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Application of functional molecules recovered from bergamot by-products: development and improvement of food systems

PH.D. THESIS

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ABSTRACT

The Calabrian citrus sector is the primary world producer of bergamot (*Citrus bergamia Risso* & Poiteau). Bergamot is cultivated in a coastal portion of Reggio Calabria primarily for industrial processing to yield essential oil. The solid by-product, known as "pastazzo", constitutes approximately 50% of processed fruit's weight, representing a significant economic challenge for the industry due to its costly disposal.

Hence, the first aim of this thesis was to convert bergamot pomace (BP) into a valuable resource. The results from the first experiment, reported in the second chapter *("Recovery of Bioactive Compounds from Calabrian Bergamot Citrus Waste: Selection of Best Green Extraction"*), optimized an extraction system using food-grade solvents demonstrated the extraction of a substantial quantity of bioactive compounds, particularly phenolic compounds. The phenolic profile of BP revealed a high flavonoid content including eriocitrin (0.25 mg g^{-1} dw BP), neoeriocitrin (13. 95 mg g^{-1} dw BP), naringin (12.47 mg g^{-1} dw BP), neohesperidin (7.35 mg g^{-1} dw BP), melitidin (2.29 mg g^{-1} dw BP) and brutieridin (5.67 mg g^{-1} dw BP).

The antioxidant extract (AE), produced with the selected extraction technique (maceration extraction at 70°C for 30 minutes) was used in Chapter 3 (*"Fortification of Vegetable Fat with natural antioxidants recovered from Bergamot Pomace to use as ingredient for the production of biscuits")* for the functionalisation of a vegetable fat (VF) used as ingredient in the formulation of a bakery product (biscuits). VF exhibited functional and qualitative improvements, with increased antioxidant properties analysed through the total polyphenols content (TPC), total flavonoids (TFC) and ABTS and DPPH assays. Chromatographic analysis (UHPLC-DAD) confirmed the enrichment, contributing to improve oxidative stability detected with an OXITEST system. Enriched biscuits were comparable to the test sample in terms of moisture, colour and Maillard products. The antioxidant compounds added with VF exhibited resistance to heat treatment, further enhancing the oxidative stability of the biscuits.

Another research investigated an edible coating (Chapter 4: *"Effect of Edible Coating Enriched with Natural Antioxidant Extract and Bergamot Essential Oil on the Shelf Life of Strawberries"*) enriched with varying percentages of AE (based on gum Arabic) applied to strawberries to estimate their shelf life in terms of weight loss, decay, TPC, bioactive compounds, organic acids, microbial contamination and maintenance of quality

parameters such as colour and texture. This application significantly improved the quality of the strawberries during the storage period.

Chapter 5 *("Microencapsulation of antioxidant extract recovered by bergamot pomace through freeze-drying methodology and application in hydrophilic and lipophilic food systems"*) focused on improving the liquid extract by microencapsulating the AE for better stability and resistance to degradation. The microencapsulate was used to enrich hydrophilic (apple juice) and lipophilic (sunflower oil) food systems, evaluating the physicochemical characteristics and the release of antioxidant compounds during storage (up to 90 days) at 25°C.

Following the principles of a circular economy, BP flour (BF) and BF after phenol compounds extraction (BFE) were used as ingredients to replace durum wheat flour in pasta production (Chapter 6: *"Bergamot pomace flour: a functional ingredient for pasta production"*). The functional, structural and sensory properties of pasta produced with two concentrations of BF and BFE (2.5 and 5%) were studied. The research results emphasized that BP can be considered an excellent processing aid for producing functional foods.

The physicochemical characteristics of BP (antioxidants, fibre, minerals) offer opportunities for develop new nutraceuticals or natural preservatives applicable to the food industry enhancing products quality and extend shelf-life valorising BP.

RIASSUNTO

Il comparto agrumicolo calabrese è il primo produttore mondiale di bergamotto (*Citrus bergamia Risso* & Poiteau). Il bergamotto è coltivato in una striscia costiera di Reggio Calabria e destinato principalmente all'industria per l'estrazione di olio essenziale. Il sottoprodotto solido, noto come "pastazzo", costituisce circa il 50% del peso del frutto lavorato, rappresentando una sfida economica significativa per l'industria a causa del suo oneroso smaltimento.

Pertanto, il primo obiettivo di questa tesi è stato quello di convertire il pastazzo di bergamotto (BP) in una preziosa risorsa. I risultati della prima sperimentazione, riportati nel secondo capitolo *("Recovery of Bioactive Compounds from Calabrian Bergamot Citrus Waste: Selection of Best Green Extraction"*), attraverso l'ottimizzazione di un sistema di estrazione con solventi di grado alimentare, hanno dimostrato come sia possibile estrarre una quantità sostanziale di composti bioattivi, in particolare di composti fenolici. Il profilo fenolico del BP ha evidenziato un elevato contenuto di flavonoidi, tra cui eriocitrina (0.25 mg g^{-1} dw BP), neoeriocitrina (13.95 mg g^{-1} ss BP), naringina (12.47 mg g^{-1} ss BP), neoesperidina (7.35 mg g^{-1} ss BP), melitidina (2.29 mg g^{-1} ss BP) e brutieridina (5.67 mg g^{-1} ss BP).

L'estratto antiossidante (AE), ottenuto con la tecnica di estrazione selezionata (estrazione per macerazione a 70°C per 30 minuti), è stato utilizzato nel capitolo 3 (*"Fortification of Vegetable Fat with natural antioxidants recovered from Bergamot Pomace to use as ingredient for the production of biscuits"*) per la funzionalizzazione di un grasso vegetale (VF) utilizzato come ingrediente nella formulazione di un prodotto da forno (biscotto). Il VF ha mostrato miglioramenti funzionali e qualitativi, con un aumento delle proprietà antiossidanti analizzate attraverso il contenuto di polifenoli totali (TPC), flavonoidi totali (TFC) e saggi ABTS e DPPH. L'analisi cromatografica (UHPLC-DAD) ha confermato l'arricchimento, contribuendo a migliorare la stabilità ossidativa rilevata con un sistema OXITEST. I biscotti arricchiti erano paragonabili al campione testimone in termini di umidità, colore e prodotti Maillard. I composti antiossidanti aggiunti al VF hanno mostrato resistenza al trattamento termico, migliorando ulteriormente la stabilità ossidativa dei biscotti.

Un'altra ricerca condotta ha studiato un rivestimento edibile (Capitolo 4: *"Effect of Edible Coating Enriched with Natural Antioxidant Extract and Bergamot Essential Oil*

on the Shelf Life of Strawberries") arricchito con varie percentuali di AE (a base di gomma arabica) applicato alle fragole per stimare la loro shelf-life in termini di perdita di peso, decadimento, TPC, composti bioattivi, acidi organici, contaminazione microbica e mantenimento di parametri qualitativi come colore e consistenza. Questa applicazione ha migliorato significativamente la qualità delle fragole durante il periodo di conservazione.

Il capitolo 5 (*"Microencapsulation of antioxidant extract recovered by bergamot pomace through freeze-drying methodology and application in hydrophilic and lipophilic food systems"*) si è concentrato sul miglioramento dell'estratto liquido mediante microincapsulazione dell'AE per una migliore stabilità e resistenza alla degradazione. Il microincapsulato è stato utilizzato per arricchire sistemi alimentari idrofili (succo di mela) e lipofili (olio di semi di girasole), valutando le caratteristiche chimico-fisiche e il rilascio di composti antiossidanti durante la conservazione (fino a 90 giorni) a 25°C.

Seguendo i principi dell'economia circolare, la farina di BP (BF) e la BF dopo l'estrazione dei composti fenolici (BFE) sono state utilizzate come ingrediente per sostituire la farina di grano duro nella produzione di pasta (Capitolo 6: *"Bergamot pomace flour: a functional ingredient for pasta production"*). Sono state studiate le proprietà funzionali e sensoriali della pasta prodotta con due concentrazioni di BF e BFE (2.5 e 5%). I risultati della ricerca hanno evidenziato che il BP può essere considerato un eccellente coadiuvante tecnologico per la produzione di alimenti funzionali.

Le caratteristiche fisico-chimiche del BP (antiossidanti, fibre, minerali) offrono l'opportunità di sviluppare nuovi ingredienti nutraceutici o conservanti naturali utilizzabili nell'industria alimentare per migliorare la qualità dei prodotti e prolungarne la shelf life, valorizzando il BP.

KEYWORDS

Bergamot pomace; phenolic compounds; vegetable fat; bakery products; edible coating; microencapsulate; enriched pasta.

1. GENERAL INTRODUCTION

Nowadays, the world is facing a challenge that affects the whole planet. Global pollution is changing the world, and human activities are responsible for this. Country governments are addressing important issues such as the overconsumption of natural resources, environmental pollution, uncontrolled waste generation (Sadhukhan et al., 2020; Greyson, 2007; Abad-Segura et al., 2021), searching sustainable solutions including the use of new technologies to reduce gas emissions, encouraging the circular economy by the entire society and zero-waste strategies (Hailemariam & Erdiaw-Kwasie, 2022; Debnath & Sarkar, 2023; Song et al., 2015).

In this context, waste generation in agro-food industry is one of the most significative problems, relating not only to the wastage but also to the huge consumption of resources. All sectors of this industry are involved in the production of waste with different percentages (Figure 1.1).

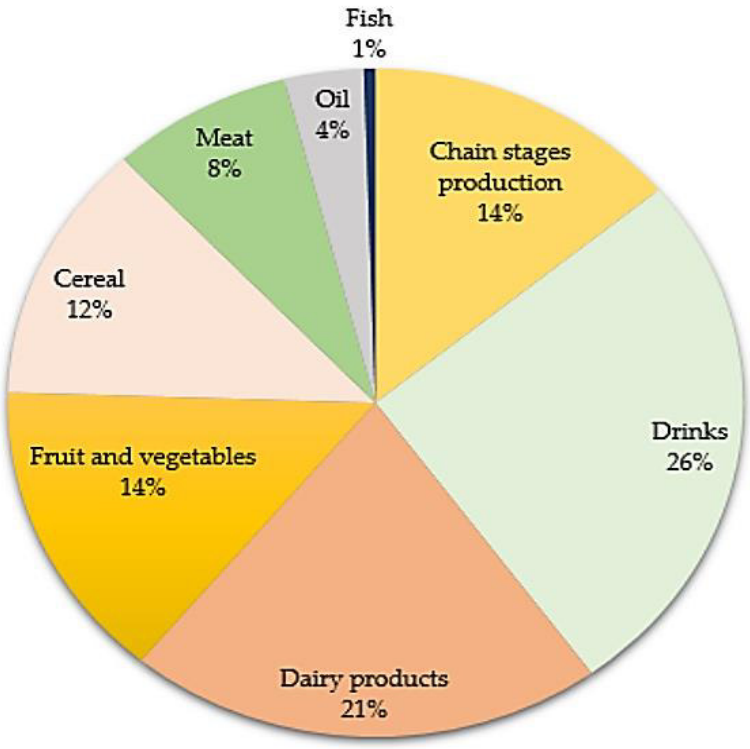


Figure 1.1. Distribution of waste production in agro-food industry (Pérez-Marroquín et al., 2023).

Among fruits and vegetables, citrus fruits are the crop largely cultivated around the world. This results in a copious amount of by products (solid residue and wastewater) from its transformation. Solid residue, also called pomace, consists of seeds, pulp residue

(membranes, juice sacs), portion of peels and albedo (Caballero et al., 2021). As is well known, citrus pomace (CP), due to its richness in phytochemical compounds, the low pH comprises between 3 and 4, the high fermentability of sugars and acids, can be a pollutant to soil and water if managed incorrectly and in disagreement with legal regulations. The common and traditional use over the last years was in feeding animals, soil conditioning, in compost production (Zema et al., 2018), in the extraction of pectin and fibre, high value-added compounds and more others. In view of this, in the last decades CP have been used also in biorefinery production (Yadav et al., 2022), in the recovery of chemical constituents usable in food, pharmaceutical, cosmetic, chemical, medicine and food industries (Figure 1.2) (Mahato et al., 2017; Sharma et al., 2018; De Albuquerque et al., 2019; Wang et al., 2018; Panwar et al., 2021).

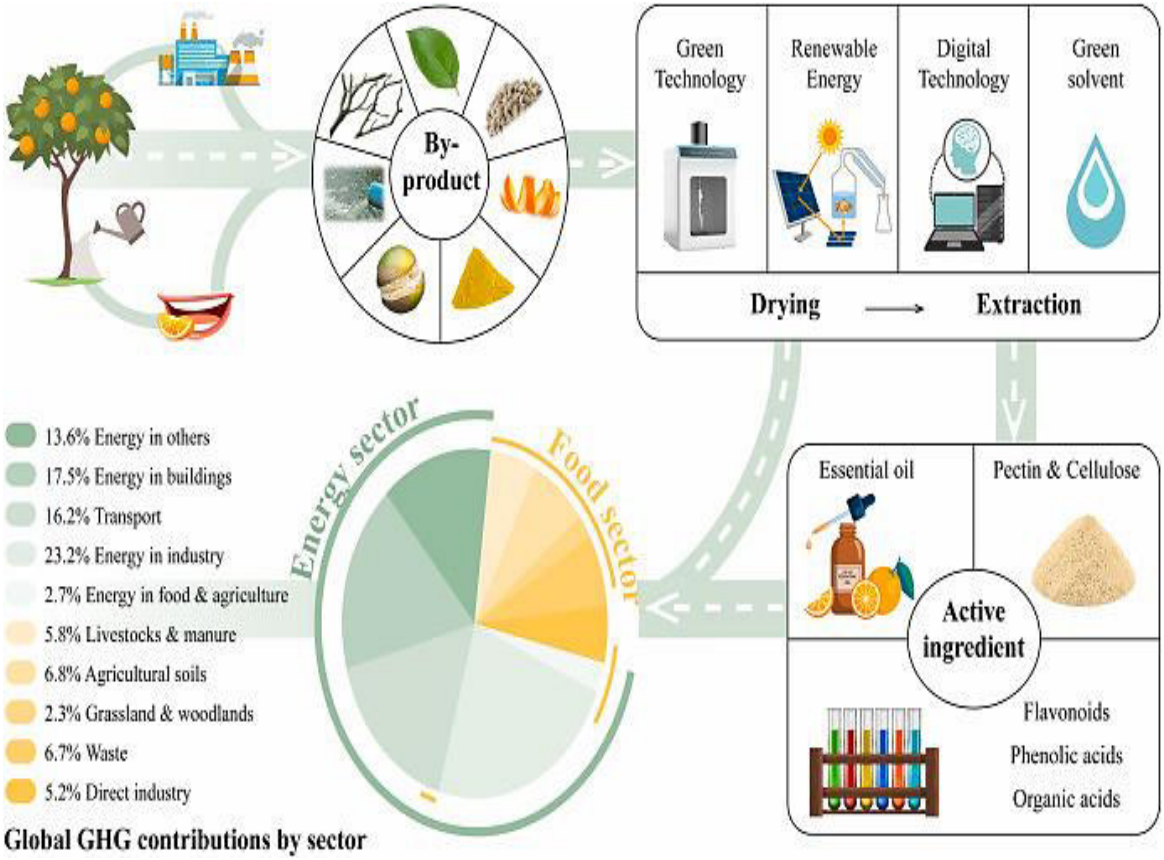


Figure 1.2. Representation of citrus by-products utilization (Xu et al., 2023).

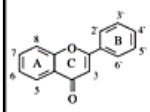
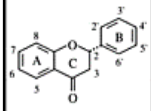
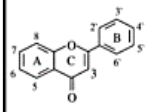
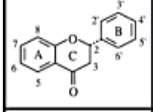
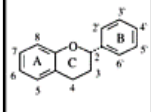
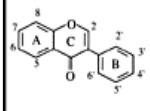
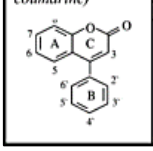
The usefulness of CP in the various sectors described above, is given by the abundant presence of several bioactive compounds including:

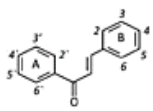
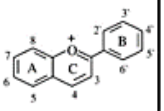
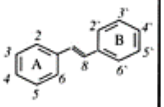
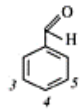
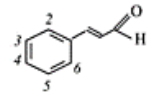
- polyphenols;

- essential oil;
- pectin;
- dietary fibre;
- fatty acids (especially in seeds).

Polyphenols, which are the main class of bioactive compound addressed in this thesis, are molecules naturally and abundantly present in plant kingdom in all parts of the plant (seeds, flowers, leaves, wood etc). The distinctive feature of polyphenols is the structure formed by hydroxyl groups and a single or double aromatic ring (Han et al., 2007). Based on the phenolic rings' number, they are differentiated into flavonoids (flavones, flavanones, isoflavones, flavonols, anthocyanidins, and flavanols); phenolic acids divided into subclasses (derived from hydroxybenzoic acids for i.e. protocatechuic, syringic, vanillic, hydroxybenzoic, gallic acids; and derived from hydroxycinnamic acid such as coumaric, chlorogenic, ferulic, sinapic and caffeic acid); stilbenes, lignans and polyphenol amides (Table 1.1.) (Singla et al., 2019; Baniwal et al., 2021).

Table 1.1. Polyphenols classification (Papuc et al., 2017).

Class name, sub-classe name and the backbone structure	Examples	Position of hydroxyl groups / substituted hydroxyl groups / other substituents												Sources
1. Flavonoids														
		2	**3**	**4**	**5**	**6**	**7**	**8**	**2`**	**3`**	**4`**	**5`**	**6`**	
Flavones 	Luteolin	–	–	–	OH	–	OH	–	–	OH	OH	–	–	Curly endive (*Cichorium endivia*), Alfalfa (*Medicago sativa*), Indian trumpet flower (*Oroxylum indicum*), Baikal skullcap
	Apigenin	–	–	–	OH	–	OH	–	–	–	OH	–	–	
	Chrysin	–	–	–	OH	–	OH	–	–	–	–	–	–	
	Baicalein	–	–	–	OH	OH	OH	–	–	–	–	–	–	
Flavanones 	Hesperetin	–	–	–	OH	–	OH	–	–	OH	OCH_3	–	–	Citrus and grapefruit peels
	Naringenin	–	–	–	OH	–	OH	–	–	–	OH	–	–	
Flavonols 	Quercetin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Propolis, honey, chamomile and linden, *Ginkgo biloba*, Blue gum eucalyptus.
	Kaempferol	–	OH	–	OH	–	OH	–	–	–	OH	–	–	
	Galangin	–	OH	–	OH	–	OH	–	–	–	–	–	–	
	Fisetin	–	OH	–	–	–	OH	–	–	OH	OH	–	–	
	Myricetin	–	OH	–	OH	–	OH	–	–	OH	OH	OH	–	
	Morin	–	OH	–	OH	–	OH	–	OH	–	OH	–	–	
	Hyperoside	–	OGal	–	OH	–	OH	–	–	OH	OH	–	–	
	Heliosin	–	OdiGal	–	OH	–	OH	–	–	OH	OH	–	–	
Flavanonols 	Taxifolin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Dahurian larch (*Larix gmelinii*), Chinese lacquer tree
	Fustin	–	OH	–	–	–	OH	–	–	OH	OH	–	–	
Flavan-3-ols 	***Monomers***													Green tea, black tea, cocoa
	(+)-Catechin	–	βOH	–	OH	–	OH	–	–	OH	OH	–	–	
	(-)-Epicatechin	–	αOH	–	OH	–	OH	–	–	OH	OH	–	–	
	(-)-Epigallocatechin	–	αOH	–	OH	–	OH	–	–	OH	OH	OH	–	
	(-)-Epicatechin-3-gallate	–	αOGallate	–	OH	–	OH	–	–	OH	OH	–	–	
	(-)-Epigallogatechin-3-gallate	–	αOGallate	–	OH	–	OH	–	–	OH	OH	OH	–	
	Polymers Proanthocyanidins or condensed tannins	Oxidative condensation of the monomeric units (a flavan-3-ols) between C4 and C6 or C8												Woody plants, fruits and beverages such as beer and wine
Isoflavones 	Genistein	–	–		OH	–	OH	–	–	–	OH		–	Leguminous family plants (soybean), red clover
	Genistin	–	–	–	OH	–	OGl	–	–	–	OH	–	–	
	Daidzein	–	–	–	–	–	OH	–	–	–	OH	–	–	
	Daidzin	–	–	–	–	–	OGl	–	–	–	OH	–	–	
	Biochanin A	–	–	–	OH	–	OH	–	–	–	OCH_3	–	–	
	Formononetin	–	–	–	–	–	OH	–	–	–	OCH_3	–	–	
Neoflavonoids (4-phenyl coumarine) 	Dalbergin		–	–	–	OH	OCH_3	–	–	–	–	–	–	Widely distributed in plant kingdom
	Calophyllolide	Complex structure												
	Inophyllums B, P, G, F	Complex structure												

