondary products (i.e., acids, ketones, epoxides, and organic acids) and these contribute to pH changes.

In addition, the color change of the patties is influenced by pH change. In meat, myoglobin exists in various forms, including deoxymyoglobin (purple), oxymyoglobin (bright red), and metmyoglobin (brown). A higher pH favors oxymyoglobin that results to a brighter red color, while a lower pH can lead to more metmyoglobin that causes a darker brown color. Consumers prefer meat products that are redder in color as this is associated with freshness.

At T3, it was observed that the decrease in pH (Figure 21) significantly changed the patties color to brown for both negative and positive controls however, it did not significantly affect the color of the beef patties with ARO as shown in Figure 15. The patties with ARO retained their redness even after T3 despite exhibiting the lowest pH among the three batches. This indicates that the bioactive compounds present in the patties have strong antioxidant properties that can retain redness longer compared to the controls. Also, these bioactive compounds, reducing acidity in meat, have antimicrobial properties that can inhibit the growth of microorganisms involved in spoiling. In fact, as this batch of patties showed the lowest pH throughout the storage period, this could be due to the presence of acidic compounds such as ascorbic acid from acerola extract. Additionally, it was observed that the beef patties with ARO exhibited a delayed microbial growth compared to the controls. Therefore, it was evident that the blend of plant extracts was effective in preventing beef patties from early meat deterioration and spoilage.

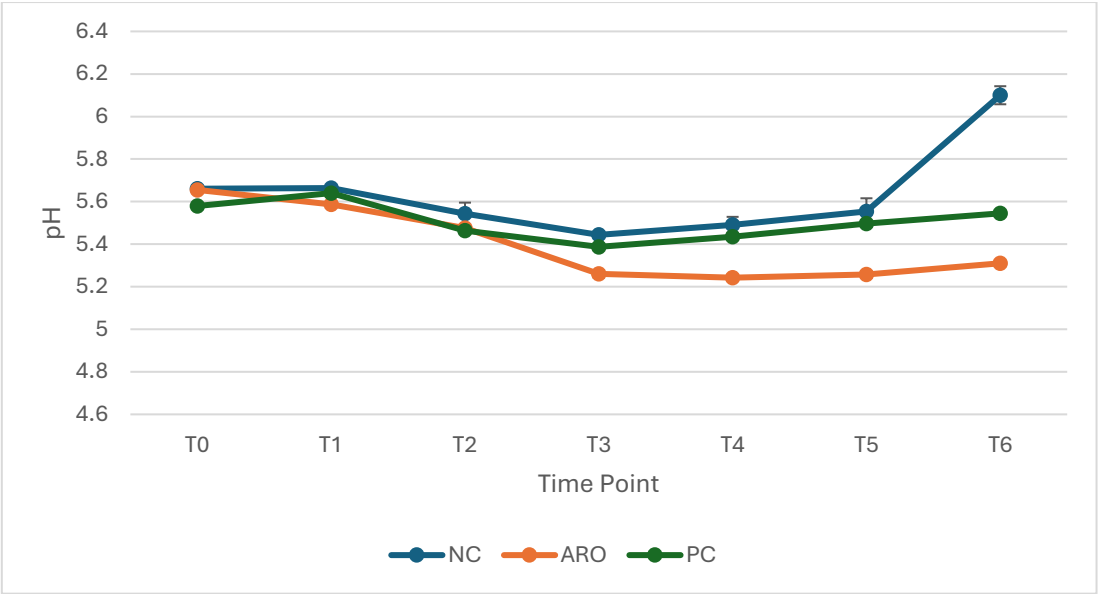


Figure 21. Comparison of the pH levels among batches. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate. Error bars represent standard deviation.

3.3 Sensory Analysis

An indirect way to measure shelf-life is through sensory analysis (Abeyrathne et al., 2021). However, in this work, this method was used for tasting to describe the perceived intensities on appearance, odor, texture, and flavor of the beef burgers as shown in Figure 22. After cooking at 70°C, the burgers were indistinguishable one from another. From the panels' perception, texture and flavor were mostly the same for the positive control (436) and the blend (187), but the overall acceptability was favored with the burger having the blend. In addition, the blend gave a different taste to the burger as most panel mentioned that there was an herbal note which was not overpowering. This suggests that the combination of plant extracts: acerola, rosemary, and olive at 0.4% concentration was favorable to taste.