Class name, sub-classe name and the backbone structure	Examples	Position of hydroxyl groups / substituted hydroxyl groups / other substituents												Sources
		2	3	4			7	8	2`	3`	4`	5`	6`	
Chalcones 	Isoliquiritigenin	–	–	OH	–	–	–	–	OH	–	OH	–	–	Apples, flowers, hop, beer
	Flavokawain A	–	–	OCH_3	–	–	–	–	OH	–	OCH_3	–	OCH_3	
	Flavokawain B	–	–	–	–	–	–	–	OH	–	OCH_3	–	OCH_3	
	Flavokawain C	–	–	OH	–	–	–	–	OH	–	OCH_3	–	OCH_3	
	Gymnogrammene	–	–	OCH_3	–	–	–	–	OH	–	OCH_3	–	OH	
Anthocyanidins 	Cyanidin	–	OH	–	OH	–	OH	–	–	OH	OH	–	–	Red and blue flowers petals, fruits and vegetables
	Pelargonidin	–	OH	–	OH	–	OH	–	–	–	OH	–	–	
	Peonidin	–	OH	–	OH	–	OH	–	–	OCH_3	OH	–	–	
	Delphinidin	–	OH	–	OH	–	OH	–	–	OH	OH	OH	–	
	Petunidin	–	OH	–	OH	–	OH	–	–	OH	OH	OCH_3	–	
	Malvidin	–	OH	–	OH	–	OH	–	–	OCH_3	OH	OCH_3	–	
	Anthocyanins													
	Cyanidin-3-glucoside	–	OGl	–	OH	–	OH	–	–	OH	OH	–	–	
	Cyanidin-3-rutinoside	–	ORu	–	OH	–	OH	–	–	OH	OH	–	–	
	Cyanin	–	OGl	–	OGl	–	OH	–	–	OH	OH	–	–	
	Pelargonidin-3-glucoside	–	OGl	–	OH	–	OH	–	–	–	OH	–	–	
2.Stilbenoids														
Stilbenoids 	*trans*-Resveratrol	–	OH	–	OH	–	–	–	–	–	OH	–	–	Red grapes, wine, blueberries, peanuts, dark chocolate, *Cajanus cajan*, sorghum
	trans-Piceatannol	–	OH	OH	–	–	–	–	–	OH	–	OH	–	
	trans-Piceid	–	OGl	–	OH	–	–	–	–	–	OH	–	–	
	trans-Pterostilbene	–	OCH_3	–	OCH_3	–	–	–	–	–	OH	–	–	
	Cajanotone	C_5H_9	OCH_3	–	OH	–	–	OH	–	–	–	–	–	
	Cajanamide	C_5H_9	OCH_3	–	OH	CO		NH	–	–	–	–	–	
3.Phenolic acids														
Benzoic acids  *C6 – C1*	***Monomers***													Fruits and vegetables
	p-Hydroxybenzoic acid	–	–	OH	–	–	–	–	–	–	–	–	–	
	Gallic acid	–	OH	OH	OH	–	–	–	–	–	–	–	–	
	Protocatechuic acid(3,4)	–	OH	OH	–	–	–	–	–	–	–	–	–	
	Ellagic acid	*Dilactone of the gallic acid*												
	Polymers *Hydrolyzable tannins (gallotannins and ellagitannins)*	*Derivatives of gallic acid or ellagic acid*												Chinese rhubarb
Hydroxycinnamic acids  *C6 – C3*	Caffeic acid	–	OH	OH	–	–	–	–	–	–	–	–	–	Fruits and vegetables, coffee
	p-Coumaric acid	–	–	OH	–	–	–	–	–	–	–	–	–	
	Ferulic acid	–	OCH_3	OH	–	–	–	–	–	–	–	–	–	
	Sinapic acid	–	OCH_3	OH	OCH_3	–	–	–	–	–	–	–	–	
	Curcumin (diferuloylmethane)	Condensation of two ferulic acid by a methylen group												Turmeric (*Curcuma longa*)
	Chlorogenic acids Chlorogenic acid 5-Caffeoylquinic acid *Caffeoylferuloylquinic acids*	Esters of hydroxycinnamic acids with quinic acid 3-O-Ester of caffeic acid with (-)-quinic acid 5-O- Ester of caffeic acid with (-)-quinic acid Esters of caffeic and ferulic acids with quinic acid												Fruits, coffee, potatoes, roselle (*Hibiscus sabdariffa*)

Their quantity and type depend on several conditions such as environment, phenological phase and functions for instance protection against microbial attack, ultraviolet radiation (UV) and predation by animals (Cutrim et al., 2018). The vast range of functions is due to the fact that polyphenols exert numerous functions such as antimicrobial, antifungal, antioxidant (Rasouli et al., 2017; Ullah et al., 2022).

Nevertheless, the structure of polyphenols, containing phenolic hydroxyl groups, rendering them susceptible to degradation when exposed to light, high temperatures, alkaline environments and negatively affecting the bioavailability (de Oliveira et al., 2016). The microencapsulation technique is used to overcome these factors which can compromise the functionality of the extract. This, allowed to bind (non-covalent/covalent binding) polyphenols to biopolymers, enhancing the stability, the bioaccessibility and preserving its antioxidative properties (Ćorković et al., 2021; Neuenfeldt et al., 2022; Fang & Bhandari 2010).

The extraction of these bioactive compounds has allowed to take advantages for the human activities. Clinical trials and scientific studies have shown its beneficial effects (direct and indirect) on human health and the possibilities to employ their peculiarities in the various industrial sectors (pharmaceutical, cosmetic, food).

In these fields polyphenols showed a great interest for their effect in the prevention of cardiovascular disease and hypertension (Grosso et al., 2022), as anti-aging agent (Wu et al., 2020), anti-neurodegenerative disease (Di Meo et al., 2020), anti-cancer and anti-diabetic (Rana et al., 2022), can improve the effect of positive bacteria such as Bifidobacterium and Lactobacillus (Lavefve et al., 2020) and many other benefits. In particular in food, they have benefit effects related to human health, but they can also exert activity against microbial growth enhancing the shelf life of products (Aguilar-Veloz et al., 2020; de Moraes Crizel et al., 2018; Yuan et al., 2015), acting as preservatives (Bouarab Chibane et al., 2019), as natural antioxidant delaying lipids and nutrients oxidation (Wang et al., 2023; Tian et al., 2022), in colour and flavour stability (Yang et al., 2023; De Francesco et al., 2020).

In view of the above, CP needs to be valorised through total and sustainable recycling of the numerous bioactive compounds, whose can be reused as natural income resources by overcoming traditional disposal methods.

This PhD research considered to valorise a CP typical of Reggio Calabria (Calabria, Italy), derived from bergamot (*Citrus bergamia Risso*) processing. Bergamot is a citrus fruit grown exclusively in the province of Reggio Calabria. It has the chemical characteristics of a common citrus fruit, that enable it to exercise many beneficial properties to the human health. In the last years, many researchers have paid attention to this fruit for its medical, perfumery, cosmetics and food interest. Large part of the bergamot fruits is intended for the processing industry for the main production of the essential oil.

The essential oil extracted from the peel, is widely used in cosmetic, pharmaceutical, and food industries (Mandalari et al., 2006), while its juice, obtained from pressing fruits, due to its bitter taste is used in combination with other fruit juices to prepare great beverages (Giuffrè et al., 2019). Moreover, bergamot is a natural source of vitamins A, C, B, sugar, pectin, fiber, and different compounds with biological properties (Postorino et al., 2001). For these features, is considered as a functional food containing antioxidants and bioactive compounds with beneficial effects on human health that have been widely studied regarding diabetes, cancer, Alzheimer's disease, insulin resistance, neuro-disease, antioxidant, anti-inflammatory, and cholesterol reducing functions (Qiu et al., 2018; Risitano et al., 2014; Ferlazzo et al., 2015; Nauman et al., 2019).

Bergamot represents a valuable source of bioactive molecules with a distinctive bitter taste, containing mainly the flavanones neohesperidin, naringin, neoeriocitrin, and minor amounts of flavones and furanocoumarins (Da Pozzo et al., 2018; Tsiokanos et al., 2021). It has been shown that flavonoids have different health properties, especially determined by their antioxidant constituents (Mandalari et al., 2007). Particularly, in recent years, some studies have focused on flavonoids of bergamot because they have important properties, such as protection against some types of cardiovascular diseases (Cappello et al., 2005; Benavente-Garcia et al., 2008). For example, neohesperidin and neoeriocitrin have efficient properties in osteoporosis treatment, while naringin possesses anti-inflammatory and anti-tumour characteristics (Liu et al., 2017; Tan et al., 2017; Li et al., 2011). Two characteristic flavonoids (C-glycosyl flavones) typical of bergamot are brutieridin and melitidin. In fact, brutieridin is named for the name of the city Cosenza (Brutium), where studied, while melitidin derives from Melito Porto Salvo, a locality with the greatest vocation for the cultivation of bergamot located in the province of Reggio

Calabria. These two statin-like flavonoids are considered as natural active components due to their capacity to lower blood cholesterol levels (Di Donna et al., 2014). Musolino et al., 2019, showed that bergamot polyphenolic fraction (BPF) produces hypolipemic effect in organisms undergoing a fat-rich diet, proving that this biomolecules fraction used as dietary supplementation in hyperlipemic disease states, antagonize cardiometabolic risk. This effect confirmed what Walker et al., 2014 reported, who studied cardiometabolic risk prevention of BPF and assuming its mechanisms of action.

Today's modern extraction machines allow to extract essential oil and juice during the same processing cycle.

As a consequence of the above and of the significant increase in new bergamot plantations, it is necessary to look for innovative, sustainable and as yet little researched solutions for the utilisation of the by-product of bergamot, reducing disposal costs, linked to the production of solid waste, and the consumption of natural resources for by-product recovery.

1.1. Research works organisation

The aim of this PhD thesis was that focus attention on the reuse of bergamot pomace (BP), through the recovery of the bioactive compounds present within them and subsequent application in food sector as ingredient/preservative useful both for the food preservation and for the daily human intake as natural elements. Specifically, the Ph.D. project was organised into main steps. The first one consisted in the selection of a food grade extraction technique of bioactive compounds (**Chapter 2: "Recovery of Bioactive Compounds from Calabrian Bergamot Citrus Waste: Selection of Best Green Extraction"; article published in the journal "*Agriculture*" 2023, *13*, 1095. https://doi.org/10.3390/agriculture13051095**). This research line aimed at selecting a method of extraction of bioactive compounds, in particular polyphenols, to optimize the extraction yield. The procedure was carried out with food-grade solvents in order to obtain an antioxidant extract (AE) containing the high biological value compounds for the food sector.

Thus, the extraction method selected was the one used in other trials in which the AE was used. In Chapter 3 **("Fortification of Vegetable Fat with Natural Antioxidants Recovered by Bergamot Pomace to Use as Ingredient for the Production of Biscuits"; article published in the journal "*Sustainable Food Technology*", Royal**

Society of Chemistry, 2023, doi.https://doi.org/10.1039/D3FB00125C), AE was used as a functional ingredient in the formulation of a vegetable fat (VF). The VF was included in the production of a bakery product, a sweet biscuit, replacing the common butter used in baked good production.

Hence, the effect of AE was tested in a dipping solution in which strawberries were coated (**Chapter 4: "Effect of Edible Coating Enriched with Natural Antioxidant Extract and Bergamot Essential Oil on the Shelf Life of Strawberries"; article published in the journal *Foods (Basel, Switzerland)*, *12*(3), 488. https://doi.org/10.3390/foods12030488**). This application allowed to extend shelf life of minimally treated strawberry fruits and to prolong the retention of quality parameters during storage time reducing quality and quantity losses.

In the chapter 5, entitled **"Microencapsulation of antioxidant extract recovered by bergamot pomace through freeze-drying methodology and application in hydrophilic and lipophilic food systems"** (Article prepared for publication), the AE was microencapsulated as a result of a previous series of tests by considering different coating agents and coating agent/extract ratios. The microencapsulate was characterised and used as a functional ingredient in two different liquid food systems (hydrophilic and lipophilic system). The study carried out monitoring the enriched products, an apple juice and a sunflower oil, during a storage period of 90 days at controlled temperature (25°C). Specifically, the antioxidant activity and the main phenolic compounds were studied following the release trend. In sunflower oil the oxidative stability was also studied.

The subsequent chapter (**Chapter 6: "Bergamot pomace flour: a functional ingredient for pasta production"**) (Article prepared for publication) investigated the use of BP flour to produce pasta, considering physicochemical, antioxidant, structural and sensorial characteristics. Moreover, BF recovered after the extraction of phenolic compounds was also used to enrich pasta.

The study cases considered in the present Ph.D. thesis are reported in Figure 1.3.

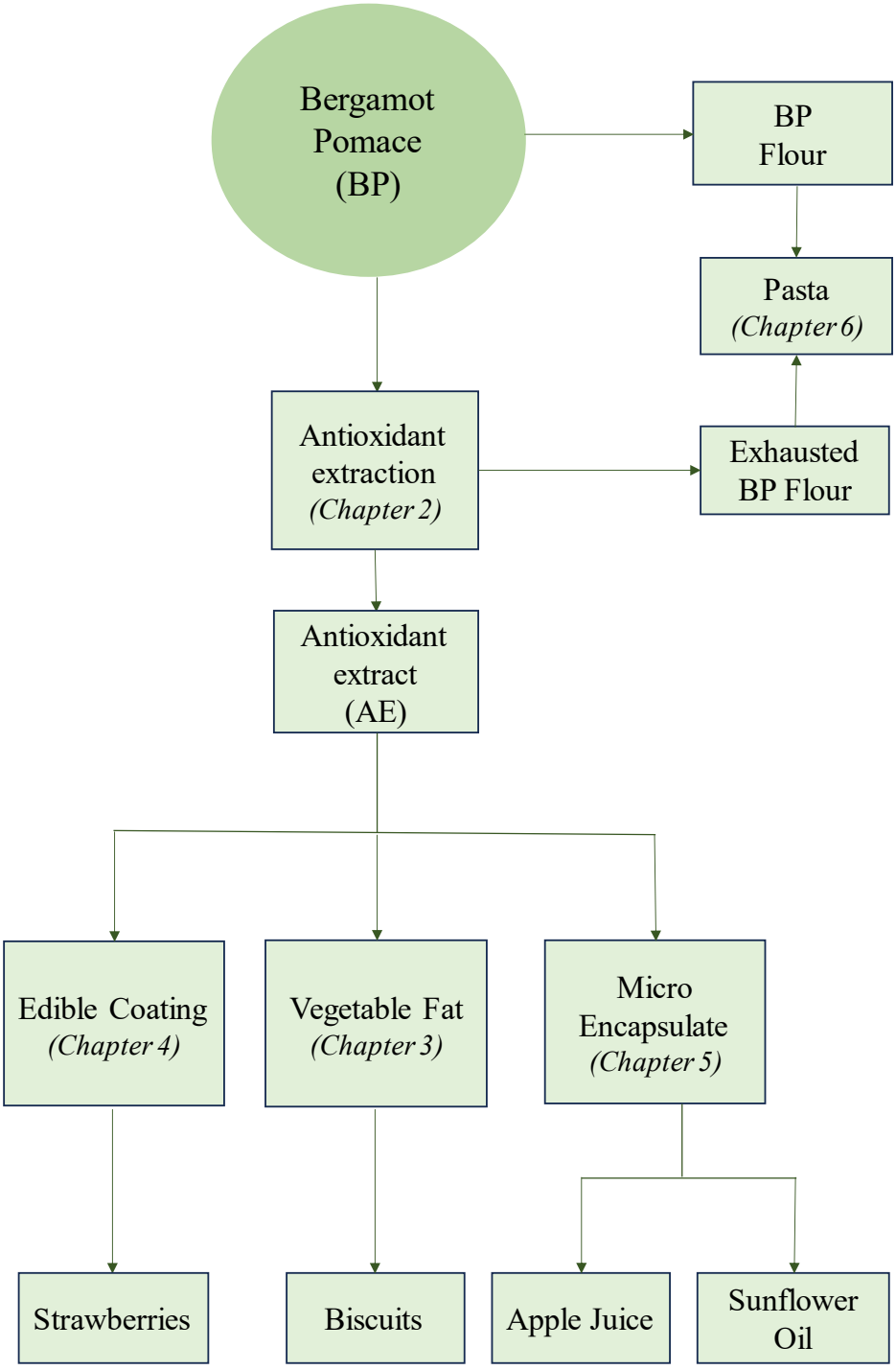


Figure 1.3. Schematic overview of the Ph.D. activities.

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2. RECOVERY OF BIOACTIVE COMPOUNDS FROM CALABRIAN BERGAMOT CITRUS WASTE: SELECTION OF BEST GREEN EXTRACTION

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Abstract

The purpose of this study was to select the best green extraction technique to recover the bioactive compounds in Calabrian Bergamot waste (Pomace). Different experimental variables such as solvent, time, and temperature were tested and the main physicochemical characteristics and antioxidant activity and constituents, such as total flavonoids, individual flavonoids, and limonoids (UHPLC-DAD) were analysed. Later, the best extraction methodology was applied to characterize the individual portions that compose the bergamot pomace (albedo/pulp, seeds, and juice) of three different Calabrian cultivars: Fantastico, Femminello, and Castagnaro. Results of this study evidence that bergamot waste possesses a high antioxidant content that can be potentially used for further applications in the food industry.

Keywords: antioxidant activity; bergamot pomace; bioactive compounds; *Citrus bergamia Risso*; green extraction; microwave assisted; ultrasound assisted; waste

Chapter 2. Graphical abstract

2.1. Introduction

Scientific studies showed that the Mediterranean diet, mainly linked to the intake of antioxidant components contained in foods, leads to a lower risk of developing age-related vascular disease, with an increase in longevity. Citrus fruits are included among the foods that contain a high content of antioxidant compounds, particularly flavonoids. One of the most important citrus fruits grown in Calabria is Bergamot which represents a valuable source of active molecules that contribute to antioxidant, anti-inflammatory, and cholesterol reduction capacities (Da Pozzo et al., 2018; Schwingshackl et al., 2020).

Citrus bergamia Risso, commonly named Bergamot, is a hybrid plant of lemon and sour orange, belonging to the Rutaceae family. About 90% of the world's production is concentrated in Italy, in the province of Reggio Calabria (Tsiokanos et al., 2021; Strano et al., 2017), characterized by a microclimate suitable for their growth. Bergamot peel is used to extract a valuable essential oil (claimed DPI since 1999 from the European Union), obtained by rasping and cold pressing the fruit peel as defined by International Organization for Standardization (ISO 9235:2021). Essential oil is widely employed in pharmaceutical, cosmetic, and food industries (Di Donna et al., 2020); while its juice, obtained from the endocarp of the fruit, in the last few years has been used to formulate beverages mixed with other fruit juices, due to its bitter taste (Giuffrè, 2019).

From the bergamot essential oil extraction process, about 50–65% become waste (peel, mesocarp, and juice) that needs to be managed by the manufacturing industry (Gabriele et al., 2017). The high amount of annually generated waste, if not properly managed, can be a major environmental problem. For this reason, the recovery of these wastes and their

use for the extraction of natural antioxidants can represent a valid sustainable alternative (Imeneo et al., 2022).

Bergamot fruits and their derivative products, called also “Pomace” have aroused much interest in the scientific community, because of their beneficial effects on human health. These, represent a valuable source of bioactive molecules with a distinctive bitter taste, containing mainly the flavanones neohesperidin, naringin, neoeriocitrin, and minor amounts of flavones and furanocoumarins [1,3]. It has been shown that flavonoids have different health properties, especially determined by their antioxidant constituents (Mandalari et al., 2007). Particularly, in recent years, some studies have focused on flavonoids of bergamot because they have important properties, such as protection against some types of cardiovascular diseases (Cappello et al.,2015; Benavente-Garcia et al., 2008). For example, neohesperidin and neoeriocitrin have efficient properties in osteoporosis treatment, while naringin possesses anti-inflammatory and anti-tumor characteristics (Liu et al., 2017; Tan et al., 2017; Li et al; 2011). Moreover, bergamot is a natural source of vitamins A, C, and B, sugars, pectin, fiber, and other compounds with biological properties (Postorino et al., 2001; Laratta et al., 2008; Ferro et al., 2020; Mandalari et al., 2016).