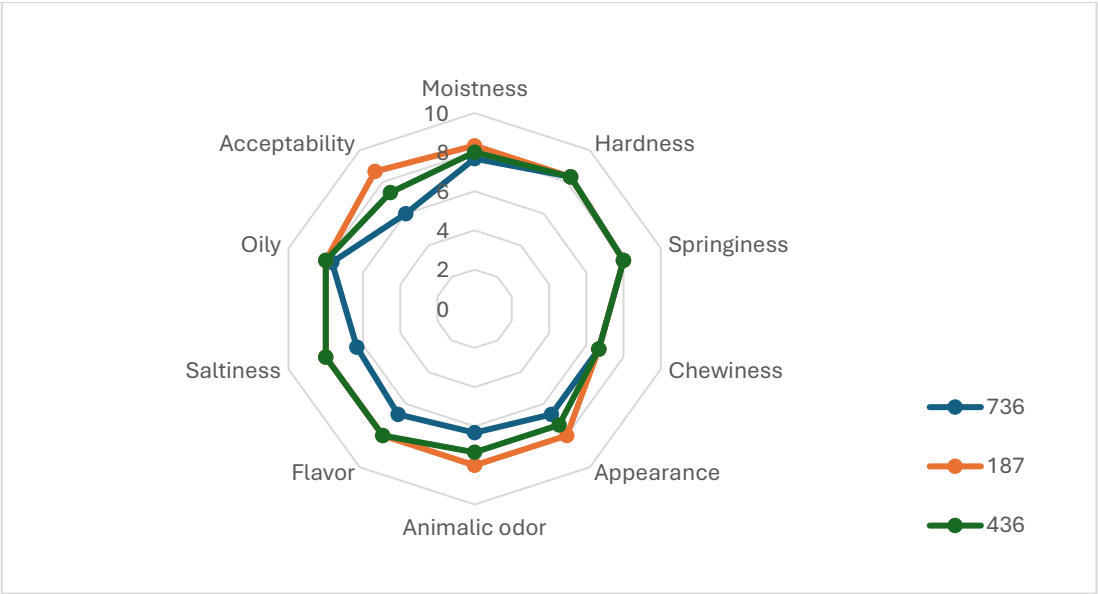


Figure 22. Spider-gram representing the perceived intensities on the beef burgers. Sample codes:736=NC, 187=ARO, 436=PC. NC: Negative Control without additives; ARO: Acerola, Rosemary, & Olive Blend; PC: Positive Control with Sodium Citrate.

3.4 Metabolomic Fingerprinting

Metabolomics, or metabolic phenotyping, is a quantitative analysis of low-molecular-weight compounds (<1 kDa) present in a biological sample. Instead of focusing on the interactions between transcriptional and translational data, metabolomics examines the final products of these processes and function as machinery that catalyzes and regulates cellular reactions (Kosmides et al., 2013). It offers the most direct and predictive link to phenotype compared to other -omics technologies (Cao et al., 2022). Additionally, it is a comprehensive analytical approach that is non-selective, universally applicable, and capable of identifying and quantifying all metabolites within a biological system. This field aims to create complete metabolic profiles, identify differences between metabolites, and develop hypotheses to explain such differences (Wolfender et al., 2015).

In metabolomics, as stated by Wolfender et al. (2015), there are various analytical strategies employed to determine the chemical composition of a biological sample or extract. One common approach is "metabolite fingerprinting" that focuses rapidly on classifying samples. These high-throughput analyses often prioritize sample classification over extensive metabolite identification and quantification. This method aims to compare patterns or "fingerprints" of metabolites and not identify individual metabolites that vary within a biological system. Also, its approach is generally untargeted and serves as a hypothesis-generating method within the context of metabolomics.

According to Alvarez-Rivera et al. (2019), plants are rich sources of secondary metabolites, which are considered as bioactive compounds (Revutska et al., 2021), with potential applications in drug discovery, cosmetics, nutraceuticals, and biomaterials. In addition, Farias et al. (2022) emphasized that there are beneficial effects brought by plant-based foods and their byproducts as these are linked to abundant and diverse polyphenols. Secondary metabolites play important roles in plant defense, signaling, and interaction against external pathogens and reactive oxygen species (ROS). More so, epidemiological studies consistently point to their potent bioactivity.

Polyphenols exert a range of bioactive properties, including antioxidant, anti-inflammatory, antimicrobial, and anticancer activities. Additionally, these bioactive compounds can influence biological processes and can provide benefits to human health by potentially reducing the risk of chronic diseases, such as cardiovascular diseases, diabetes, and certain types of cancer.

While several techniques have been used to characterize phenolic profiles in different plant matrices, metabolomics has been proven to be an effective and reliable tool (Farias et al., 2022). Among its advantages are: 1) the ability to elucidate details of plant natural product biosynthesis, 2) the capacity to evaluate and identify metabolites even when there is limited

information, 3) the ability to detect and quantify metabolic changes, and 4) there is reduced costs (Weston et al., 2015).

High-resolution mass spectrometry (HRMS) is a versatile analytical tool, particularly suited for the analysis of complex natural products. Its ability to be coupled with several chromatographic techniques and ionization sources enables rapid and sensitive screening and identification of secondary metabolites. Additionally, its analysis gives both positive and negative ions of analytes of the samples. Direct analysis in real time (DART) is an ambient plasma ionization method. Together with mass spectrometry, it has been applied to various fields from food chemistry to pharmaceutical studies. DART-HRMS has proven to be effective in analyzing and identifying secondary metabolites from plant sources such as phenolic compounds, alkaloids, saccharides, and iridoid glycosides. This technique is valuable for its rapid screening, bio-distribution studies, fingerprint analysis and quality control of natural products (Alvarez-Rivera et al., 2019). Moreover, it has been applied remarkably to food authenticity, quality, and safety analyses (Rubert et al., 2015).

3.4.1 DART-HRMS in positive ion mode

Analysis through the positive ion mode of DART-HRMS detected several metabolites from the plant extracts as shown in Figure 23. High abundance of ascorbic acid was found in the acerola extract. Conversely, the rosemary extract was characterized by the presence of high levels of flavonoids, mainly salvigenin, cirsimaritin, and hesperetin.

Salvigenin, a natural polyphenolic compound, was identified with neuroprotective, antitumor cytotoxic, and immunomodulatory properties. Whereas cirsimaritin was found to exhibit various biological effects, including antimicrobial, anti-inflammatory, and anti-proliferative properties. While hesperetin, which is the aglycone of hesperidin, was observed along with hesperidin itself. Both flavonoids possess numerous biological properties, particularly

antioxidant and anti-inflammatory activities. Recent findings suggest that their antioxidant activity extends beyond mere radical scavenging, also augmenting cellular antioxidant defenses through the ERK/Nrf2 signaling pathway (Tata, 2024).

Generally, flavonoids are plant pigments that also enhance plant resistance and contribute to the quality of food through their preservative properties. These polyphenols have bioactive properties and can modulate the function of key cellular enzymes, leading to their widespread use in the production of pharmaceuticals, nutraceuticals, medical products, and cosmetics (Revutska et al., 2021).

In the olive extract, high relative intensities of the antioxidant tyrosol were observed. Tyrosol is a scavenger of ROS such as peroxyl radicals, and it is a simple phenol with nutraceutical properties against insulin resistance, obesity, coronary heart disease, chronic heart failure, hypertension, and atherosclerosis (Cuffaro et al., 2023). Additionally, a high relative abundance of pantothenic acid and vinylphenol were found. Pantothenic acid, also known as vitamin B5, contributes to lowering cholesterol and triglycerides in the body. Notably, pantothenic acid serves as the most crucial component of coenzyme A, which plays a role in various metabolic pathways, including fat transfer within the body. However, vinylphenol (4-vinylphenol) is one of the compounds that makes olive oils unacceptable. The other compounds were cinnamic acid ethyl ester and styrene. A study suggested that 4-vinylphenol likely originates from p-coumaric acid via decarboxylation during olive storage, and its presence and the cinnamic acid ethyl ester cause off-flavors in olive oils (Tata, 2024).