Over the years, several researchers have proposed different extraction methods for bioactive compounds, but nowadays, is essential that these extraction techniques are environmentally friendly, safe, and non-toxic, namely, “green”. Green technology can be defined as a method useful to improve not only the production processes but also the environmental impact, for this reason, the application of solvents with improved characteristics such as pressurized and supercritical fluids, ionic liquids, deep eutectic solvents or high static pressure, were widely studied (Abeysinghe et al., 2007; Rodríguez-Rojo et al., 2021; Choi et al., 2019). Other applied techniques, instead, use nonconventional energies, such as ultrasound and microwave-assisted extraction, and are very versatile and applicable to different matrices, with ease of application and low consumption (Banerjee et al., 2017; Chemat et al., 2017; Imeneo et al., 2022). The adoption of alternate extraction systems is helpful to reduce the drawbacks connected to the use of methodologies too expensive and not economically convenient and to increase the extraction yields.

The present study aimed to select the best green extraction useful to maximize the recovery of phytochemical compounds present in bergamot pomace. In this regard, several extraction procedures were tested, and the obtained extracts were investigated for their antioxidant properties both spectrophotometrically and through ultrahigh-performance liquid chromatography (UHPLC). Subsequently, the best extraction technique was selected to analyze the individual portions that constitute the fruit in the three Calabrian cultivars of bergamot: Castagnaro, Femminello, and Fantastico. Castagnaro is a cultivar that allows the harvest of the fruits for a longer time, but the quality of essential oil (EO) is the least valuable and the production is not constant in different production years; Femminello is characterized by a low yield of EO, but excellent quality; Fantastico represents a hybrid of the first two varieties and it is the most cultivated (Valussi et al., 2021).

Despite bergamot pomace representing industrial waste, at the same time, it is very rich in antioxidant compounds that could later be reused as natural additives in the food industry. The obtained results will provide information to guide future utilization of bergamot, and in general citrus waste as an important source of bioactive compounds to reuse in the food field as nutritional and/or preservative compounds.

2.2. Materials and Methods

2.2.1. Raw Materials

Bergamot (*Citrus bergamia Risso*) pomace (BP) represented by skins, pulp, and seeds, has been found at a citrus farm processing situated in Reggio Calabria (Italy). This company works the fruits mainly for the production of essential oil and in a small part of juice. After the transport to the Food Technology laboratory of the Mediterranea University of Reggio Calabria, BP was subjected to dehydration (at 50 °C) to reduce the moisture content (up to 12%) and powdered to facilitate the extraction process.

In the second phase, twenty bergamot fruits *(BF*) of three different cultivars (Castagnaro, Femminello, and Fantastico), harvested on a farm situated in the province of Reggio Calabria (Italy), were analyzed. After peel abrasion for essential oil extraction, the parts of bergamot fruits remaining, which included albedo/pulp, juice, and seeds, were individually separated and the antioxidant compounds were extracted using the best extraction process following the selected method (Section 2.2.3).

2.2.2. Chemicals and Apparatus

Ethanol (96% *v/v* (F.C.C.), Food grade, PanReac)) and drinking water were used for the extraction process. Folin–Ciocalteu's phenol reagent, 2,2'-azino-bis acid (3-ethylbenzothiazolin-6-sulfonic acid) (ABTS), 2,2- diphenyl-1-picrylhydrazyl (DPPH), and Trolox were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, ultrapure water, and acetonitrile (UHPLC grade, Carlo Erba, Italy) were used for chromatographic analysis. Eriocitrin, Neoeriocitrin, naringin, and neohesperidin were purchased from Merck (Darmstadt, Germany).

A heating magnetic stirrer (Velp Scientifica, Usmate Velate (MB), Italy); a Sonoplus Ultrasonic homogenizers (BANDELIN, Ultraschall seit 1955); and a Microwave Digestion System (ETHOS EASY, Millestone, Bergamo, Italy) were used.

For spectrophotometric analyses, a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV-Vis λ2, Waltham, MA, USA) was used.

A UHPLC system (Knauer, Berlin, Germany), a Kinetex® 2.6 µm Biphenyl 100 Å column 100 × 2.1 mm (Torrance, CA, USA) coupled with a Photo Diode Array Detector (PLATINblue, Knauer) and Clarity software were used for the quali-quantitative analysis of individual bioactive compounds.

2.2.3. Extraction of Antioxidant Compounds

The experimental procedure was performed during the crop season of 2021 and a schematic overview is shown in Figure 2.1.

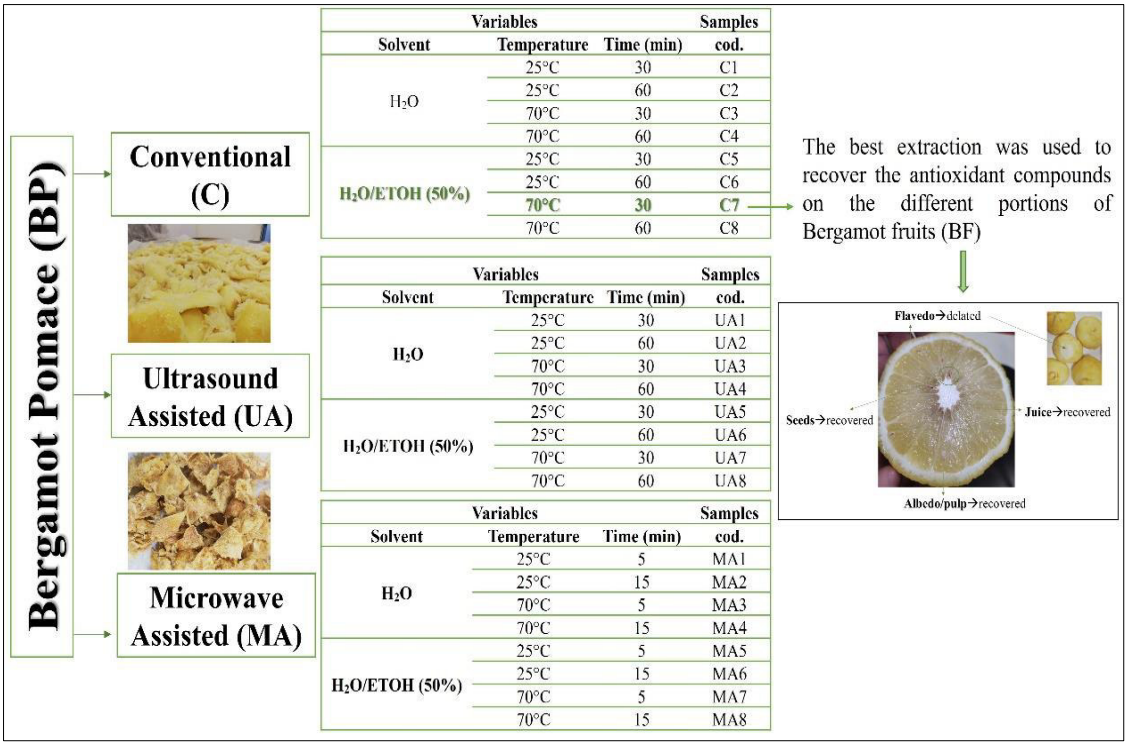

Figure 3.1. Schematic overview of the experimental procedure.

With the aim to obtain an extract with high antioxidant power, different techniques were carried out, following the methods reported in another of our recent works (Imeneo et al., 2022). We applied: conventional extraction (C); ultrasound extraction (UA); and microwave extraction (MA) (Table 2.1.).

Table 2.1. Extraction technique performed.

Conventional solid–liquid extraction	Using a magnetic stirrer equipped with a temperature control device
Ultrasound-assisted extraction	The Ultrasonic were applied for 30 and 60 min (25 °C: ω= 10%, pulsation time on 1 s off 15 s and 70 °C: ω = 50%, pulsation time on 1 s off 1 s), frequency of 59 kHz
Microwave-assisted extraction	The applied microwave power (Watt) was: 250 W corresponds to 25 °C and 800 W corresponds to 70 °C.

Different food grade solvents/water (H_2O) and water/ethanol (H_2O:EtOH, 50%), different temperatures: 25 and 50 °C; and different times: 30 and 60 min were tested. Only for MA extraction the extraction time was different (5 and 15 min).

For all types of extraction was used 10 g of powdered BP and 50 mL of solvent (1:5, sample: solvent ratio). The samples were named with numbers from 1 to 8 matched by the initial related to the extraction system (C, UA, and MA), as shown in Figure 2.1.

The best-selected extraction technique was applied also for the different portions of BF (albedo/pulp, seeds, juice).

2.2.4. Analytical Methods

2.2.4.1. Total Phenolic Content (TPC)

For TPC determination the method reported by González-Molina et al., 2009, (appropriately modified) was followed. 0.2 mL of each extract (BP and BF and Juice, diluted 1/5, *v/v*), 5 mL of deionized water and 1 mL of Folin-Ciocalteu reagent were placed in a volumetric flask (25 mL) and mixed. After 8 min, 10 mL of Na_2CO_3, 20% were added. The reaction mixture was left to dark for two hours first to read the absorbance at 765 nm. The results were compared with a gallic acid calibration curve and were expressed as mg of gallic acid g^{-1} (mg GAE g^{-1} dw) of the dry weight of pomace and mg of gallic acid 100 mL^{-1} for juice (mg GAE 100 mL^{-1}).

2.2.4.2. Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined following the method described by Papoutsis et al., 2018. In a volumetric flask (5 mL), 0.5 mL of each extract (BP and BF and Juice, diluted 1/5, *v/v*), 1 mL of water, and 0.15 mL of $NaNO_2$, 5% were mixed and incubated for 6 min. Then, 0.15 mL of $AlCl_3$ 10% was added and after 6 min, 2 mL of NaOH 4% and 0.7 mL of deionized water were mixed. The mixture was incubated in the

dark for 15 min and then the absorbance was measured at 510 nm. The results were compared with a catechin calibration curve and were expressed as mg of catechin g^{-1} (mg CE g^{-1} dw) of the dry weight of pomace and mg of catechin 100 mL^{-1} for juice (mg CE 100 mL^{-1}).

2.2.4.3. Total Antioxidant Activity (DPPH and ABTS Assays)

For the determination of total antioxidant activity (TAA), DPPH and ABTS assays were performed following the methods reported by De Bruno et al., 2021. The DPPH assay involves the reaction between the radical (2,2-diphenyl-1-picrylhydrazyl) and the bioactive compounds present in the samples to analyze, generating a discolouration of the reaction solution (Brand-Williams et al., 1995); 50 µL of the extract or filtered juice and 2950 µL of methanol solution of DPPH (6×10^{-5} M) were mixed in a cuvette and let us react for 30 min in darkness, and after the absorbance was measured at 515 nm.

For the ABTS assay, 25 µL of extract or filtered juice and 2975 µL of $ABTS^{+}$ solution (7 mM) were mixed in a cuvette and let us react for 6 min, and after the absorbance was measured at 734 nm.

For both the assays the results were reported as mM Trolox equivalents g^{-1} (mM TE g^{-1} dw) of the dry weight of pomace and for juice as mM Trolox equivalents L^{-1} for juice (mM TE L^{-1}).

2.2.4.4. Chromatographic Conditions and Validation Methods for the Evaluation of Individual Bioactive Compounds

Chromatographic determination of bioactive components was performed following the method reported by Romeo et al., 2019, using a UHPLC system. The chromatographic conditions are shown in Figure 2.2., whereas the following elution solvents were used: water (acidified with formic acid to pH 3.10, A) and acetonitrile (B) and 5 µL of the sample.

	Time (min)	A(%)	B(%)	Flow (mL/min)
1	Initial	95.0	5.0	0.400
2	3.00	95.0	5.0	0.400
3	17.00	40.0	60.0	0.400
4	15.50	0.0	100.0	0.400
5	20.00	95.0	5.0	0.400
6	21.00	95.0	5.0	0.400

Figure 2.2. The antioxidant's chromatographic conditions (elution gradient).

External standards (eriocitrin, neoeriocitrin, naringin, neohesperidin, melitidin, and brutieridin) were used for the quantification and the results were expressed as mg g^{-1} of dry weight (mg g^{-1} dw), and mg L^{-1} for juice.

For limonoids determination was followed the method described by Celano et al., 2019, using a UHPLC-DAD system equipped with a Kinetex 2.6 µm Biphenyl 100 Å. The chromatographic conditions are shown in Figure 2.3, in which the mobile phase consisted of water (A) and acetonitrile (B).

	Time (min)	A(%)	B(%)	Flow (mL/min)
1	Initial	95.0	5.0	0.400
2	15.00	75.0	25.0	0.400
3	20.00	40.0	60.0	0.400
4	21.00	2.0	98.0	0.400
5	25.00	2.0	98.0	0.400
6	28.00	95.0	5.0	0.400
7	30.00	95.5	5.0	0.400

Figure 2.3. Limonoids' chromatographic conditions (elution gradient).

External standards (limonin and nomilin) were used for the quantification of limonoids and the results were reported as mg g^{-1} of dry weight (mg g^{-1} dw).

2.2.5. Statistical Analysis

The results obtained in this experimentation were reported as mean value ± standard deviation. For the statistical elaboration, One-way analysis of variance (ANOVA), and Multivariate analysis with Tukey's post hoc test at $p < 0.05$ were performed with the SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA). For the determination of

correlation coefficients (r) among TPC, TFC, DPPH, and ABTS assays Pearson's correlation test was carried out.

2.3. Results and Discussion

2.3.1. Bergamot Pomace (BP): Antioxidant Characterization

Different Food grade solvents (H_2O and H_2O: EtOH), times, temperatures, and systems to assist the extractability were applied to optimize the extraction yield of bioactive compounds present in the BP. All the obtained extracts were analyzed by spectrophotometric methods, for the determination of total bioactive components, in particular: total polyphenol content (TPC), flavonoid content (TFC), and total antioxidant activity (DPPH and ABTS). Statistical differences were analyzed among all the studied variability (ANOVA). Multivariate analysis carried out on the antioxidant extracts, showed significant differences ($p < 0.01$) for extraction methods and used solvents, considering all the total antioxidant assays, and the results are reported in Table 2.2.. However, significant differences were also highlighted for extraction * solvent, extraction * temperature, and extraction * solvent * temperature ($p < 0.01$).

Table 2.2. Multivariate statistical analysis of different extraction methods.

Dependent Variable	DPPH	ABTS	TPC	TFC
Extraction	**	**	**	**
Solvent	**	**	**	**
Temperature	n.s.	n.s.	**	**
Time	*	n.s.	**	**
Extractions* Solvent	**	**	**	**
Extraction * temperature	**	**	**	**
Solvent * temperature	n.s.	**	**	**
Extration * Solvent * temperature	**	**	**	**
Extration * Time	*	n.s.	**	**
Solvent * Time	n.s.	*	**	**
Extration * Solvent * Time	*	n.s.	**	**
Temperature * Time	n.s.	**	n.s.	n.s.
Extraction * Temperature * Time	n.s.	**	**	**
Solvent * Temperature * Time	n.s.	**	**	n.s.
Extraction * Solvent * Temperature * Time	n.s.	**	n.s.	**

n.s. not significant; ** Significance at $p < 0.01$; * Significance at $p < 0.05$.

Significative differences in TPC ($p < 0.01$) were observed among the different extraction systems (C, UA, MA) and for the different studied variables within the same extraction system (Table 2.3.).

Conventional and Ultrasound assisted extraction has provided important results in terms of total phenolics recovery. The highest values were measured for the conventional extraction system (C), particularly for C7 and C8 samples (26.30 and 26.06 mg GAE g^{-1} dw, respectively), also if the Ultrasound assisted system (UA) led to a good recovery of TPC (about 23.64 mg GAE g^{-1} dw in UA5 extract). The obtained values are higher than those reported by Gabriele et al., 2017 (17.44 ± 0.40 mg GAE g^{-1} dw) on lyophilized bergamot fruits, and by Multari et al., 2020 (410 ± 36.8 mg kg^{-1}) on bergamot pomace. Moreover, the content of total phenols was higher compared with other citrus (lemon and orange peel), as reported by Gorinstein et al., 2001. Among all the evaluated extracts, those obtained through microwave-assisted (MA), showed less extractability (between 10 and 19 mg GAE g^{-1} dw).

Regarding the TFC the obtained values were reported in Table 2.3.. The amount of determined TFC showed great variability in accordance with the applied extraction procedure ($p < 0.01$). This spectrophotometric assay showed a different trend compared with the TPC assay; indeed, great extractability was obtained through the application of UA treatment, particularly in the samples prepared using water as an extraction solvent. As demonstrated by some authors, the application of ultrasound to facilitate the extraction of bioactive compounds is very useful, indeed, this technique adopts an acoustic cavitation system that combines ultrasound and traditional solvent extraction. The sound waves induce forces that can break the cell walls intensifying the release of constituents (Imeneo et al., 2022). In comparison to water, ethanol is characterized by a higher heating efficiency when applied in an aqueous mixture and it is preferred thanks to its better capacity in solving the phenolic compounds. Higher values were estimated in the samples UA1 and UA2 (7.52 and 7.81 mg CE g^{-1} dw, respectively). In all the other situations was found a lower content of TFC. Nevertheless, a similar recovery of TFC was evidenced for the sample obtained with a hydroalcoholic mixture (7 and 8) for all the extraction systems (C, UA, and MA).

Table 2.3. Total phenolic content and Total flavonoid content of different BP extracts.

TPC (mg GAE g^{-1} dw)	C	UA	MA	Sign.
1	12.00 ± 0.43 ^{eC}	22.72 ± 0.37 ^{abA}	13.61 ± 0.16 ^{cB}	**
2	19.60 ± 0.29 ^{cB}	21.76 ± 0.36 ^{abcA}	13.42 ± 0.22 ^{cdC}	**
3	15.59 ± 0.04 ^{dB}	18.63 ± 0.69 ^{dA}	12.27 ± 0.07 ^{deC}	**
4	23.66 ± 0.22 ^{bA}	20.79 ± 0.65 ^{bcB}	13.51 ± 0.43 ^{cdC}	**

5	19.24 ± 1.22 ^{cA}	23.64 ± 0.27 ^{aA}	9.53 ± 0.24 ^{fB}	**
6	21.97 ± 0.76 ^{bA}	23.28 ± 0.55 ^{aA}	11.82 ± 0.41 ^{eB}	**
7	26.30 ± 0.09 ^{aA}	20.52 ± 0.62 ^{cdB}	19.44 ± 0.07 ^{aB}	**
8	26.07 ± 0.62 ^{aA}	21.10 ± 0.15 ^{bcB}	17.73 ± 0.63 ^{C}	**
Sign.	**	**	**	
TFC (mg CAE g^{-1} dw)	**C**	**UA**	**MA**	**Sign.**
1	2.67 ± 0.32 ^{dC}	7.52 ± 0.00 ^{abA}	5.14 ± 0.06 ^{bB}	**
2	4.93 ± 0.35 ^{bcB}	7.81 ± 0.05 ^{aA}	4.47 ± 0.08 ^{cB}	**
3	5.58 ± 0.10 ^{abB}	6.43 ± 0.09 ^{cdA}	4.03 ± 0.09 ^{dC}	**
4	5.72 ± 0.39 ^{abB}	7.13 ± 0.00 ^{bA}	4.14 ± 0.03 ^{cdC}	**
5	4.16 ± 0.04 ^{cB}	6.59 ± 0.25 ^{cA}	2.92 ± 0.09 ^{eC}	**
6	4.51 ± 0.16 ^{cB}	6.24 ± 0.12 ^{cdA}	3.01 ± 0.10 ^{eC}	**
7	6.18 ± 0.12^{aA}	6.02 ± 0.04 ^{dAB}	5.58 ± 0.14 ^{aB}	*
8	5.61 ± 0.26 ^{abAB}	6.11 ± 0.17 ^{cdA}	5.27 ± 0.13 ^{abB}	*
Sign.	**	**	**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$.