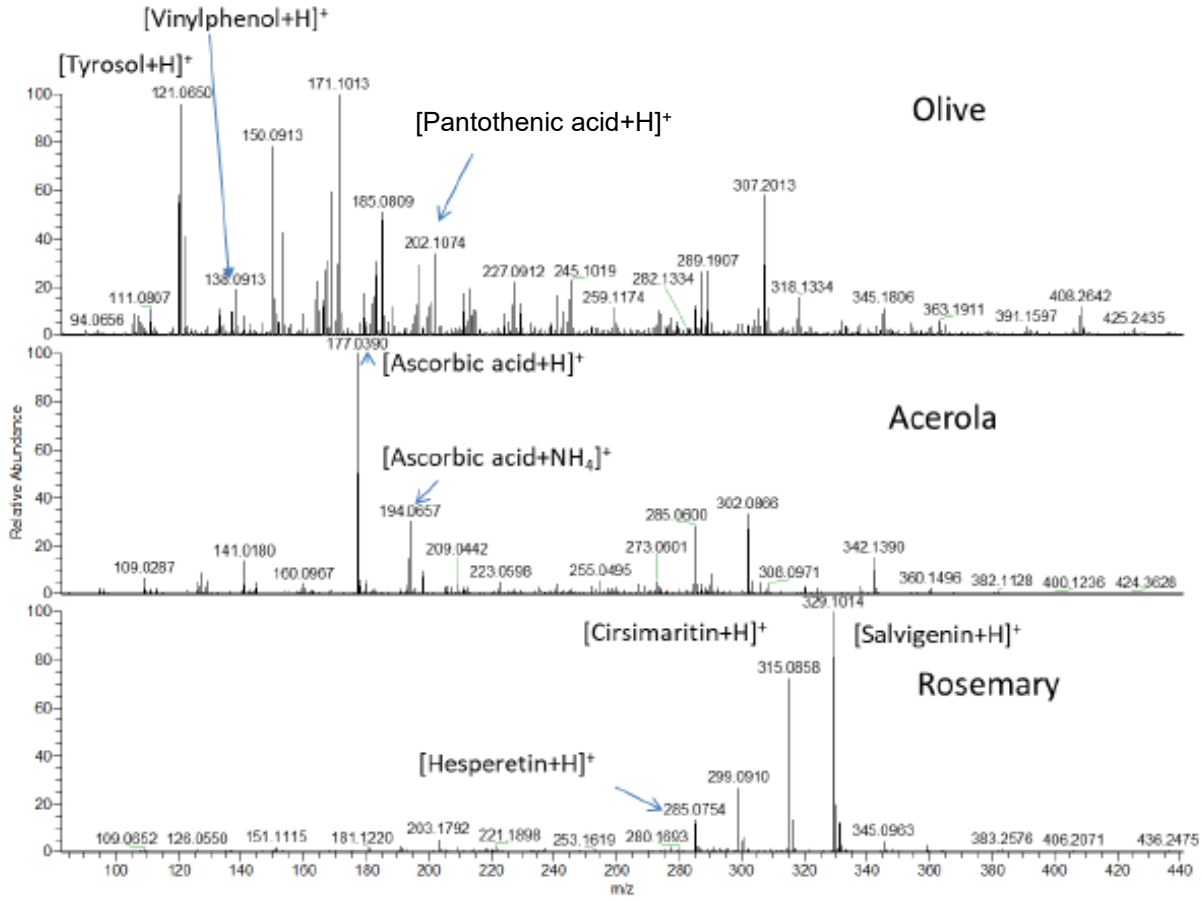


Figure 23. Secondary metabolites of olive, acerola, and rosemary extracts acquired in positive ion mode.

Besides the abundant bioactive compounds mentioned, other detected molecules in the positive ion mode are listed in Table 4. The metabolites were measured based on the mass-to-charge ratio (m/z) of the ion. This is a principle of MS in measuring the mass of a molecule (Rubert et al., 2015). Once the m/z of metabolites were detected, these were compared to theoretical m/z from an online library to identify the metabolites. Thus, the metabolites were putatively annotated by interrogating the online library www.foodb.ca.

Table 4. List of metabolites, positively ionized and observed in olive, acerola, and rosemary extracts. The metabolites were putatively annotated by interrogating the online library www.foodb.ca