The antioxidant ability of the different obtained extracts was analyzed in vitro by applying two common spectrophotometric assays: DPPH, and ABTS (Figure 2.4.a,b). These tests are based on the ability of an antioxidant matrix (represented by the antioxidant extract) to reduce a radical species. The antioxidant molecules present in the extracts react against the radicals and causing oxidation of the reacting molecule, with a related decrease in the solution absorbance (discolouration reaction).

The DPPH assay on the different obtained extracts showed highly significant differences among the samples ($p < 0.01$), with values that ranged between 0.71 and 3.21 mM TE g^{-1} dw. As clearly shown in Figure 2.4.a, the antioxidant extracts recovered through the use of a hydroalcoholic solvent (H_2O: EtOH, 50%) highlighted the highest total antioxidant activity ($p < 0.01$), particularly, the extracts recovered with ultrasound.

Concerning the ABTS assay, the results are shown in Figure 2.4.b, where is possible to observe that the obtained values highlighted a higher antioxidant activity compared to the DPPH assay, with values that ranged between 3.22 and 16.93 mM TE g^{-1} dw. The best extraction in terms of total antioxidant activity was obtained by applying the hydroalcoholic solution (H_2O-EtOH, 50%) as extraction solvent and assisted by a conventional maceration (samples C6 and C7). The two different antioxidant assays, respond differently to extracts, this trend is probably due to the different interaction of the two free radicals with antioxidant molecules.

For all the analyzed extracts, the two total antioxidant assays showed different results, with higher concentrations of total antioxidants highlighted by the ABTS assay. It could be dependent on the fact that ABTS assay generally is a more sensitive hydrophilic and lipophilic antioxidant, while DPPH assay is more sensitive to lipophilic antioxidants (Kim et al., 2002). This affinity was also described by Zacarías-García et al., 2021 which highlighted the highest value for ABTS compared to DPPH in the hydrophilic fraction.

As already reported in other works, the extractability of bioactive compounds is strongly dependent on different applied extraction variables, such as solvent, time, temperature, etc. (De Bruno et al., 2018), as also evidenced in this work.

In addition, positive correlations were found between TPC/DPPH, particularly for the conventional extraction method ($r > 0.7$), while the ABTS assay was related to both TPC and TFC ($r > 0.95$) especially for microwave extraction.

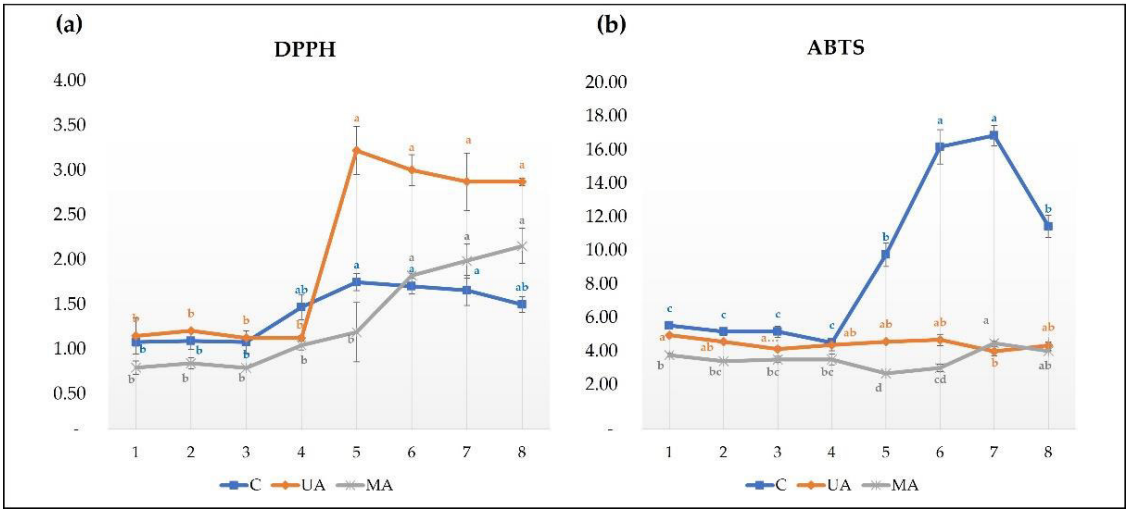

Figure 2.4. Results of total antioxidant activity (**a**, DPPH; **b**, ABTS) of different BP extracts (mM TE g^{-1} dw). Small letters show significant differences as assessed by Tukey's post hoc test.

2.3.2. Quantification of Individual Bioactive Compounds through Liquid Chromatography

The quali-quantitative analysis of the prevalent neohesperidose flavanones (eriocitrin, neoeriocitrin, naringin; neohesperidin, metilidin, and brutieridin) present in bergamot pomace extracts was performed by UHPLC system.

In Table 2.4., the validation of chromatographic analysis was reported. The applied methodology was developed by the injection of different concentrations of standard solutions. Particularly, limits of detection (LOD) and quantification (LOQ); the correlation coefficient (R^2) and regression equations for each antioxidant standard were studied.

While for Metilidin and Brutieridin compounds the Naringin was used as standard, and the results are expressed as naringin equivalent (mg g^{-1} dw).

Table 2.4. UHPLC validation method.

Compounds	Regression Equation	R^2	LOD µg g^{-1}	LOQ µg g^{-1}
Eriocitrin	y = 41.62x − 15.40	0.9999	0.0625	0.2245
Neoeriocitrin	y = 37.66x − 17.39	0.9999	0.0536	0.2777
Naringin	y = 42.08x − 13.67	0.9993	0.0635	0.2326
Neohesperidin	y = 51.28x + 8.76	0.9997	0.1290	0.2129
Limonin	y = 41.28x + 47.93	0.9998	0.0361	0.9984
Nomilin	y = 38.40x − 19.25	0.9996	0.0932	0.2465

Figure 2.5. shows the chromatographic profiles of the obtained extracts and the main identified compounds. Particularly, seven phenolic compounds were detected: eriocitrin, neoeriocitrin, narirutin, naringin, neohesperidin, melitidin and brutieridin.

Neoeriocitrin, naringin, neohesperidin, and brutieridin were the main flavonoids detected in BP (Table 2.5.), as also reported by other authors (Giuffrè, 2019; Bartella et al., 2022) and represent the typical compounds that determine the bitterness in the fruit (Russo et al., 2022).

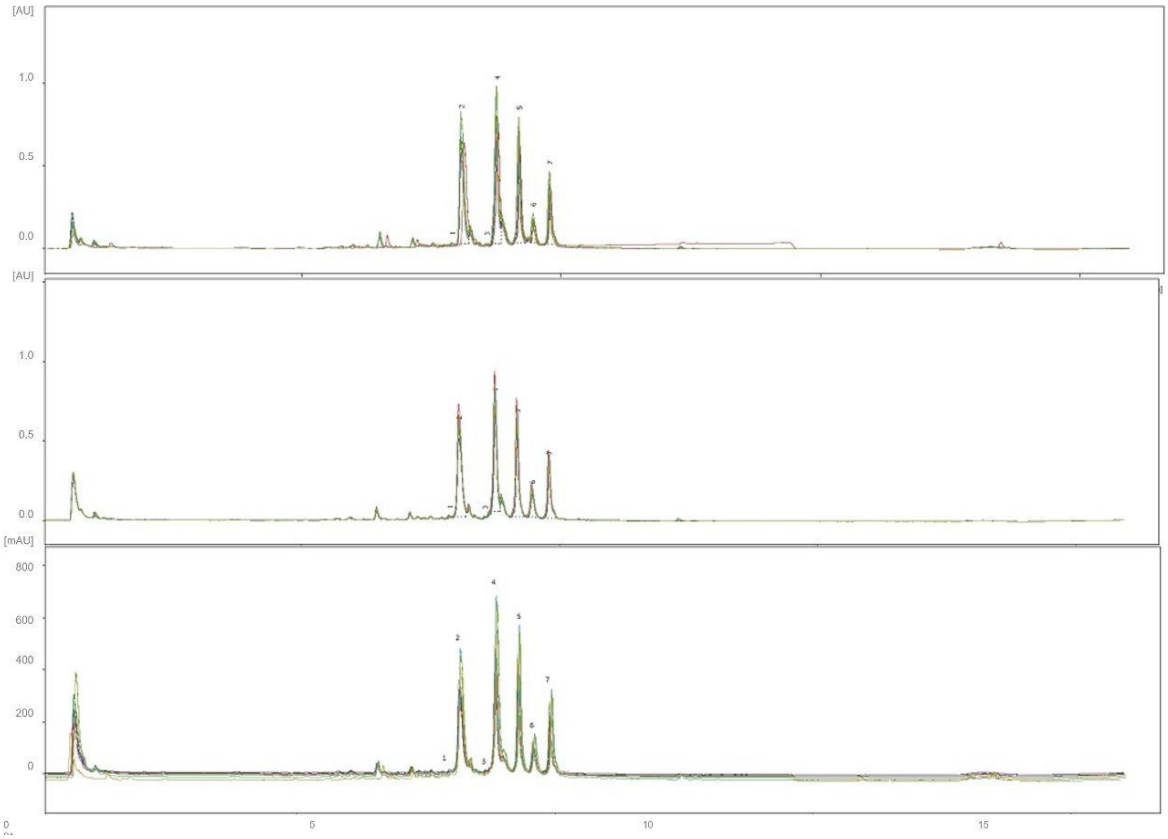

Figure 2.5. Example of chromatographic profiles of different BPs injected by UHPLC.
(1) eriocitrin; (2) neoeriocitrin; (3) narirutin; (4) naringin; (5) neohesperidin; (6) melitidin and (7) brutieridin.

Among these, neoeriocitrin was the most abundant in BP extract ranging from 5.03 to 14.14 mg g^{-1} dw; in particular, it showed its highest amounts (13.95 and 14.14 mg g^{-1} dw) in the C7 and C8 samples, obtained by conventional extraction at 70 °C for 30 and 60 min. Regarding the other prevailing compounds, the range were 4.69–12.87 mg g^{-1} dw for naringin; 2.84–7.35 mg g^{-1} dw for neohesperidin, 2.05–5.67 mg g^{-1} dw for brutieridin, and 1.12–2.67 mg g^{-1} dw for melitidin. Lesser quantities of eriocitrin and narirutin were instead determined in all samples.

All the assayed extracts were significantly different for the phenolic content, related to the extraction system (C, UAE, and MA, $p < 0.01$) and variables of extraction (times and temperatures, $p < 0.01$). The conventional extraction system produced the greatest extractability of this class of antioxidants with similar values in C7 and C8 samples. As observed previously in other food matrices, the hydroalcoholic solvent is the best choice to obtain the maximum yield of antioxidant compounds (Imeneo et al., 2022; Bartella et al., 2022), when combined with 70 °C temperature (Imeneo et al., 2022).

Table 2.5. Results of the prevalent flavanones on the different BP extracts (mg g^{-1} dw).

	Eriocitrin				**Neoeriocitrin**				**Naringin**			
Samples	**C**	**UA**	**MA**	**Sign.**	**C**	**UA**	**MA**		**C**	**UA**	**MA**	
1	0.19 ^{efB}	0.26 ^{aA}	0.10 ^{aC}	**	9.83 ^{dB}	11.58 ^{bA}	7.31 ^{cC}	**	8.54 ^{fB}	9.49 ^{cA}	7.32 ^{cC}	**
2	0.19 ^{eB}	0.26 ^{aA}	0.09 ^{aC}	**	9.64 ^{dB}	11.28 ^{bcA}	6.08 ^{deC}	**	8.61 ^{fB}	9.67 ^{cA}	5.85 ^{dC}	**
3	0.22 ^{cA}	0.18 ^{bB}	0.05 ^{bC}	**	11.08 ^{cA}	9.26 ^{eB}	5.03 ^{fC}	**	10.02 ^{eA}	7.76 ^{eB}	5.13 ^{fC}	**
4	0.23 ^{bcA}	0.13 ^{eB}	0.05 ^{bC}	**	11.85 ^{bA}	10.09 ^{dB}	6.42 ^{dC}	**	10.47 ^{dA}	8.60 ^{dB}	5.63 ^{eC}	**
5	0.19 ^{fA}	0.18 ^{bB}	0.04 ^{bC}	**	10.16 ^{dB}	13.10 ^{aA}	5.04 ^{fC}	**	9.73 ^{eB}	11.03 ^{aA}	4.69 ^{gC}	**
6	0.24 ^{bA}	0.15 ^{cB}	0.04 ^{bC}	**	12.15 ^{bA}	11.30 ^{bcB}	5.93 ^{eC}	**	11.00 ^{cA}	10.18 ^{bB}	5.31 ^{fC}	**
7	0.25 ^{aA}	0.14 ^{dB}	0.04 ^{bC}	**	13.95 ^{aA}	11.04 ^{cB}	9.72 ^{aC}	**	12.47 ^{bA}	9.45 ^{cB}	8.32 ^{aC}	**
8	0.21 ^{dA}	0.11 ^{fB}	0.04 ^{bC}	**	14.14 ^{aA}	10.43 ^{dB}	9.01 ^{bC}	**	12.87 ^{aA}	8.71 ^{dB}	7.99 ^{bC}	**
Sign.	**	**	**		**	**	**		**	**	**	
	Neohesperidin				**Melitidin**				**Brutieridin**			
Samples	**C**	**UA**	**MA**		**C**	**UA**	**MA**		**C**	**UA**	**MA**	
1	5.13 ^{eB}	5.98 ^{bA}	3.98 ^{cC}	**	1.65 ^{eB}	2.46 ^{bA}	1.60 ^{bC}	**	3.59 ^{fB}	4.60 ^{bA}	2.97 ^{cC}	**
2	4.29 ^{fB}	5.61 ^{cA}	3.58 ^{dC}	**	1.49 ^{fB}	2.59 ^{abA}	1.41 ^{cC}	**	3.63 ^{fB}	4.73 ^{bA}	2.69 ^{dC}	**
3	5.81 ^{cdA}	4.80 ^{eB}	2.84 ^{fC}	**	1.83 ^{dA}	1.82 ^{eA}	1.22 ^{efB}	**	4.38 ^{dA}	3.52 ^{fB}	2.21 ^{fC}	**
4	6.03 ^{bcA}	5.21 ^{dB}	3.56 ^{dC}	**	1.99 ^{cA}	1.10 ^{dA}	1.38 ^{cdB}	**	4.50 ^{dA}	3.98 ^{eB}	2.71 ^{dC}	**
5	5.43 ^{deB}	6.78 ^{aA}	2.77 ^{fC}	**	1.87 ^{cdB}	2.67 ^{aA}	1.12 ^{fC}	**	3.88 ^{eB}	5.00 ^{aA}	2.05 ^{fC}	**
6	6.28 ^{bA}	6.03 ^{bA}	3.28 ^{eB}	**	2.12 ^{bB}	2.54 ^{abA}	1.28 ^{deC}	**	4.71 ^{cB}	4.95 ^{aA}	2.47 ^{eC}	**
7	7.35 ^{aA}	5.65 ^{cB}	5.03 ^{aC}	**	2.29 ^{aA}	2.29 ^{cA}	2.00 ^{aB}	**	5.67 ^{aA}	4.35 ^{cB}	3.95 ^{aC}	**
8	7.11 ^{aA}	5.49 ^{cB}	4.78 ^{bC}	**	2.28 ^{aA}	2.12 ^{dB}	1.94 ^{aC}	**	5.12 ^{bA}	4.13 ^{dB}	3.57 ^{bC}	**
Sign.	**	**	**		**	**	**		**	**	**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$.

Additionally, the limonoid content was evaluated on Bergamot pomace extract, as it represents an interesting index to evaluate, indeed, its contribution to the bitter taste of citrus fruits, as also reported by Shi et al., 2020. In addition, limonoids from Citrus are getting a lot of scientific interest for their bioactive properties in vitro and in vivo such as antitumor, antioxidative, and antibacterial compounds (Zhang et al., 2013). The chromatographic analysis showed that also the highest yield of the two mains determined limonoids (limonin and nomilin) was obtained through conventional extraction technique C7 (Figure 2.6).

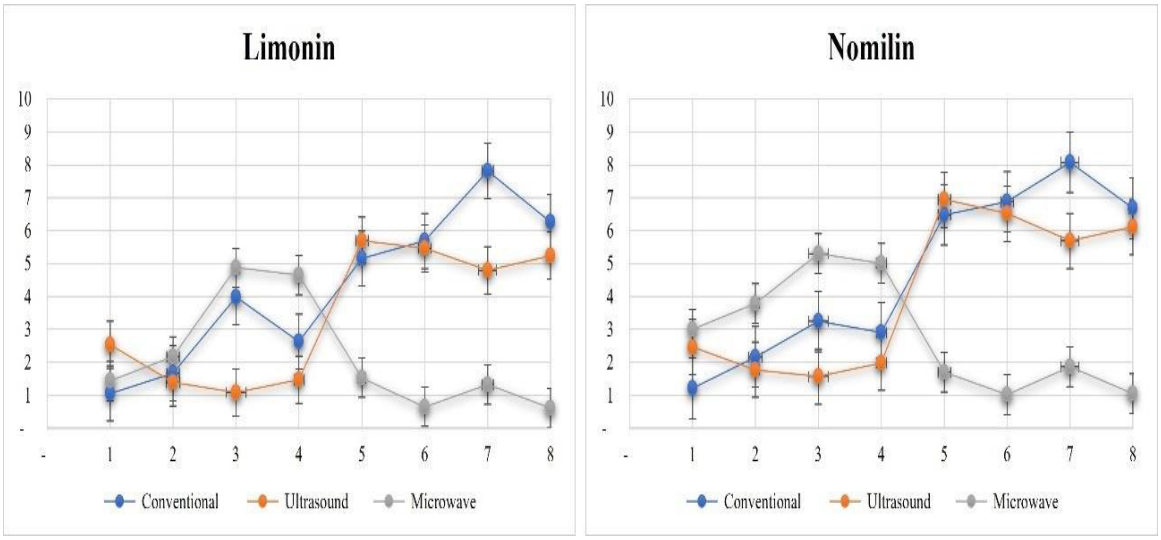

Figure 2.6. Concentration values (mg g^{-1}) of Limonin and Nomilin content in bergamot pomace extracts.

The statistical analysis carried out on the obtained results for limonoid content showed significant differences ($p < 0.01$) among all the studied variables (Table 2.6.).

Table 2.6. ANOVA limonoids content.

	Limonin				Nomilin			
	C	**UA**	**MA**	**Sign.**	**C**	**UA**	**MA**	**Sign.**
1	hC	eA	deB	**	gC	cB	dA	**
2	gB	fC	cA	**	fB	cC	cA	**
3	eB	gC	aA	**	dB	cC	aA	**
4	fB	fC	bA	**	eB	cC	bA	**
5	dB	aA	deC	**	cB	aA	fC	**
6	cA	bB	fC	**	bA	bA	gB	**
7	aA	dB	eC	**	aA	bB	eC	**
8	bA	cB	fC	**	bA	abB	gC	**
Sign.	**	**	**		**	**	**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$.

After identifying the best extraction system, namely conventional maceration for 30 min at 70 °C and using H_2O/EtOH 50% as extraction solvent, a correlated study on the antioxidant properties of the different portions of bergamot fruits was carried out. Specifically, this extraction technique was applied to test separately the individual portions of each fruit (albedo/pulp, seeds, and juice) belonging to the three cultivars Fantastico, Femminello, and Castagnaro.

The amounts of total flavonoid content quantified in the albedo/pulp portion showed significant differences ($p < 0.01$) among the three cultivars as shown in Figure 2.7. The highest content was 46.61 mg CE g^{-1} in Fantastico, and the lowest one in Castagnaro (19.11 mg CE g^{-1}). The same trend was observed for TPC with values that ranged between 61.76 mg GAE g^{-1} (Castagnaro) and 129.44 mg GAE g^{-1} (Fantastico).

The antioxidant activity of the albedo/pulp portion was studied by DPPH and ABTS radical scavenging assays. Both assays revealed a higher activity than those shown by BP sample. Higher results were expressed by ABTS assay, with statistical differences ($p < 0.05$) among the samples with ranges from 15.35 mM TE g^{-1} dw (Castagnaro) and 24.54 mM TE g^{-1} dw (Fantastico). Lower values were found in the DPPH (2.38 mM TE g^{-1} dw in Castagnaro and 4.22 mM TE g^{-1} dw in Fantastico) without statistical differences.

The antioxidant determinations on seeds revealed significant differences for TFC, with higher amounts in Castagnaro and Fantastico (9.16 and 8.64 mg CE g^{-1}, respectively) than in Femminello cultivars (4.59 mg CE g^{-1}).

The distribution of TPC among the cultivars' seeds was different compared to the albedo/pulp portion. TPC was quantified in the range of 16.57, 21.20, and 37.40 mg GAE g^{-1}, respectively on Femminello, Fantastico, and Castagnaro seeds. TPC in seeds was comparable with those reported by Al et al., 2020.

The free radical scavenging activity measured with DPPH assay for seed extracts did not show differences with values of about 1 mM TE g^{-1} dw.

The highest level of scavenging activity based on ABTS assay was determined in Castagnaro seed extract (8.69 mM TE g^{-1} dw), and the lowest (4.42 mM TE g^{-1} dw) in Femminello, exhibiting the same trend of TPC, with a high correlation ($r = 0.999$).

Figure 2.7 also showed the different amounts of total flavonoid content in tested juices. Although the highest value was found in Castagnaro (160.08 mg CE L^{-1}), the replications

conducted, and the statistical analysis suggested that there are no statistical differences among the analyzed samples.

The phenolic content in the juice was in accordance with the amounts detected by Xu et al., 2008and Chen et al., 2020, who analyzed respectively the juice of fifteen and twenty-seven different varieties of citrus fruit. Their contents were 78.94 mg GAE 100 mL^{-1} in Femminello, 79.33 mg GAE 100 mL^{-1} in Fantastico, and 98.73 mg GAE 100 mL^{-1} in Castagnaro. The antioxidant activity, evaluated with the DPPH assay was more correlated to the TPC assay (r = 0.768) compared with the other assays. Cautela et al., 2019, in fact, reported relevant differences in the chemical composition and functional properties of juices obtained with different processing methods.

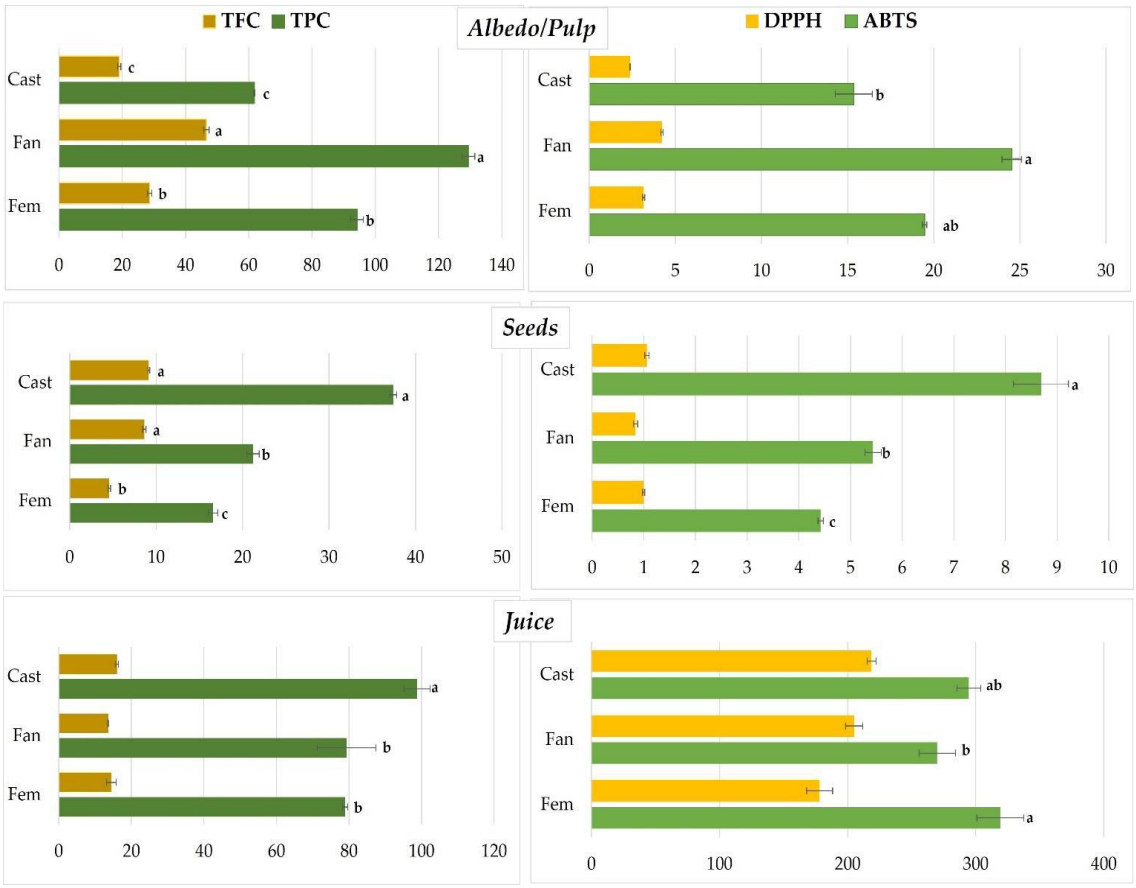

Figure 2.7. Total antioxidant activity of the different bergamot portions.
(Albedo/pulp and seeds: TFC: mg CE g^{-1} dw, TPC: mg GAE g^{-1} dw; ABTS and DPPH: mM TE g^{-1} dw; Juice: [1], TFC: mg CE 100 mL^{-1}, TPC: mg 100 mL^{-1}, ABTS and DPPH: mM TE L^{-1}). Small letters show significant differences as assessed by Tukey's post hoc test.

Bergamot portions (albedo/flavedo, seeds, juice) showed high concentrations of flavanones, including eriocitrin, neoeriocitrin, naringin, neohesperidin, melitidin, and brutieridin (Table 2.7.), as also reported by Walker et al., 2014.

Neoeriocitrin and naringin represent the prevalent flavonoid compounds in the albedo/pulp portion, with values that ranged between 33.19–88.91 and 31.72–58.02 mg g^{-1} dw, respectively. Lower content of this class of compounds was found in the seeds.

The chromatographic analysis of Bergamot Juice resulted in a high content of neoeriocitrin, naringin, and neohesperidin. They showed values different from those reported by Leporini et al., 2021, but more or less comparable with those reported by Walker et al., 2014, Da Pozzo et al., 2018, and Baron et al., 2021. The juice of Fantastico cv. showed the highest content in neoriocitrin (196.02 ± 19.13 mg L^{-1}) while naringin and neohesperidin were higher in Femminello cv. Brutieridin ((hesperetin 7-(200-R-rhamnosyl-600-(30000-hydroxy-30000-methylglutaryl)-β-glucoside)) and melitidin (naringenin7-(200-R-rhamnosyl-600-(30000-hydroxy-30000-methylglutaryl)-β-glucoside)), which have anticholesterolemic activity, exhibiting statin-like properties (Cai et al., 2017; Fiorillo et al., 2018), were found either in the albedo/pulp, seeds and juice of the three different bergamot cultivars showing higher concentration for brutieridin than melitidin as reported by Di Donna et al., 2009).

Table 2.7. Individual phenolic content in the different bergamot portions (Albedo/pulp and seeds mg g^{-1} dw; Juice mg L^{-1}).

		Eriocitrin	Neoeriocitrin	Naringin	Neohesperidin	Melitidin	Brutieridin
Albedo/pulp	FA	1.06 ± 0.04 ^{a}	88.91 ± 1.34 ^{a}	58.02 ± 0.58 ^{a}	27.67 ± 1.14 ^{a}	13.61 ± 0.99 ^{a}	26.09 ± 0.87 ^{a}
	FE	0.70 ± 0.03 ^{b}	58.37 ± 0.40 ^{b}	32.42 ± 1.92 ^{b}	11.41 ± 1.20 ^{b}	10.01 ± 0.64 ^{b}	18.30 ± 0.67 ^{b}
	CA	0.61 ± 0.03 ^{b}	33.19 ± 0.76 ^{b}	31.72 ± 0.39 ^{b}	13.85 ± 0.45 ^{b}	8.17 ± 0.34 ^{c}	12.45 ± 0.72 ^{c}
	Sign.	**	**	**	**	**	**
Seeds	FA	0.10 ± 0.01 ^{b}	1.96 ± 0.08 ^{c}	2.37 ± 0.10 ^{c}	0.91 ± 0.04 ^{c}	0.82 ± 0.05 ^{c}	1.5 ± 0.09 ^{c}
	FE	0.09 ± 0.01 ^{b}	4.27 ± 0.09 ^{b}	3.94 ± 0.08 ^{b}	1.71 ± 0.06 ^{b}	1.47 ± 0.09 ^{b}	2.76 ± 0.10 ^{b}
	CA	0.16 ± 0.02 ^{a}	9.04 ± 0.22 ^{a}	8.16 ± 0.18 ^{a}	3.71 ± 0.18 ^{a}	2.90 ± 0.23 ^{a}	5.32 ± 0.46 ^{a}
	Sign.	**	**	**	**	**	**
Juice	FE	5.43 ± 0.42 ^{a}	167.80 ± 6.03 ^{b}	186.70 ± 16.34 ^{a}	101.39 ± 6.78 ^{a}	57.59 ± 8.31	171.45 ± 8.33
	FA	4.59 ± 0.74 ^{b}	196.02 ± 19.13 ^{a}	141.83 ± 23.54 ^{b}	79.45 ± 10.84 ^{b}	47.70 ± 6.14	165.98 ± 7.39
	CA	4.46 ± 0.56 ^{b}	168.66 ± 17.01 ^{b}	161.48 ± 22.09 ^{ab}	81.88 ± 7.29 ^{ab}	48.56 ± 4.98	164.59 ± 4.96
	Sign.	**	**	*	**	ns	ns

Small letters within a column show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$; n.s., not significant; FA: Fantastico; FE: Femminello; CA: Castagnaro.

The two major limonoid aglycones, limonin, and nomilin, were also detected in the different parts of bergamot fruits. They are responsible for bitterness in citrus fruits. Concerning the limonoids content evaluated on individual bergamot portions. Femminello cv showed a high amount of limonin and nomilin on albedo/pulp (6.77 and 8.79 mg g^{-1} dw, respectively). Bergamot seeds possess a higher content of limonin, particularly those of Castagnaro cv (13.29 mg g^{-1} dw). The different content of limonin

is explained by the variability due to the state of ripeness of the fruit, the variety, and the part of the fruit (Sharma et al., 2017).

2.4. Conclusions

This work reports a careful study carried out on the recovery of antioxidant compounds by bergamot fruits and their by-products. The evaluation of data is helpful for the selection of food-grade solvent extraction in bergamot by-products also related to the different parts of fruit and to the three varieties. Overall, the present work has shown the possibility to valorize this waste with sustainable methods minimizing environmental impact offering an important source of bioactive compounds such as polyphenols, flavonoids, and limonoids that could be recovered.

The discovery of the beneficial effect and antioxidant activity of these extracts obtained from the portions of fruit or bergamot pomace could lead to the development of new products such as nutraceuticals and/or natural preservatives to apply in the food industry to extend the shelf life of products.

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3. FORTIFICATION OF VEGETABLE FAT WITH NATURAL ANTIOXIDANTS RECOVERED BY BERGAMOT POMACE TO USE AS AN INGREDIENT FOR THE PRODUCTION OF BISCUITS

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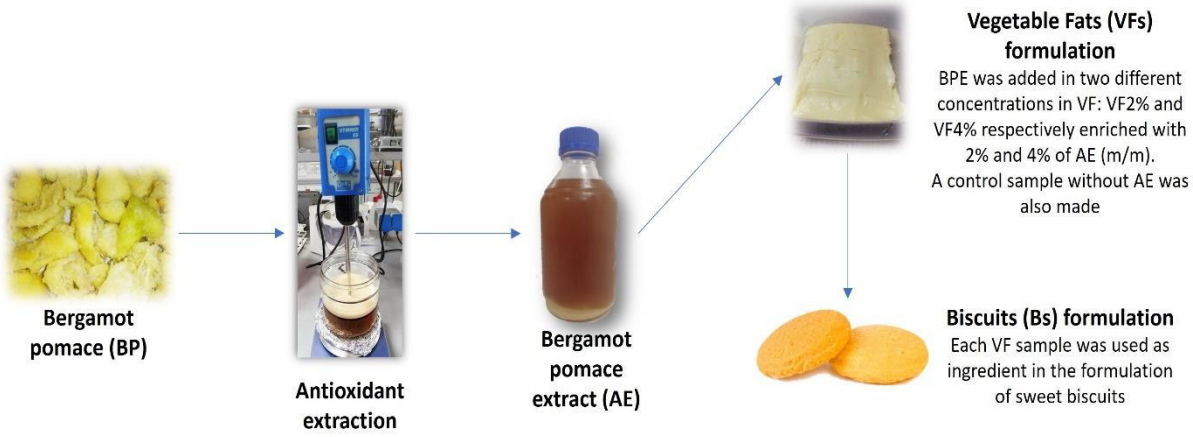
Abstract

Modern consumers are increasingly interested in eating healthy food and paying attention to the reduction of synthetic preservatives and the increased use of natural preservatives. For this reason, the aim of this work was to assess the effect of the addition of natural antioxidants extracted from Bergamot Pomace on the fortification of vegetable fat to improve its functional and qualitative characteristics. Furthermore, vegetable fat of neo formulation was used as an ingredient for the formulation of baked products (biscuits). The main physicochemical, microbiological, sensorial and antioxidant properties were evaluated for the different samples (fats and biscuits) to define the possible application of antioxidant compounds recycled from citrus waste. The effect of the fortification was demonstrated by the good results obtained regarding the enhancement of the oxidative stability; indeed, the addition of antioxidants to the biscuits protected them against the oxidation that could happen during the baking process. The

antioxidant and preservative effects of the flavonoids resulted in an increase of the oxidative stability of the biscuits with a potential extension of shelf life.

Keywords: Antioxidant, Bergamot, Biscuits, Fortification, Pomace, Recovery, Vegetal Fat.



Chapter 3. Graphical abstract

3.1. Introduction

In the last few decades, plant-based milk products have been constantly growing in response to the increase in diseases related to animal milk consumption. Several studies have reported allergies (El-Agamy, 2007; Crittenden & Bennett, 2005), lactose intolerance (Shafi & Husain, 2022; Szilagyi & Ishayek, 2018), and hypercholesterolemia due to the high amount of fats (Ney, 1991) and antibiotic residues (Rahman et al., 2021) associated with milk consumption. Furthermore, animal production has a negative impact on the environment concerning all factors linked to livestock, e.g., through the utilization of resources such as land and water, eutrophication, and gas emissions resulting in pollution and climate change. For these reasons, the demand for milk replacements has increased and consumers of a vegan diet have influenced the market trend in support of such sustainable food productions (Sethi et al., 2016). In fact, this social situation has encouraged researchers to come up with novel solutions in milk and milk dairy products, and to study differences among alternatives like coconut milk, which has a good taste and low calories, almond milk, soymilk which is rich in protein, or ricemilk. The preferred alternative with the best nutritional intake in the human diet is soymilk (Vanga & Raghavan, 2018). Considering these positive contributions and in light of increasing consumer demand, there has been a surge in research and development of technology to

increase and improve the range of plant-based foods. For example, plant-based milks are widely used to produce analogue milk products such as yogurt, ice-cream or cheese.

Nevertheless, these products that are rich in polyunsaturated fats and micronutrients, possess high oxidation (Valencia-Flores et al., 2013) and microbial growth, and for this reason, food industries need to employ strategies such as adding antioxidants, preservatives and stabilizers to improve the final products for consumers.

Modern consumers are increasingly interested in the consumption of healthy foods and are paying more and more attention to the reduction of synthetic preservatives and the increased use of natural preservatives/antioxidants (De Bruno et al., 2022). In addition, it is important that these natural antioxidants be obtained with green, environmentally friendly and natural strategies. This is done for health reasons, and because it is ethical, sustainable and provides different functions: antibacterial (Martins et al., 2013; Carvalho & Conte-Junior, 2021), insecticidal and anti-fungal activities (Silva et al., 2021), antioxidants (Abeyrathne et al., 2021; De Bruno et al., 2021) and preservatives (Bensid et al., 2022; Santos-Sànchez et al., 2017).

A great source of natural antioxidants is represented by food processing wastes and by-products. Food wastes are an extraordinary source of antioxidant compounds, that if appropriately recovered can be recycled back into the production process. By means of the recovery of these fractions, the sustainability of the entire process can be increased by improving wastewater management. In fact, bioactive compounds that are present in these extracted fractions could reduce the biodegradability of waste. This is an environmental, economic and social challenge when trying to find a solution through responsible practices to reduce the environmental impact. In this way, the recovery of agri-food waste avoids pollution, and the use of natural resources such as water, energy, land and much more that could be needed to produce nutrients supporting a circular economy in favor of waste instead of raw materials. In order to follow a model of a circular and environmentally friendly economy, it is essential to apply green extraction techniques. Advances in green solutions have been pursued in recent years considering ionic liquids, and supercritical and subcritical fluids (Perna et al., 2020), but also water and ethanol as good substitutes of organic solvents used in the past (Chemat et al., 2019; Nutrizio et al., 2021).

For example, great interest has been attracted by Bergamot Pomace (BP), the waste resulting from the processing of bergamot. Bergamot (*Citrus bergamia,* Risso) is a citrus belonging to the Rutaceae family, cultivated mainly in the province of Reggio Calabria (Tsiokanos et al., 2021; Strano et al., 2017), and used mostly for the production of essential oils (claimed DPI since 1999 from the European Union), and juice. BP consists of skins, pulp and seeds, and is concretely an important source of bioactive molecules that are of considerable interest at the scientific level due to their beneficial effects on health (Gattuso et al., 2023). These recovered bioactive compounds that show antioxidant properties as demonstrated by De Bruno et al., 2023 can be applied for the formulation/fortification of other food products or food ingredients. For example, they could be used to improve the qualitative aspects of plant-based foods. For this reason, the aim of this paper was to investigate the possible application of natural antioxidants recovered from bergamot processing waste for the fortification of vegetable fat for application as an ingredient for the formulation of baked products (biscuits). The fortification had a dual purpose: improving the sustainability of the entire citrus processing and creating new foods for consumer needs.

3.2. Materials and methods

3.2.1. Materials and chemicals

Bergamot Pomace (BP) was collected by a company located in Reggio Calabria (Italy). Sunflower oil, soy milk, wheat flour, sugar, salt, and chemical baking powder (disodium diphosphate, sodium hydrogen carbonate and cornstarch) were purchased from a local supermarket.

3.2.2. Extraction of antioxidant compounds from bergamot pomace

The antioxidant extract (AE) was obtained following the method reported by Gattuso et al., 2023. Briefly, 200 g of powdered BP (powder was obtained by grinding of BP with a laboratory blender, 12% of moisture content) was mixed with 800 mL of an hydro-alcoholic mixture (ethanol : water; 1 : 1, v/v) and kept under a heater and continuous stirring for 30 minutes at 70 °C. Afterward, the mixture was centrifuged (9000 rpm, 10 min, 4 °C) in a refrigerated centrifuge (NF 1200R, Nüve, Ankara, Turkey) and the liquid extract was concentrated with a Rotavapor to remove the ethanol (97 mbar of vacuum and at 25 °C).

3.2.3. Physicochemical and antioxidant evaluation of the AE

The AE was characterized for physicochemical and antioxidant characteristics; in particular, the analyses that have been carried out were as follows.

pH was measured using a Crison pH-meter, basic model 20, according to the AOAC International Method (14.022) (AOAC, 1980).

Colour was measured using a Minolta CM-700d Spectrophotometer, with reference to the CIE L* a* b* coordinates (where L* represent the brightness; for a* positive values indicate redness, and negative values indicate greenness; and for b* positive values indicate yellow and negative values indicate blue) using a D65 illuminant.

Total Polyphenol Content (TPC) was determined by following the method reported by Gonzalez-Molina et al., 2009 with appropriate modification. For the assay, 0.2 mL of diluted (1:20) AE were place in a volumetric flask (25 mL) with 5 mL of distilled water and 1 mL of Folin–Ciocalteu reagent. After 8 min, 10 mL of Na_2CO_3 (20%) was added and brought to volume with distilled water. The mixtures were incubated for two hours at room temperature in the dark. Gallic acid solution was used as a reference standard and absorbance was measured at 765 nm in a double beam ultraviolet-visible spectrophotometer (PerkinElmer UVVis 2, Waltham, MA, USA).