Plant source	Observed *m/z*	Theoretical *m/z*	Error ppm	Type of ion	Number of charges	Putative annotation	Formula
Rosemary	329.1014	329.1020	2	[M+H]+	1	Salvigenin	$C_{18}H_{16}O_6$
	315.0858	315.0863	2	[M+H]+	1	Cirsimaritin	$C_{17}H_{14}O_6$
	285.0754	285.0757	3	[M+H]+	1	Hesperetin	$C_{16}H_{12}O_5$
	299.0910	299.0914	1	[M+H]+	1	Methylflavonoid	$C_{17}H_{14}O_5$
Acerola	177.0390	177.0392	1	[M+H]+	1	Ascorbic acid	$C_6H_8O_6$
	194.0657	194.0657	3	[M+NH4]+	1	Ascorbic acid	$C_6H_8O_6$
	285.0600	285.0611	4	[M+H-H2O]+	1	Phenolic glycoside	$C_{12}H_{14}O_9$
Olive	121.0650	121.0654	3	[M+H-H2O]+	1	Tyrosol	$C_8H_{10}O_2$
	138.0913	138.0913	0	[M+NH4]+	1	Vinylphenol	C_8H_8O
	202.1074	202.1080	2	[M+H-H2O]+	1	Pantothenic acid	$C_9H_{17}NO_5$
	345.1806	345.1806	1	[M+NH4]+	1	Quinolin alkaloid	$C_{19}H_{21}NO_4$

Principal component analysis (PCA) was employed to statistically analyze the metabolic fingerprints of the three plant extracts. Mass spectrometry and statistical analysis facilitated a clear differentiation of the extracts in both positive (Figure 24) and negative (Figure 26) ion modes. A score plot generated by the PCA analysis visually depicted the discrimination of these extracts based on the first two components. It is viewed that acerola (red cluster), rosemary

(blue cluster), and olive (green cluster) are distant from one another and clustered closely together indicating that they are distinct and contain different bioactive compounds.

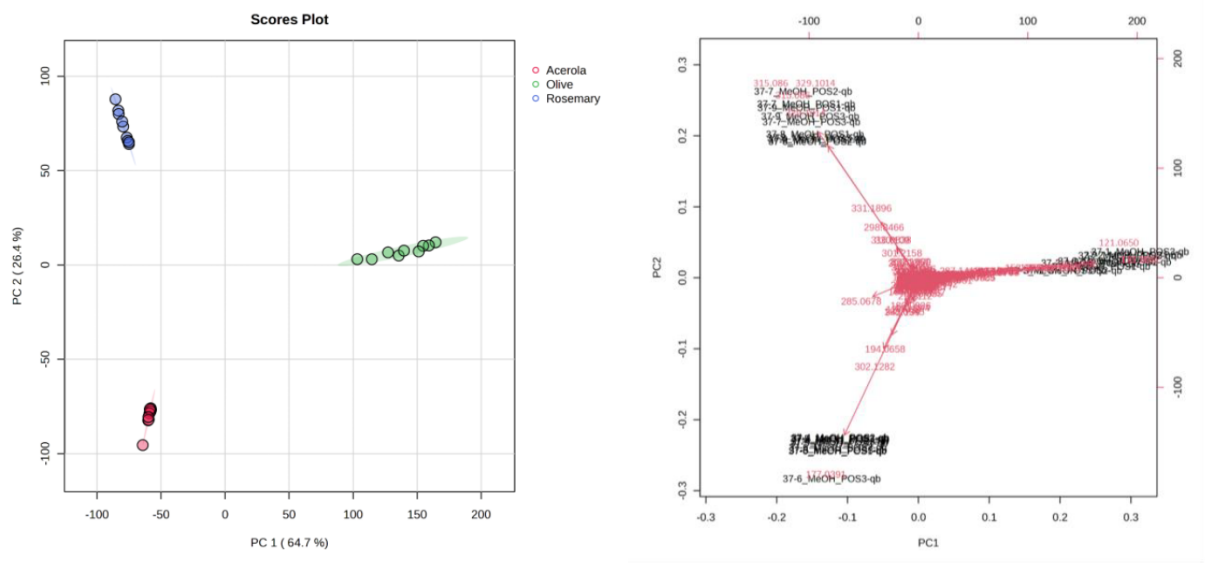


Figure 24. Statistical discrimination of olive, acerola, and rosemary extracts based on the (+) DART-HRMS data.

3.4.2 DART-HRMS in negative ion mode

The analysis in the negative ion mode (Figure 25) detected few metabolites. Olive extracts were found to contain quinic acid and threonic acid and mannitol. Threonic acid is a major breakdown product of ascorbic acid and is commonly used as a food additive. While quinic acid has bioactive properties that may improve metabolic health such as its effects on pancreatic beta-cell functions by controlling blood glucose levels (Heikkilä et al., 2019).

Malic acid and ascorbic acid were identified as characteristic components of the negative spectra of acerola. This ascorbic acid is recognized as an antioxidant.