Calibration standard solution (mg/L) =

$$\frac{s\ x\ V}{25\ mL}$$

where s is the concentration of the gallic acid solution and V is the volume of the stock standard solution.

The results are expressed as mg of gallic acid equivalents on 100 mL^{-1} of antioxidant extract (mg GAE 100 mL^{-1} AE).

$$TPC = c\ x \frac{V}{m}$$

where c is the concentration of gallic acid obtained from the calibration curve in mg L^{-1}; V is the volume of the extract in mL, and m is the mass of the extract in grams.

Total Flavonoid Content (TFC) was determined following the method reported by Cerda-Bernad et al., 2022 with slight modifications. Briefly, 0.300 mL of sample was mixed with 1 mL of water and 150 µL of sodium nitrite (5%, w/v) solution. After 6 min,

150 µL of aluminium trichloride (10%) solution was added, and 6 min after that, 2 mL of sodium hydroxide (1 M) solution was added. The volume was adjusted to 5 mL with water. A solution without sample, was used as a blank and the absorbance was measured at 510 nm. The results were expressed as mg of catechin equivalents 100 mL^{-1} of antioxidant extract (mg CE 100 mL^{-1} AE).

Calibration standard solution (mg/L) =

$$\frac{s\ x\ V}{25\ mL}$$

where s is the concentration of the catechin solution and V is the volume of the stock standard solution.

$$TFC = c\ x \frac{V}{m}$$

where c is the concentration of catechin obtained from the calibration curve, V is the volume of extract in mL, and m is the mass of extract in grams.

Total antioxidant activity (TAA) was analysed in vitro through two different assays, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) following the methods reported by Mafrica et al., 2023 with appropriate modifications. For DPPH, the reaction was prepared by mixing in a cuvette 20 µL of diluted (1 : 20) AE with 2980 µL of 6 × 10−5 mmol L^{-1} of DPPH solution and left in the dark under continuous stirring for 30 min. The decrease in absorbance was measured at 515 nm. For the ABTS assay, 20 µL of diluted AE was added to the ABTS solution to achieve a final volume of 3 mL and left in the dark for 6 min in a cuvette. The absorbance was measured at 734 nm. The results of both assays were expressed as mmol Trolox per L of antioxidant extract.

Calibration standard solutions (mg/L) =

$$\frac{s\ x\ V}{25\ mL}$$

where s is the concentration of the Trolox solution and V is the volume of the stock standard solution.

$$\text{TAA} = \left(C\ \frac{Vc}{\text{v}}\right) / 1000$$

where C is the concentration of the Trolox solution obtained from inhibition percentage, Vc is the total volume in the reaction cuvette (3 mL) and v is the volume of the sample.

Polyphenol profile and quantification with ultra-high performance liquid chromatography (UHPLC) was conducted applying the same conditions as described by Gattuso et al., 2023 using a UHPLC PLATINblue (Knauer, Berlin, Germany), equipped with a binary pump system using a Knauer blue orchid column C18 (1.8 mm, 100 × 2 mm) coupled with a PDA-1 (Photo Diode Array Detector) PLATINblue (Knauer, Germany). First, 5 µL of filtered sample was injected and the eluents were water (A) acidified with formic acid (pH 3.1) and acetonitrile (B). The gradient elution program consisted of 0–3 min, 95% A and 5% B; 3–15 min, 95–60% A and 5–40% B; 15–15.5 min, 60–0% A and 40–100% B; and finally returning to the initial conditions (column temperature: 30 °C). External standards (concentration between 1 and 100 mg L^{-1}) were analyzed for quantification and Clarity 6.2 software was used. The results were expressed as mg mL^{-1} of AE.

3.2.4. Preparation of vegetable fats (VFs) and biscuits (Bs)

3.2.4.1. Vegetable fat (VF)

The experimental plan provided the production of three samples: two VFs enriched with AE (2 and 4% AE) and one control sample (VFC, without AE). VFs were obtained by mixing with a blender the ingredients reported in Table 3.1.. In VF2% and VF4%, an aliquot of soymilk was replaced by AE. Subsequently, they were centrifuged (8000 rpm, 10 min, 4 °C) in a refrigerated centrifuge (NF 1200R, Nüve, Ankara, Turkey) and the supernatant was removed. The VFs were stored at 4 °C until further use and analytical characterization.

In the experimental procedure, VFs were used after their characterization, as ingredients in biscuit (B) formulations. For each VF sample, a biscuit sample was made. Biscuits were prepared in the laboratory of Food Technology of the Mediterranean University of Reggio Calabria (Italy). The ingredients of the biscuit's and their denominations are reported in Table 3.2. and the only variable is the VF used in their production. The dough was prepared using a mixer (Bimby TM31, Vorwerk, Wuppertal, Germany), mixing the ingredients (at about 3200 rpm) until a homogeneous dough is obtained. Then, the dough was rolled out with a rolling pin calibrated to obtain a

homogeneous thickness of 3 mm. The baking process was carried out in an electric oven (Angelo Po Combistar FX, Carpi, Modena, Italy) at a temperature of 180 °C for 10 minutes (Figure 3.1). Baked samples were subject to characterization.

Table 3.1. VFs denomination

Ingredient (g)	VFC[1]	VF2%[2]	VF4%[3]
Sunflower oil	66.6	66.66	66.66
Soymilk	33.33	31.33	29.33
AE	-	2	4

[1] Control sample; [2]VF enriched with 2% of AE; [3] VF enriched with 4% of AE

Table 3.2. Biscuits denomination

Ingredient (g)	CB[1]	B2%[2]	B4%[3]
Wheat Flour	400	400	400
VFC	100	-	-
VF2%	-	100	-
VF4%	-	-	100
H_2O	96	96	96
Baking powder	8	8	8
Sugar	96	96	96

[1] Control sample; [2] Biscuits formulated with VF2%; [3] Biscuits formulated with VF4%.

Figure 3.1. Biscuits preparation

3.2.5. Characterization of the physicochemical properties of the VFs

3.2.5.1. Physicochemical evaluation

The moisture content (MC) in the VFs was determined for 5 g of the sample using a Sartorius Moisture Analyzer MA37 thermal balance by the gravimetric method at 105 °C until constant weight. The results were expressed as a percentage (MC%). The water activity (a_w) was determined at 25 °C using a hygrometer (Aqualab LITE, Decagon, Nelson Court, Pullman, Washington). A few grams of sample were inserted into a high-density polyethylene container and subsequently placed into the instrument cell for analysis. Each measurement took about 10 min. The AquaLab system was verified using a_w standard solutions. The pH and colour determination of the VF samples were performed as previously reported in Section 3.2.3. The VF colour was analysed by homogenizing the sample into a glass vessel. Chroma and Hue angle (h°) were calculated from the a* and b* values according to the equations:

$$\text{Chroma (C): } (a^2 + b^2)^{1/2}$$

$$\text{Hue angle (h°): } \arctan (b^*/a^*)$$

Total acidity was calculated according to Official and standards (AOCS) methods (Ca 5a 40; Cd 8–53; Ch 5-91) as % of oleic acid. (AOCS, 2017; AOCS, 2017; AOCS, 1989). It was calculated as follows:

$$\%\ \text{oleic acid} = \frac{(\text{V}\ x\ \text{c}\ x\ \text{M})}{(10\ x\ m)}$$

Where:

V= Volume in mL of standard sodium hydroxide used for titration;

c= Normality of the sodium hydroxide solution;

M= Molecular Mass of Oleic Acid (282.47 g/mol);

m= Weight of the sample in g

Peroxide value (PV) was also carried out to evaluate the antioxidant effect of AE addition. It was performed according to the standard method of AOCS (965.33.12) (AOCS, 2000). PV was expressed as meq O_2 kg^{-1} and calculated with the following equation:

$$PV = \frac{(1000\ x\ V\ x\ c)}{m}$$

Where:

V= Volume in mL of $Na_2S_2O_3$ used for titration;

c= Normality of $Na_2S_2O_3$;

m= Weight of the sample in g

Oxidative stability was determined using an Accelerated Storage Test (OXITEST). VFs were submitted to high oxidative conditions in an OXITEST reactor. This test detects the time necessary to reach an end point of oxidation that corresponds to a detectable rancidity or a rapid change in the oxidation rate and allows the sample Induction Period (IP) to be obtained within a short time. The method recommended by the AOCS International Standard Procedure (Cd 12c–16) for the determination of oxidation stability of food, fats, and oils (AOAC) (AOCS, 2017) was followed. In order to determine the oxidative stability, samples were treated under conditions of accelerated oxidation, monitoring the oxygen uptake of the reactive constituents of the food samples in an oxidation test reactor (VELP Scientifica, Usmate Velate, MB, Italy). Briefly, 10 g of sample were distributed homogenously in a hermetically sealed titanium chamber; oxygen was purged into the chamber up to a pressure of 6 bar. The reactor temperature was set at 90 °C. These reaction working conditions allow the sample Induction Period (IP) to be obtained within a short time. The OXITEST allows the modification of absolute pressure inside the two chambers to be measured and, through the OXISoftTM Software (Version 10002948 Usmate Velate, MB, Italy), the IP is automatically generated, expressed as hours, by the graphical method.

3.2.5.2. Extraction and antioxidant evaluation of phenolic fraction.

The recovery of the phenolic fraction from the VF samples was carried out as reported by Baiano et al., 2009 with slight modification. First, 10 g of VF was mixed in a vortex with 5mL of methanol : water (70 : 30, v/v) and 5 mL of hexane for 10 min. Then, the hydro-alcoholic phase was separated from the lipid phase in a refrigerated centrifuge apparatus at 6000 rpm, 4 °C for 10 min. Hydro-alcoholic extracts (VFEs) were recovered, filtered through a 0.45 mm nylon filter of diameter 15 mm (Thermo Fischer Scientific, Waltham, MA, USA) and stored until evaluation of the phenolic compounds and antioxidant activity.

In order to determine the total phenolic content (TPC), 0.300 mL of extract was mixed with 0.300 mL of Folin reagent, 0.250 mL of distilled water and, after 4 min, 2.4 mL of an aqueous solution of Na_2CO_3 (5%). The mixture was maintained in a 40 °C water bath for 20 min and TPC was determined at 750 nm. The results were expressed as mg of gallic acid equivalent on 100 g^{-1} of VF.

The total flavonoid content (TFC) was evaluated following the method described in Section 3.2.3. The results are expressed as mg of catechin equivalents 100 g^{-1} of vegetable fat (mg CE 100 g^{-1} VF).

Radical scavenging activity was tested with two different assays: DPPH and ABTS. In vitro assays were performed following the methods reported in Section 3.2.3. The results are expressed as mmol Trolox per kg of VF.

Identification and quantification of phenolic compounds in VFs was carried out as described in Section 3.2.3, and the results are expressed as mg 100 g^{-1} of sample.

3.2.6. Characterization of physicochemical and antioxidant properties of biscuits (Bs)

3.2.6.1. Physicochemical evaluation

The moisture content (MC), water activity, pH, colour parameters and oxidation stability were evaluated for the biscuits following the methods described in Section 3.2.5.1. Colour values were used to calculate the Browning Index (BI) as reported by Phatak et al., 2017 using the following equation:

$$BI = \frac{[100\ (X - 0.31)]}{0.17}$$

$$\text{Where } X = \frac{(a+1.75L)}{m(5.645L + a - 3.012b)}$$

3.2.6.2. Maillard reaction products (MRPs)

MRPs were measured spectrophotometrically at three different wavelengths: 280 nm, 360 nm and 420 nm. This is because early low molecular weight compounds were monitored at 280 nm, a pool of more advanced ones at 360 nm and high molecular weight compounds, such as melanoidins, with chromophore groups at 420 nm, determined by their absorption. MRPs were determined as suggested by Delgado-Andrade et al., 2010 with some modifications. Briefly, 1 g of biscuit was vortexed for 30 s with 20 mL of distilled water in a centrifuge tube. Then, the tube was sonicated (IKA, Staufen, Germany)

for 10 min, vortexed and centrifuged at 7000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.45 mm RC filter (Thermo Fischer Scientific, Waltham, MA, USA), and measured. Distilled water was used as the blank and the analysis was carried out in triplicate and due to the unavailability of calibration standards, the results were expressed as absorbance units referring to 1 g dry sample (AU per g dw).

3.2.6.3. Rheological analyses on doughs and cocked biscuits

A TA-XT.plus texture analyzer (Stable Micro Systems Ltd., Godalming, UK) equipped with the Exponent software 6.1.4.0 (Stable Micro Systems Ltd., Godalming, UK) was used to evaluate the rheological characteristics of the dough and biscuit samples as described by Merlino et al., 2022 with slight modification. The tests were carried out on ten replicates for each sample.

The doughs were subjected to stickiness tests. These tests were performed on 20 g of sample employing a Chen and Hoseney probe (A/DSC) (Stable Micro Systems Ltd., Godalming, UK). To evaluate the forces of insertion and withdrawal from the dough the following parameters were used: pre-test speed, 0.50 mm s^{-1}; test speed, 0.50 mm s^{-1}; post-test speed, 10.00 mm s^{-1}; distance, 5.0 mm; trigger force, 5.0 g; data acquisition rate, 500 pps.

The entire biscuit was used to perform a TPA test using a 100 mm compression plate (P/100) probe (Stable Micro Systems Ltd., Godalming, UK) with the following parameters: pre-test speed, 1.00 mm s^{-1}; test speed, 5.00 mm s^{-1}; post-test speed, 5.00 mm s^{-1}; distance: 20.0 mm, trigger force, 5.0 g; data acquisition rate, 400 pps. The test results expressed different textural characteristics, such as cohesiveness, gumminess, chewiness, springiness, and resilience.

A three-point bending test (TPB) was carried out using a three-point bend ring probe (HDP/3PB). The sample was placed on the two adjustable supports and the cutting probe was lowered until it touched the sample, imparting a force that was increased until the biscuit breaks. The maximum peak force was used to calculate the hardness value. For this test, the operative conditions were: pre-test speed, 1 mm s^{-1}; test speed, 3 mm s^{-1}; post-test speed, 10.00 mm s^{-1}; distance, 7.5.0 mm; trigger force, 5.0 g; data acquisition rate, 400 pps.

3.2.6.4. Total antioxidant activity assays and individual phenolic compounds in Bs

These tests were performed following the methods described in Section 3.2.5.2.

3.2.7. Statistical elaboration

All the analysis were performed in triplicate (n = 3), and the experimental results were expressed as mean value ± standard deviation. The significant differences ($p < 0.05$) among mean values were determined by one-way analysis (ANOVA, Analysis of Variance), applying SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA). A series of multiple comparisons, with Tukey's post hoc test, was performed to determine individual significant differences ($p < 0.05$).

3.3. Results and discussion

3.3.1. Antioxidant characteristics of bergamot pomace extract (AE)

Citrus by-products and in particular bergamot pomace are characterized by a high antioxidant content, and for this reason can be reused for different aims, such as natural preservatives for food production. Indeed, in this study, the BP was used for extracting the antioxidant compounds to apply for the formulation of vegetable fat.

For the extraction process, a procedure was followed that was described in our previous work (De Bruno et al., 2023) using a hydroalcoholic mixture to obtain a liquid extract rich in phenolic compounds. The green solvents used were ethanol and water (50 : 50, v/v). Ethanol is environmentally friendly and allows a good extraction of polyphenols to be obtained (Gil-Martìn et al., 2022). This extraction allows citrus waste to be converted into a source of value-added products to use in functional food as widely demonstrated in the literature (Andrade et al., 2023). The liquid extract showed a low pH of 3.31. In general, many foods are chemically acidified to hinder the growth of microorganisms, which cause contamination and loss of foodstuffs. Because of the low pH value of the extract, it could be a good basic matrix that when added to food also has acidifying power.

With the aim to determine the aliquots of extract to be added to the vegetable fat, the extract was characterized spectrophotometrically for total polyphenol content (TPC) and total flavonoid content (TFC). Its radical scavenging activity was also evaluated. As reported in Table 3.3., the AE exhibited a high value of TPC of 776.24 mg GAE 100 mL^{-1} and TFC of 267.42 mg CE 100 mL^{-1}. The total antioxidant activity level of the AE was investigated with ABTS and DPPH assays, which showed values of 795.06 and 992.2 mmol Trolox per L, respectively.

The main individual phenolic compounds evaluated through the chromatographic analysis (UHPLC-DAD) were reported in order of retention time (Table 3.3). They were (from the one with the highest concentration to the lowest) neoeriocitrin (3.64 ± 0.29 mg mL^{-1}), naringin (3.21 ± 0.17 mg mL^{-1}), neohesperidin (1.83 ± 0.19 mg mL^{-1}), brutieridin (1.48 ± 0.14 mg mL^{-1}), melitidin (0.73 ± 0.05 mg mL^{-1}), p-coumaric acid (0.1 ± 0.02 mg mL^{-1}), eriocitrin (0.06 ± 0.01 mg mL^{-1}), ferulic acid (0.05 ± 0.01 mg mL^{-1}), and narirutin (0.04 ± 0 mg mL^{-1}). The major flavonoid compounds detected were in accordance with Nogata et al., 2006 and Bartella et al., 2022.

Table 3.3. Bergamot Phenolic Extract (AE) characterization

Physicochemical	pH	3.31 ± 0.05
	L*	49.35 ± 0.14
	a*	0.57 ± 0.07
	b*	2.85 ± 0.14
Total Antioxidant assays	TPC (mg GAE 100 mL^{-1})	776.24 ± 16.05
	TFC (mg CE 100 mL^{-1})	267.42 ± 7.04
	ABTS (mmol Trolox L^{-1})	795.06 ± 115.98
	DPPH (mmol Trolox L^{-1})	992.2 ± 124.08
Phenolic acids (mg mL^{-1})	p-coumaric acid	0.1 ± 0.02
	Ferulic acid	0.05 ± 0.01
Flavonones (mg mL^{-1})	Eriocitrin	0.06 ± 0.01
	Neoeriocitrin	3.64 ± 0.29
	Narirutin	0.04 ± 0
	Naringin	3.21 ± 0.17
	Neohesperidin	1.83 ± 0.19
C-glycosyl flavones (mg mL^{-1})	Melitidin	0.73 ± 0.05
	Brutieridin	1.48 ± 0.14

3.3.2. Physicochemical and antioxidant characteristics of enriched vegetable fats (VFs)

The physicochemical characteristics of the different vegetable fat samples (enriched samples compared to control) were reported in Table 3.4.. Water activity (a_w), moisture content (MC%), L* and a* colour parameters did not show significant differences (p >

0.05) among the different vegetable fats (VFs). In contrast, high significant differences were shown for b*; in fact, there was a positive increase of this character, which highlights the yellow colour in the VF, probably due to the presence of carotenoids and other pigments abundantly present in citrus peel extract (Montero-Calderon et al., 2019). It increased from 6.69 ± 0.41 in VFC to 7.23 ± 0.03 in VF4%. This was also found by Kneifel et al., 1992 who observed changes in b* values ascribed to the carotene content in butter samples. The chroma (C*) value or saturation describes the vividness or colourfulness (Mahajan & Bandyopadhyay, 2020) and for this character, a statistical difference was noted, with the highest values recorded in VF2% and VF4%, 7.17 ± 0.11 and 7.24 ± 0.03, respectively. The hue angle (h°) is specified as 0°/360° for red/magenta, 90° for yellow, 180° for green, and 270° for blue or purple, as well as intermediate hues between adjacent pairs of these fundamental colours. It specifies the relative proportions of redness and yellowness (Korley & Tuah, 2015). The results of h° of the VF samples did not reveal any statistical differences, and the values are similar to those reported by Chudy et al., 2022 who investigated colour in various butter samples and obtained comparable h° results, showing a similarity between these and those studied in this work.

In Table 3.4., the results of total acidity (TA), peroxide value (PV), and induction period (IP) are also reported. The statistical analysis carried out on the samples showed that TA and PV did not show significant differences among the VFC, VF2%, and VF4% samples.

Regarding the evaluation of oxidation stability determined with the Oxitest system, the results were expressed as hours of induction period (IP), and the mean of the values is 10 : 37 for VFC, 12 : 36 for VF2%, and 13 : 08 for VF4% with statistical differences among the samples ($p < 0.01$). The results showed that the VFC had the shortest IP, followed by VF2%, and VF4% had the longest IP. This means that VF4% is more stable and could have a longer shelf life compared to the other two vegetable fats. In addition, the IP also increased in VF2% compared to the control, which confirmed the antioxidant effect of the added extract, in accordance with El-aal & Halaweish, 2010 who found that orange peel extract improved the oxidative stability of soybean oil. Moreover, the impact of distinct plant extracts on the induction period (IP) was examined in fat samples and validated the aforementioned effect (Gramza-Michalowska et al., 2007).

Table 3.4. Physicochemical characteristics of vegetable fats (VFs)

	VFC	VF2%	VF4%	Sign.
a_w	0.981±0.001	0.984±0.002	0.985±0.003	n.s.
MC%	21.13±2.09	22.01±3.75	23.22±0.83	n.s.
L*	88.56±3.25	88.78±0.78	87.68±1.09	n.s.
a*	-0.15±0.19	-0.26±0.03	-0.23±0.02	n.s.
b*	$6.69±0.41^{b}$	$7.16±0.11^{a}$	$7.23±0.03^{a}$	**
C*	$6.69±0.41^{b}$	$7.17±0.11^{a}$	$7.24±0.03^{a}$	**
h°	91.24±1.84	92.13±0.23	91.84±0.17	n.s.
TA (% oleic acid)	6.87±0.55	5.76±0.52	7.44±0.23	n.s.
PV (meq O_2 kg^{-1})	4.09±0.95	2.38±0.04	3.25±0.43	n.s.
IP (hh:mm)	$10:37±0.12^{c}$	$12:36±0.15^{b}$	$13:08±0.08^{a}$	**

Small letters show a significant difference by Tukey's post hoc test. Abbreviations: **, Significance at $p<0.01$; n.s., not significant

For the purpose of verifying the antioxidant activity in VF samples, different total antioxidant assays were performed and the results are reported in Table 3.5.. Specifically, VF2% and VF4% have significantly higher levels of TPC, ABTS, and DPPH antioxidant activity than VFC, indicating that the antioxidant capacity of the vegetable fats increased with the addition of the antioxidant extract. The increase in antioxidant capacity was more evident in the VF4% sample, which showed the highest values of TPC, ABTS and DPPH. Overall, these data suggested that the addition of the antioxidant extract to vegetable fat can significantly increase its antioxidant capacity, which may have important implications for food and nutrition industries seeking to improve the antioxidant content of their products. However, it is important to note that the specific antioxidant extract used can impact the results and should be carefully considered when interpreting the findings.

Table 3.5. Antioxidant characteristics of vegetable fats (VFs)

	VFC	VF2%	VF4%	Sign.
TPC (mg GAE 100 g^{-1})	75.82 ± 3.19^{c}	210.61 ± 8.89^{b}	286.31 ± 2.74^{a}	**
TFC (mg CE 100 g^{-1})	19.88 ± 2.98^{c}	38.48 ± 0.7^{b}	55.75 ± 0.64^{a}	**
ABTS (mmol TE kg^{-1})	50.69 ± 2.11^{c}	115.45 ± 19.16^{b}	147.25 ± 6.19^{a}	**
DPPH (mmol TE kg^{-1})	2.69 ± 0.29^{c}	9.71 ± 0.38^{b}	14.65 ± 1.65^{a}	**

Small letters show a significant difference by Tukey's post hoc test. Abbreviations: **, Significance at p<0.01

In addition to the determination of total antioxidants, a more detailed analysis for the identification of the individual phenolic compounds has also been carried out. The phenolic compound concentrations detected showed a trend of increasing in relation to the quantities of added extract. In Table 3.6., the results of a comparison between VF2% and VF4% obtained by liquid chromatographic analysis performed with the UHPLC-DAD system were reported. The results exhibited that VF4% contained significantly higher levels of p-coumaric acid, neoeriocitrin, naringin and neohesperidin compared to

VF2%. The differences in the levels of these compounds between VF2% and VF4% are statistically significant ($p < 0.01$) except for narirutin, melitidin and brutieridin ($p < 0.05$). Furthermore, the total phenolic content revealed by UHPLC as significantly higher in VF4% than in VF2%, with a respective content of 35.63 ± 3.1 and 19.67 ± 1.15 mg 100 g^{-1} of VF.

Table 3.6. Individual phenolic compounds revealed on vegetable fats (VFs)

mg 100 g^{-1} of VF	VF2%*	VF4%*	Sign.
p-coumaric acid	0.06 ± 0.02	0.18 ± 0.04	**
Neoeriocitrin	6.15 ± 0.64	11.47 ± 1.22	**
Narirutin	0.06 ± 0.01	0.1 ± 0.02	*
Naringin	6.08 ± 0.2	11.14 ± 1.33	**
Neohesperidin	3.86 ± 0.69	6.33 ± 0.44	**
Melitidin	1.05 ± 0.09	2.04 ± 0.51	*
Brutieridin	2.41 ± 0.28	4.36 ± 0.86	*
Total	19.67 ± 1.15	35.63 ± 3.1	**

Abbreviations: **, Significance at p<0.01; *, significance at p<0.05

3.3.3. Physicochemical and antioxidant characteristics of biscuits

After the enriched vegetable fat preparation and characterization, these ingredients were applied for the formulation of a bakery product, specifically "biscuits". The physicochemical characteristics of the biscuit samples (Table 3.7.), such as moisture content, water activity and colour of the samples, were studied. MC and aw are important factors affecting the quality and stability of food products, as they can affect their colour, texture, and microbial growth. Regarding MC, the results suggested that there was no statistical difference for the three samples compared. Concerning the aw, its measurement is crucial in the bakery sector because it is associated with the stability and safety of food products over their storage duration (Mathlouthi, 2001). The results showed significant differences between the aw values, with B4% having the lowest mean value (0.333 ± 0.007) and BC having the highest value (0.488 ± 0.001). This effect could be due to the presence of phenolic compounds, which lowered the water activity of the bakery product by binding to water molecules and diminishing the free water levels.

Table 3.7. Physical characteristics of biscuits

	BC	B2%	B4%	Sign.
MC%	4.94 ± 0.21	5.53 ± 0.17	5.36 ± 0.11	n.s.
a_w	0.488 ± 0.001[a]	0.397 ± 0.011[b]	0.333 ± 0.007[c]	**
L*	73.9 ± 2.85	72.09 ± 6.22	72.53 ± 5.91	n.s.
a*	4.87 ± 3.05	6.71 ± 4.05	6.26 ± 4.39	n.s.
b*	22.41 ± 2.72	23.19 ± 2.25	22.86 ± 2.81	n.s.
C*	23.05 ± 3.29	24.37 ± 3.07	23.97 ± 3.75	n.s.
h°	78.56 ± 6.07	74.81 ± 8.39	75.99 ± 8.84	n.s.
BI	40.84 ± 10.14	46.21 ± 12.56	44.92 ± 13.99	n.s.

Small letters show a significant difference by Tukey's post hoc test. Abbreviations: **, Significance at $p<0.01$; n.s., not significant.

As regards the rheological properties of biscuit dough, the results shown in Table 3.8. reported the changes due to the addition of enriched VFs. Statistical analysis showed differences among the samples with similar results for the Bs formulated with enriched VFs (B2% and B4%). The lower values of stickiness, work of adhesion and ratio of dough strength/ cohesiveness for samples B2% and B4% were probably due to the presence of antioxidant compounds which could modify the surface by decreasing the hydrophilicity of the dough.

Table 3.8. Dough Stickiness Test

	Stickiness (g)	Work of Adhesion (g. sec)	Dough Strength/ Cohesiveness (mm)
BC	24.97 ± 3.52[a]	0.78 ± 0.13[a]	0.45 ± 0.07[a]
B2%	18.26 ± 3.08[b]	0.54 ± 0.08[b]	0.4 ± 0.02[b]
B4%	19.31 ± 4.12[b]	0.59 ± 0.11[b]	0.4 ± 0.03[b]
Sign.	**	**	*

The results showed high statistical differences in springiness, cohesiveness, and resilience ($p < 0.01$), and statistical differences ($p < 0.05$) in gumminess (Table 3.9.). Chewiness was not affected by the different formulations. These values were lower in B2% and B4% compared to BC, for retarding starch retrogradation due to the addition of polyphenols, in accordance with those reported by Li et al., 2016. This finding showed that there is a good potential of enriched VFs used as enhanced bakery ingredients in improving texture quality and the durability of the products.

Table 3.9. TPA test.

	Springiness[1]	Cohesiveness[1]	Gumminess[2]	Chewiness[2]	Resilience[1]
BC	0.81 ± 0.06[a]	0.82 ± 0.04[a]	375.51 ± 54.24[a]	305.09 ± 64.93	0.72 ± 0.05[a]
B2%	0.71 ± 0.06[a]	0.74 ± 0.05[b]	317.71 ± 47.38[b]	249.78 ± 39.84	0.62 ± 0.07[b]
B4%	0.76 ± 0.04a[b]	0.77 ± 0.03[b]	341.8 ± 38.19[ab]	260.8 ± 42.25	0.65 ± 0.04[b]
Sign.	**	**	*	n.s.	**

1: dimensionless characteristics; 2: N.

Three-point bending is a textural test commonly used for bakery goods such as biscuits, bread, crackers or other food products to evaluate mechanical properties. In this study, the hardness of the biscuits was evaluated (Figure 3.2.). This value decreased with the addition of phenolic compounds, with the lowest value for B4% (54.79 ± 12.74 N), the highest for BC (78.04 ± 13.97 N) and an intermediate value for B2%. The results can be explained by the added phytochemical extracts affecting the properties of wheat starch as reported by Zhu et al., 2009.

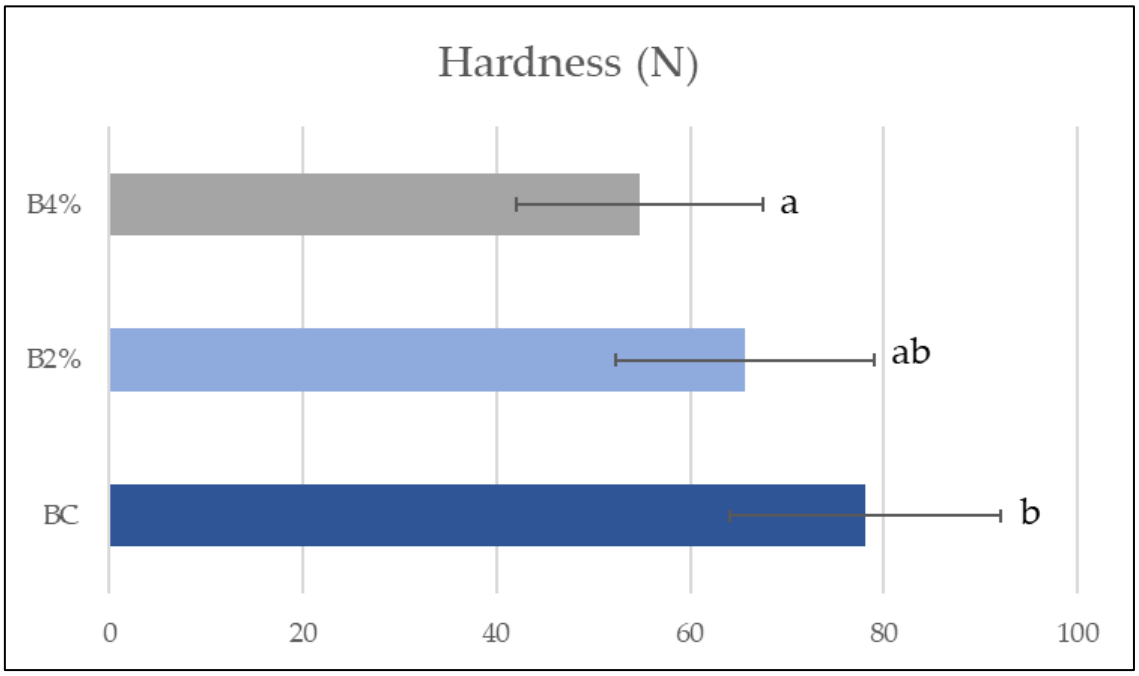


Figure 3.2. Hardness of biscuit samples

The oxidation stability test showed different induction periods among B2%, B4% and BC (Figure 3.3.). Considering these differences, it is possible to hypothesize that these variations are probably due to the presence of antioxidants that carry out their function. In fact, it is well-known that antioxidants work by inhibiting or delaying the oxidation process, which can lead to rancidity and other negative changes in the quality of the products. There is also a correlation to the aw of biscuit samples. As reported by Conte et al., 2021 water can act as an antioxidant at low levels of aw forming a barrier that protects sensitive sites from reacting with oxygen and also decreases the rate of free radical formation by increasing hydration of hydroperoxides and promoting recombination of free radicals. Additionally, it lowers metal catalytic activity. However, at higher aw values, water can have a pro-oxidant role acting as a plasticizing agent, promoting mobility and solubilization of the catalysts, and inducing matrix swelling, which exposes new reactive sites. This can lead to increased oxidation in the product.

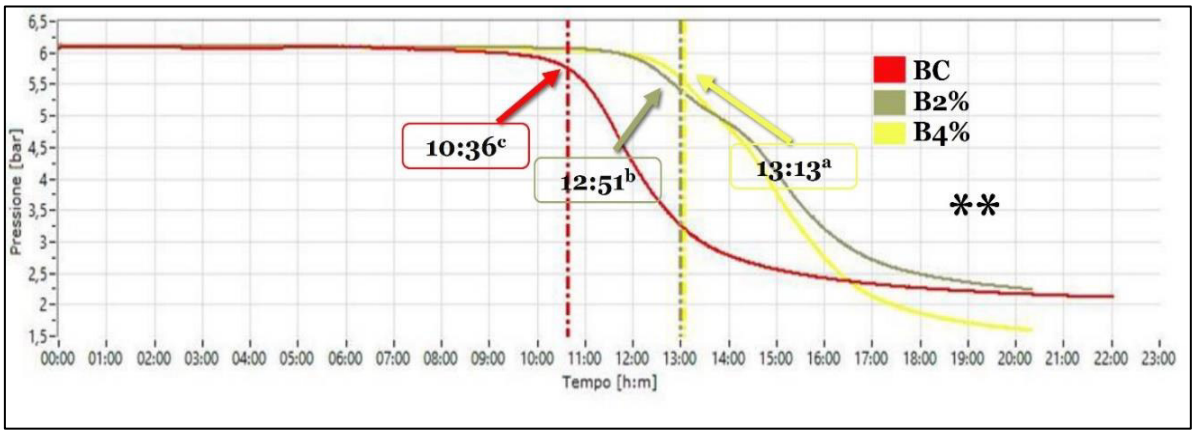


Figure 3.3. Oxidation stability response on biscuits

Another important aspect evaluated for biscuits is the Maillard reaction products. These products were measured considering low molecular weight compounds without colour that are formed early on (280 nm), intermediate molecular weight compounds with more advanced products (360 nm) and high molecular weight, coloured compounds that are known as melanoidins (420 nm) (Giovanelli & Cappa, 2021). The results reported in Table 3.10. show that there were no significant differences in MRP content among the samples, as the values for each wavelength were similar across all three samples. The MRP content in each sample ranged from 0.62 to 0.7 AU per g dw, suggesting that the addition of vegetable fats did not impact the formation of MRP in the biscuits.

The Maillard reaction is a non-enzymatic browning reaction that occurs between amino acids and reducing sugars under heat treatment. This reaction is responsible for the formation of flavor and aroma compounds, as well as the characteristic brown colour of baked goods, including biscuits. In moderate amounts, Maillard Reaction Products (MRPs) can contribute positively to the flavor and colour of biscuits, enhancing their sensory properties. However, excessive formation of MRPs can lead to negative health effects, as some MRPs have been associated with the development of chronic diseases (Bastos et al., 2012); they also result in a reduction of the nutritional value due to the loss of lysine and other amino acids through thermal degradation, as well as decreased protein bioavailability. For this reason, MRP quantity needs to be maintained equal to standard products, and it is important to limit excessive formation in order to reduce potential negative health effects.

Table 3.10. Maillard reaction products of biscuits (AU g^{-1} dw)

	λ	BC	B2%	B4%	Sign.
MRP	280 nm	0.69 ± 0.03	0.7 ± 0.02	0.62 ± 0.06	n.s.
	360 nm	0.15 ± 0.01	0.13 ± 0.01	0.12 ± 0.02	n.s.
	420 nm	0.11 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	n.s.

Abbreviations: n.s., not significant.

Like for the vegetable fats, also for the biscuits the determination of the total antioxidant activity and the individual phenolic compounds was performed, and the results are shown in Table 3.11. and Figure 3.4. The results indicated that samples B2% and B4% had higher TPC and antioxidant capacity values compared to the control one (BC). The differences between the samples were statistically significant, with B2% and B4% showing significantly higher values for all three characteristics. These findings demonstrated that the total phenolic content determined by the Folin–Ciocalteu method, and the antioxidant capacity in vitro analyzed with the ABTS and DPPH free radical scavenging methods, increased when the biscuits were formulated with vegetable fat containing phenolic compounds.

Table 3.11. Total antioxidant results of biscuits

	BC	B2%	B4%	Sign.
TPC (mg GAE 100 g^{-1})	14.25 ± 0.16^{c}	29.63 ± 1^{b}	32.96 ± 6.82^{a}	**
ABTS (mmol TE kg^{-1})	17.73 ± 2.09^{c}	35.24 ± 4.42^{b}	51.03 ± 11.32^{a}	**
DPPH (mmol TE kg^{-1})	156.58 ± 2.44^{b}	185.24 ± 3.77^{ab}	200.71 ± 27.17^{a}	*

Small letters show a significant difference by Tukey's post hoc test. Abbreviations: **, Significance at $p<0.01$; *, significance at $p<0.05$

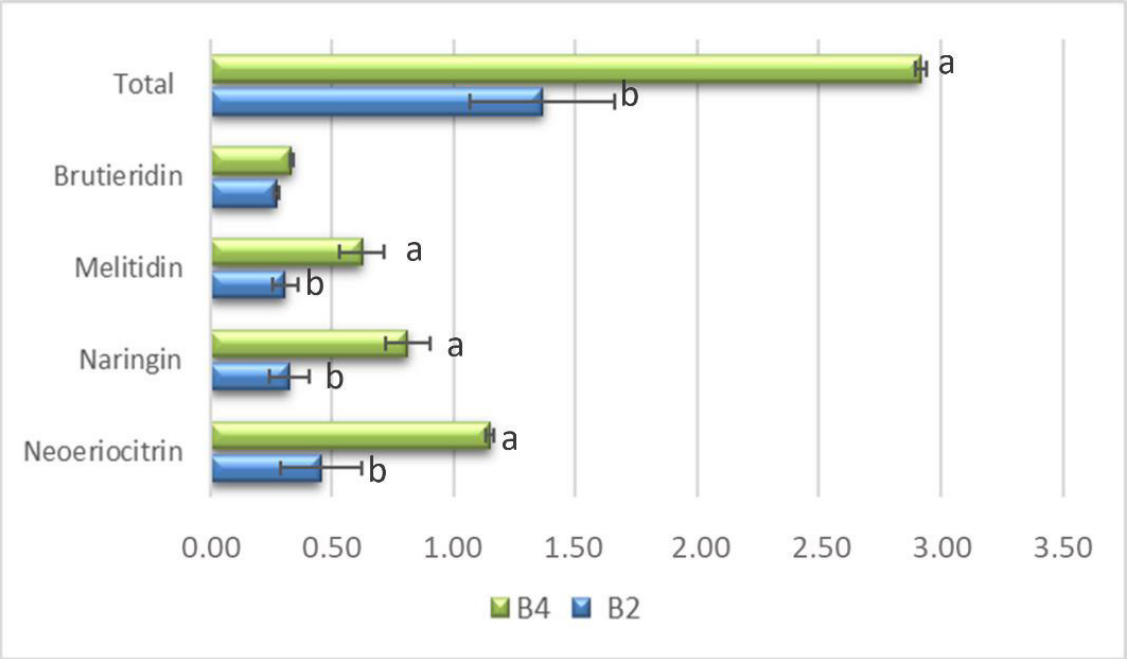


Figure 3.4. Individual phenolic compounds in Biscuits

The persistence of phenolic compounds in the biscuits after baking is confirmed by the phenolic compounds identified with UHPLC. Not all the phenolic compounds present in the VFs were identified in the biscuits, probably due to the low quantity or even because in part they were damaged by the baking heat. he results reported in Figure 4 show that there were statistical differences between the samples for the total content of flavonoids, melitidin, naringin and neoeriocitrin, but not for brutieridin that was found in a similar concentration in both biscuit samples.