Carnosic acid and isorosmanol were found in the negative spectra of rosemary. Carnosic acid, a natural benzenediol abietane diterpene, is found in both rosemary (*Rosmarinus officinalis*)

and common sage (*Salvia officinalis*). It possesses phenolic (catecholic) properties and exhibits antioxidant and antimicrobial activities. Likewise with the positive ion mode, the list of identified metabolites based on their molecular features is shown in Table 5.

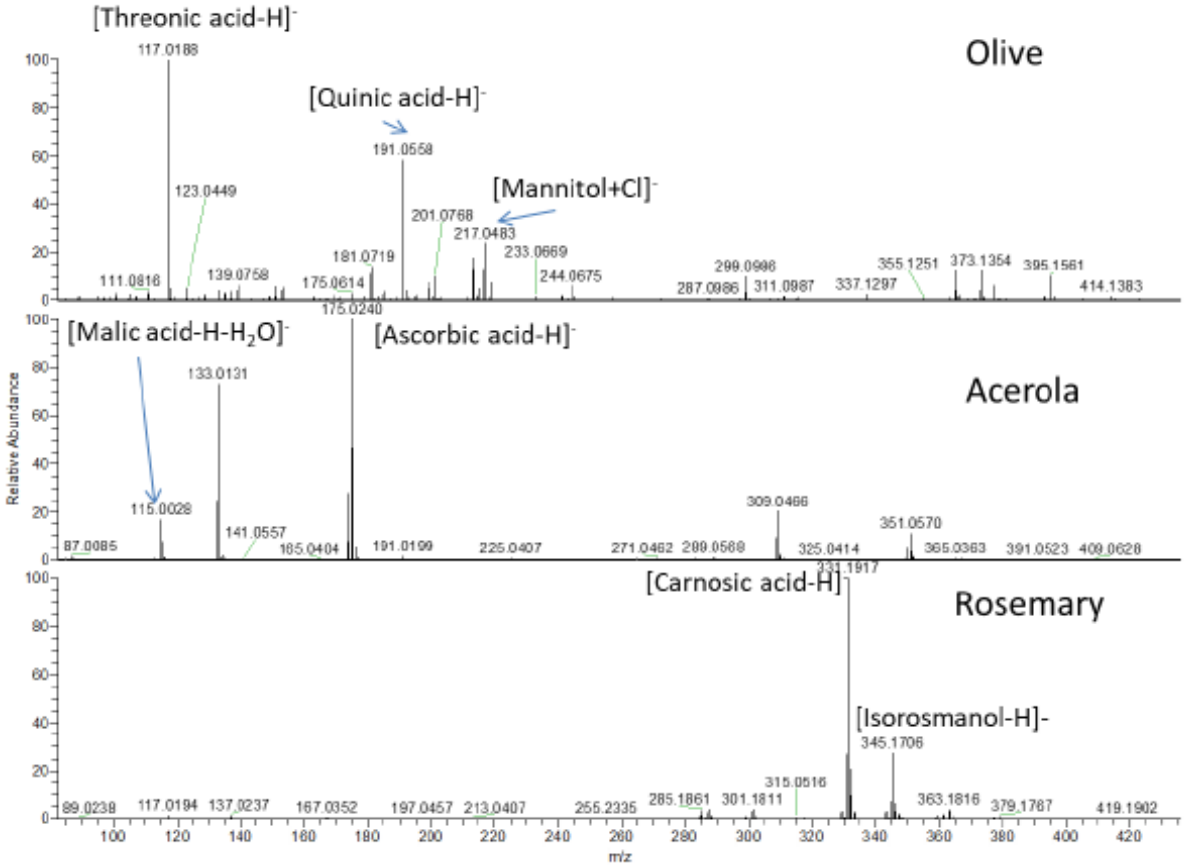


Figure 25. Secondary metabolites of olive, acerola, and rosemary extracts acquired in negative ion mode.

Table 5. List of metabolites, negatively ionized and observed in olive, acerola, and rosemary extracts. The metabolites were putatively annotated by interrogating the online library www.foodb.ca

Plant source	Observed *m/z*	Theoretical *m/z*	Error ppm	Type of ion	Number of charges	Putative annotation	Formula
Olive	117.0188	117.0188	0	[M+H-H2O]-	1	Threonic acid	$C_4H_8O_5$
	191.0558	191.0561	2	[M-H]-	1	Quinic acid	$C_7H_{12}O_6$
Acerola	115.0028	115.0031	3	[M-H-H2O]-	1	Malic acid	$C_4H_6O_5$
	175.0240	175.0248	5	[M-H]-	1	Ascorbic acid	$C_6H_8O_6$
Rosemary	331.1917	331.1915	1	[M-H]-	1	Carnosic acid	$C_{20}H_{28}O_4$

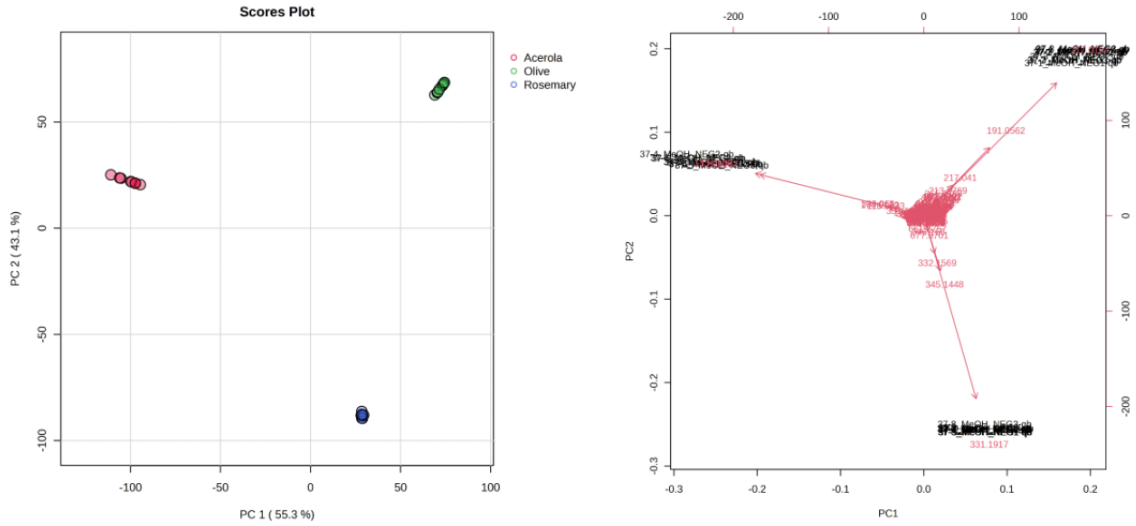


Figure 26. Statistical discrimination of olive, acerola, and rosemary extracts based on the (-) DART-HRMS data.

Box plots in Figure 27 were generated from a non-parametric ANOVA test with FDR adjustment. These box plots reveal that each of the three plant extracts were characterized by statistically different abundances of antioxidant compounds. The statistical significance of these three metabolites is supported by adjusted p-values of ≤ 0.05. Specifically, the adjusted p-values for ascorbic acid from acerola (m/z 177.0391), tyrosol from olive (m/z 121.0650), and carnosic acid from rosemary (m/z 331.1917) were determined to be 1.7535e-05, 1.7535e-05, and 2.566e-05, respectively. A comparison of the spectra from the three aliquots of each extract was conducted. The three aliquots were found to exhibit highly similar and reproducible profiles.

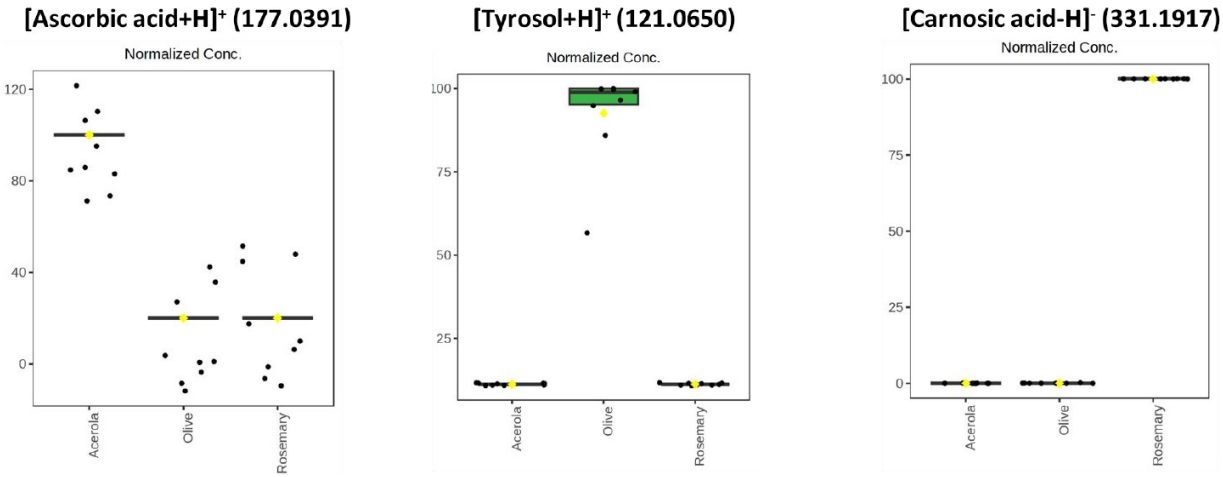


Figure 27. Box plots showing the differences in relative abundance of the three antioxidants that characterize each of the extracts.

3.5 Synergy of Acerola, Rosemary, and Olive (ARO) in Beef Patties

Metabolic fingerprinting is a fast approach to identify bioactive compounds and their abundances exhibiting antioxidative properties beneficial for the shelf-life of meat. The abundance of ascorbic acid from acerola, tyrosol from olive, and carnosic acid from rosemary indicated that these antioxidant compounds mainly inhibited lipid oxidation in beef patties. While the other bioactive compounds detected, though less in abundance, have antioxidative and even antimicrobial properties that also delayed oxidation rate and growth of spoilage microorganisms. The synergy of these compounds from the three plant extracts contributed to prolong freshness and redness of the patties compared to the positive control with sodium citrate. A summary of the detected antioxidants per plant extract is shown in Table 6.

Table 6. Bioactive compounds present in the plant extracts that exhibit antioxidative properties.

ANTIOXIDANTS		
OLIVE	**ACEROLA**	**ROSEMARY**
TYROSOL	ASCORBIC ACID	HESPERETIN
QUINIC ACID	MALIC ACID	CIRSIMARITIN
PANTOTHENIC ACID		CARNOSIC
		ISOROSMANOL

Previous studies have shown that synergism with rosemary extract provided better results such as the combined effect of ascorbic acid and rosemary extract enhanced the rosemary antioxidant activity in lard (Shahidi, 2015). Also, olive extract with rosemary extract prevented food spoilage and contamination with *Listeria monocytogenes* in meat (Bubonja-Sonje et al., 2011).

Additionally, in this research, the blend of plant extracts enhanced the flavor of the cooked burgers, demonstrating a synergistic effect. Rosemary contributed a distinct herbal note, but the combination of all three extracts created a balanced and well-rounded flavor profile. Acerola, known for its ability to reduce off-flavors, likely played a role in enhancing the overall acceptability of the burgers.

CONCLUSION

The growing consumer preference for clean-label foods and the shift towards natural antioxidants as food preservatives reflect an increasing awareness of the potential health risks associated with synthetic additives. The use of natural antioxidants offers a promising approach to enhance both the quality and shelf life of meat products.

This research project demonstrated that the blend of plant extracts from acerola, rosemary, and olive (ARO) at 0.4% concentration was effective in inhibiting lipid oxidation of beef patties. Also, it was able to prolong the redness of the patties compared to the controls. This indicates that the formulated natural solution is efficient for preserving meat, and this is attributed to the bioactive compounds exhibiting antioxidative and antimicrobial properties. Moreover, the blend added an herb flavor to the burger, perceiving it as acceptable to the trained panelists.

The use of DART-HRMS is a technological advantage in determining secondary metabolites such as natural antioxidants beneficial for maintaining quality and prolonging shelf-life of the beef burgers. This reliable tool provides rapid screening, metabolite fingerprinting, and quality control checking of natural products, making it a remarkable technology in the food industry.

As this study focused on the synergistic effects of the natural antioxidants in comparison to the standard (Sodium citrate, E331), future studies could investigate the individual effects of the same plant extracts on beef patties to provide the latest information on their individual antioxidant activities and intensities in varying concentrations. Another study should focus on clinical diagnostics to investigate the health impact of beef burgers with the blend of extracts to consumers. An example of the identified bioactive compound, cirsimaritin, in this study not only has antioxidative properties but also antimicrobial, anti-inflammatory, and anti-proliferative properties that are beneficial to human health. Other detected bioactive compounds exhibit similar and unique properties, and their combined effects might have the

potential to counteract cardiovascular diseases, cancer, type 2 diabetes, and other non-communicable diseases.

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