3.4. Conclusions

This study provides information regarding the possible reuse of bergamot pomace, a by-product rich in antioxidant compounds, which are known for their beneficial effects on human health. These compounds have many useful properties in the food sector, for example as antioxidants, nutraceuticals and antimicrobials. The antioxidant extract obtained from bergamot pomace was used to formulate enriched vegetable fat. The enriched fat samples exhibited an increase of the antioxidant properties (TPC, TFC, ABTS and DPPH assays) showing statistical differences compared with control samples. The effect of the enrichment was confirmed by the antioxidant compounds identified with UHPLC and by a better oxidative stability compared to the control sample. Regarding the application of enriched vegetable fats as an ingredient for the formulation of biscuits, the results have highlighted that the physical characteristics of biscuits such as moisture,

colour and MRP have not undergone great variations compared with the control. One difference with a positive effect was revealed in aw, which decreased with the increase of extract in VF with well-known advantageous effects on the final products. The presence of antioxidant compounds in the enriched samples provided protection against heat treatment during the baking process and an increment of the oxidative stability of the biscuits, with a potential extension of the shelf life.

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4. EFFECT OF EDIBLE COATING ENRICHED WITH NATURAL ANTIOXIDANT EXTRACT AND BERGAMOT ESSENTIAL OIL ON THE SHELF LIFE OF STRAWBERRIES

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Abstract

In this study, the effects of the application of edible coatings on the shelf life of the strawberry were evaluated, with the aim of extending the fruit's availability and shelf life while preserving its qualitative characteristics. In particular, the application of edible coatings enriched with a natural antioxidant to strawberries was evaluated for their physicochemical, microbial, and structural properties, during a storage period (up to 14 days) at refrigerated temperature. The experimental plan provided the formulation for edible coatings enriched with different concentrations of a natural antioxidant extract obtained from bergamot (Citrus bergamia Risso) pomace (1, 2.5, and 5%), bergamot essential oil (0.1% v/v and 0.2% v/v), and a synthetic antioxidant, butylated hydroxytoluene (BHT, 100 ppm). Moreover, a control test with untreated strawberries was considered. The enriched gum Arabic coatings provided good results related to the preservation of the qualitative parameters of the strawberries. The samples coated with the antioxidant extract (2.5%, sample D) and bergamot essential oil (0.1%, sample F) showed the best maintenance of the qualitative parameters after 14 days, showing lower

decay rates (36% D and 27% F), good acceptability by consumers (between 5 and 6), and good retention of ascorbic acid (>30 mg 100 g^{-1}).

Keywords: antioxidant; bergamot essential oil; citrus bergamia; coated fruits; pomace; edible coating; strawberry

Chapter 4. Graphical abstract.

4.1 Introduction

The consumption of fruits and vegetables has significantly increased in the last few years; indeed, diets rich in natural antioxidants are increasingly recommended for their beneficial effects on human health (Duthie et al., 2018).

The problem with fruits and vegetables is their high perishability, which corresponds to a very short postharvest shelf life. They are very susceptible to postharvest quality losses due to mechanical damage, high respiration rates, microbiological damage, water loss, and natural physiological decay.

In relation to this, the correct management of the postharvest period and proper application of alternative practices leading to an extended shelf life are useful to improve the marketability of these natural and nutraceutical foods.

Strawberries (*Fragaria ananassa*) are among the most cultivated berries in the world. Recent data from 2022 reported 8,861,381 tons of production, a harvested area of 384,668 ha, and a yield of 230,364 hg/ha, showing increased cultivation in recent years all over the world (FAOSTAT, 2022). Strawberries are commercially desirable worldwide due to their sweetness, flavor, and juiciness, and are largely consumed fresh or processed (Xu et

al., 2014; Klopotek et al., 2005). As reported by several authors (Šamec et al., 2016; Ornelas-Paz et al., 2013), usually consumers prefer fruit with good appearance, flavor, durability, texture, and chemical characteristics. Strawberries are a good source of bioactive compounds such as organic acids, anthocyanins, phenols, flavonoids, sugars, vitamins, and minerals (Giampieri et al., 2015; Nunes et al., 2020; Sicari et al., 2020), which play an important role in human health. Strawberries are considered a "functional food", offering different health benefits for human health, mainly attributed to their high content of phytochemicals and high antioxidant activity, acting directly on the modification of the etiology of chronic diseases (Basu et al., 2015). In fact, their consumption helps in the prevention of cardiovascular and cardiometabolic diseases (Paquette et al., 2017; Park et al., 2022), mainly by improving insulin resistance. Afrin et al., 2016 reported a large amount of bioactive compounds in strawberries, with important clinical aspects and great importance in human nutrition; as such, this fruit can be considered a functional food. Moreover, in view of the wide demand and consumption rates and for the qualitative and quantitative losses, in scientific research more and more attention are being paid the postharvest shelf life extension and to developing new alternative techniques to maintain quality, prevent losses and waste, and obtain lasting consumer acceptability in terms of the sensorial properties of the final product.

Strawberries, a being non-climatic fruit, have limited shelf-life ranges of 1–2 days at room temperature and 5–7 days at refrigerated temperatures (Nguyen et al., 2021; Ayala-Zavala et al., 2004; Agapito-Ocampo et al., 2021). Strawberries are highly sensitive to chemical and microbial deterioration; indeed, they may be contaminated during different phases such as harvesting, post- harvesting, or processing. The most common pathogens are hepatitis A virus (Ayala-Zavala et al., 2004) and enteric bacteria such as Salmonella and Escherichia coli (Saddozai et al., 2012). Different technologies have been implemented with the aim of extending the shelf life of strawberries, such as refrigeration, modified atmosphere packaging, and alternative packaging, but in the last few years a lot of attention has been paid to edible coatings (Agapito-Ocampo et al., 2021).

The application of a coating on the fruit's surface generates a proximate zone with a modified atmosphere, which preserve the safety and nutritional quality because it delays ripening and protects the fruit from microbial disease and physiological senescence (Muley et al., 2020). The performance of the edible coatings depends on their

composition; they generally consist of biodegradable materials, such as plant extracts, proteins, lipids, and polysaccharides or blends of these materials (Tahir et al., 2019). Moreover, the edible coatings maintain the physicochemical (weight loss, pH, TSS, etc.) and antioxidant properties (phenols, vitamins, etc.) of the treated fruits and vegetables for longer periods (Blancas-Benitez et al., 2022).

Gum Arabic is a mixture of polysaccharides and glycoproteins (GPs) that is obtained from the Acacia Senegal tree and can be used as a glue and binder. For this reason, it is widely used in the food, beverage, pharmaceutical, and cosmetic industries as an emulsifier and a thickening agent (Tahir et al., 2019; Mariod et al., 2018).

Usually, synthetic or inorganic additives are used in coating applications, with good results, but modern consumers are more health-conscious and aware of the problems linked to toxicity for humans and the environment (Ong et al., 2020).

The actual trend is the application of natural coating materials and the addition of natural additives such as plant extracts as "green" additives to enhance the performance of the coatings (Maria et al., 2019). These natural extracts can be obtained using different plant parts such as leaves (Difonzo et al., 2019) and fruit peels (Singh et al., 2014), or can be derived from by-products (De Bruno et al., 2018; Imeneo et al., 2022) and waste (Romeo et al., 2019), and are rich in bioactive compounds with antioxidants properties (Singh et al., 2017).

Lotus leaf extract incorporated into edible coatings promoted the shelf life of goji berries more than a control sample treated with 1-methylcyclopropene (1-MCP) for about 4 days (Fan et al., 2018). Other studies (Tahir et al., 2019; Kim et al., 2018) including the use of natural extracts in coatings reported the extension of fruit shelf-life periods by combining the effect of the coating as a barrier against transpiration and the effects of the natural phenolic extract or essential oil incorporated into the polysaccharide matrix, with known antioxidant, antimicrobial, natural preservative effects (Zhang et al., 2022; Tügen et al., 2020; Iftikhar et al., 2022). Additionally, the application of essential oils (EO) is very important because they have a wide range of functional actions against foodborne and postharvest pathogens, as reported by Sanchez-Gonzalez et al., 2011. In particular, in this study bergamot essential oil (BEO) was used, which is obtained by rasping and cold-pressing the fruit peel. This essential oil was assigned the protected designation of origin "*Bergamot of Reggio Calabria*" (PDO, bergamot essential oil, 2015). BEO is

characterized by an intense fragrance and freshness and is applied in several sectors, such as in perfumes, cosmetics, food, and confectionery. Moreover, BEO has shown antimicrobial, anti-inflammatory, analgesic, and antiproliferative properties (Gioffrè et al., 2020; Navarra et al., 2015).

The purpose of this research was to investigate the effects of edible coatings based on gum Arabic enriched with natural antioxidants on the qualitative properties of strawberries. In the context of the circular economy and sustainability, the new edible coating was formulated using natural antioxidants recovered from products and by-products derived from the bergamot production cycle, with the purpose of valorizing the citrus waste and extending the strawberries' shelf life by improving the safety and quality during postharvest cold storage.

4.2. Materials and Methods

4.2.1. Materials and Chemicals

Strawberries (Cv. Camarosa) were collected in a local farm situated in Reggio Calabria (Italy) in April 2022, transported to the FoodTec laboratory of the University of Reggio Calabria, and immediately submitted to treatments. Firstly, the fruit were selected for uniformity in terms of their size, colour, and weight, while the defective ones were discarded. Then, the fruit were dipped in a sodium hypochlorite solution (0.5%) for 2 min, washed with distilled water, and left to dry for 1 h near a UV area in a laminar vertical flow hood (UV lamp 30 W, mod. ASALAIR 1200 FLV, Asal Srl, Milano, Italy) at room temperature under forced air (20 °C).

Both bergamot "pomace" (BP) and bergamot essential oil from Reggio Calabria DPI (BEO) were sourced by a company located in Reggio Calabria (Italy). BP is a by-product of citrus fruit processing (*Citrus bergamia Risso*) during juice production, comprising the peel, pulp, and seeds (Figure 4.1.). The BP was dehydrated at 50 °C in a tangential air-flow cabinet ("Scirocco" model, Società Italiana Essiccatoi, Milan, Italy) until reaching a 12% moisture content and was then powdered. The BEO was obtained by rasping and cold-pressing the fruit peel.

The butylhydroxytoluene (BHT) was purchased from Merck KGaA (Darmstadt, Germania).

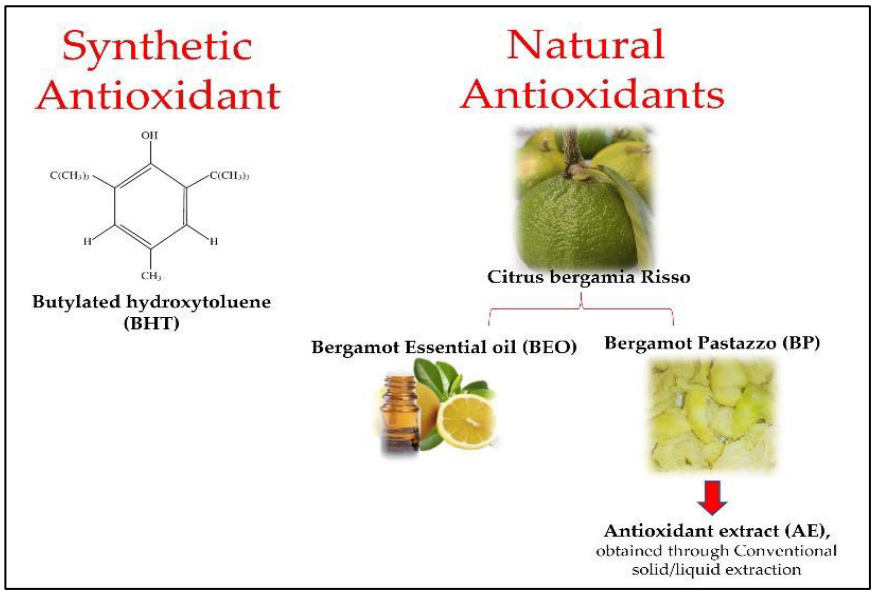


Figure 4.1. Synthetic and natural antioxidants used to enrich the edible coatings.

4.2.2. Preparation and Characterization of Antioxidant Compounds from Bergamot Pomace (AE) and Bergamot Essential Oil (BEO)

The antioxidant extract (AE) was obtained following the method reported by Imeneo et al., 2022 and appropriately modified. Briefly, 100 g of BP was mixed with 400 mL of ethanol/water (1:1, v:v) solution and kept under continuous stirring (30 min, 70 °C) using a conventional solid–liquid extraction method. Subsequently, the AE was centrifuged (8000 rpm, 10 min, 4 °C) in a refrigerated centrifuge (NF 1200R, Nüve, Ankara, Turkey), filtered through 0.45 µm filter paper, and stored at 4 °C until use. The AE was characterized for its total phenolic and flavonoid contents, and the antioxidant activity was measured following the method reported by Imeneo et al., 2022.

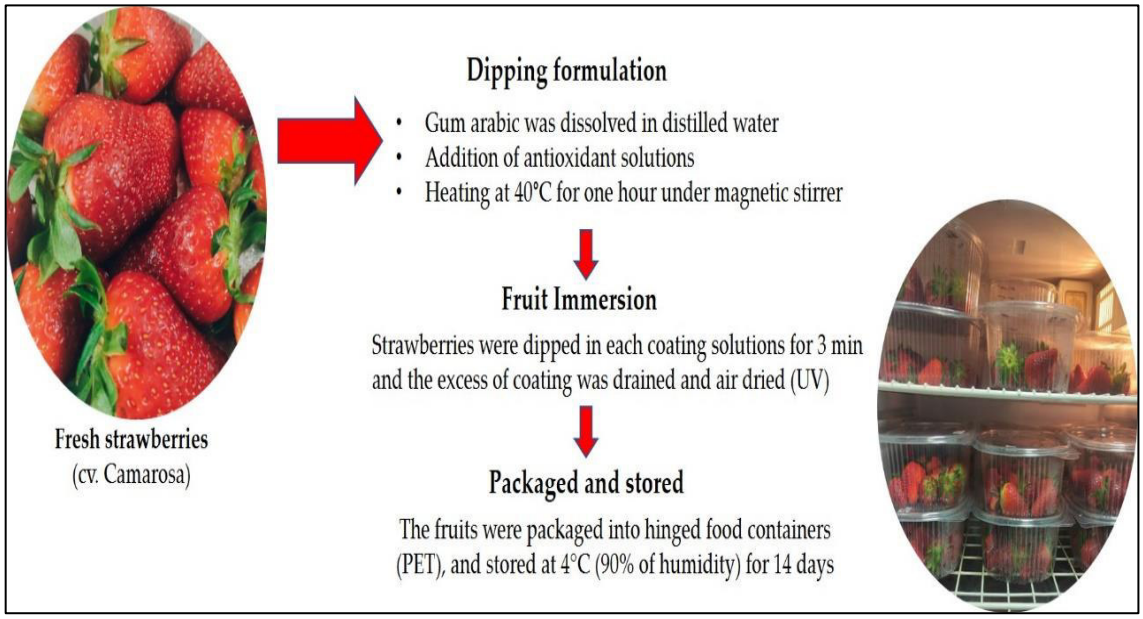
The bergamot essential oil (BEO) characterization process was carried out according to the modified methodology reported by Gionfriddo et al., 2003. Briefly, the composition was determined through a GC2010 gas chromatograph equipped with a flame ionization detector (FID) and a capillary column of fused silica (DB-5MS). The temperature program was 70 °C for 10 min, then heating at 3 °C/min to 120 °C, heating from 130 °C to 220 °C at 4 °C/min, maintenance for 5 min at 220 °C, heating from 220 °C to 280 °C at 15 °C/min, and maintenance for 10 min at 280 °C. The operative conditions were as follows: split ratio of 1:60 at 230 °C, helium as the carrier gas, with a flow rate of 1.5 mL/min; FID 250 °C; injection volume of 0.2 µL, manually injected in split mode. The

main constituents were identified by comparing their RI (retention index) values with those provided in the literature and the internal standards.

4.2.3. Coating Preparation and Application on Surfaces of Strawberries

As reported in Figure 4.2., the experimental plan involved the formulation of six different tests, in addition to the control conditions (untreated sample: CTRL).

Figure 4.2. Schematic overview of the sample preparation process.



The coating was prepared as follows (Tahir et al., 2018). The gum Arabic (2% concentration, *w/v*) was dissolved in distilled water until it was completely dissolved, after which the AE, BEO, and BHT solutions were added and heated at 40 °C for one hour with a magnetic stirrer. Subsequently, 1% glycerol (*v/w*) was added as s plasticizer to improve the strength and flexibility of the coating solutions. Tween 20 (0.5% *v/v* OE tween 20/OE) was added to promote the dispersion of the essential oil. The concentrations of AE, BEO, and BHT added to the coatings are reported in Table 4.1. and here: 100 ppm of BHT (sample B); 1% AE (sample C); 2.5% AE (sample D); 5% AE (sample E); 0.1% BEO (sample F); 0.2% BEO (sample G).

Table 4.1. Sample denomination.

Sample	Enriching Antioxidant Compounds
A	none
B	BHT (100 ppm)
C	AE (1%)
D	AE (2.5%)

E	AE (5%)
F	BEO (0.1% + Tween 20)
G	BEO (0.2% + Tween 20)

The strawberries were dipped in each coating solution for 3 min and the excess of the coating was drained and air-dried (under UV and at room temperature to prevent environmental contamination). The fruit samples were packaged into hinged food containers (PET) and stored at 4 °C. Each treatment contained three replicates.

Before the application of the coating on the strawberries, the different solutions were analyzed for their antioxidant components to define the real transfer or enrichment of the bioactive compounds.

4.2.4. Effect of Edible Coating Application based on the Physicochemical Analysis

All coated strawberries were subjected to a physicochemical analysis at different times (1, 3, 7, 10, and 14 days) during the storage period.

4.2.4.1. Weight Loss Percentage

The strawberries were individually weighed (11 reruns for each sample) at the storage times. The weight loss was calculated as the difference between the initial and final weights of the fruit and the values were reported on a percentage basis in accordance with the AOAC standard method (AOAC, 1994).

4.2.4.2. Decay Percentage

The decay percentage (DC) was evaluated at each storage time following the method reported by D'Acquino et al., 2022). The strawberries were visually evaluated and considered decayed when visible damage, described as brown spots, softening, or mold growth, was detected. The DC was calculated using the following equation (Equation (1)):

$$DC = (NIF/INF) \times 100 \quad (1)$$

where NIF is the number of infected fruit samples and INF is the initial number of all fruit samples.

4.2.4.3. Surface Colour Measurement

The surface colour was measured at ten points for each sample using a Minolta CM-700d Spectrophotometer, with reference to CIE L*a*b* coordinates using a D65

illuminant (Mafrica et al., 2023). These values were then used to calculate the hue degree (h°) and chroma (C*), as reported by Hernández-Muñoz et al. [43].

4.2.4.4. Texture Analyses: Penetration Test

The strawberry fruit texture was determined using a TA-XT Plus Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK), following the method reported by Doving et al., 2005 with appropriate modifications. Data acquisition and curve integration were carried out using Exponent software 6.1.4.0 (Stable Micro Systems Ltd., Godalming, UK).

The penetration test outlined a mechanical force displacement using a 5 kg loading cell and with a cylindrical flat head probe with a diameter of 5mm (P/5) entering the fruit (placed on the plate with the receptacle cavity upright to the compression probe to assess its firmness). The mechanical profiles were acquired with a data acquisition rate of 300 pps with the following instrumental settings: pretest speed: 10.00 mm/s; test speed: 5 mm/s; post-test speed: 10.00 mm/s; trigger force: 2.0 g. For each sample, ten replicates were used.

4.2.4.5. Sensorial Analysis

The sensorial analysis test was based on perceptions of visual appearance (hue, brightness, integrity), aroma intensity (fruity, aromatic intensity, citrus, fermented), taste (sweetness, acidity, bitterness, aftertaste), and texture (turgidity, juiciness) and the overall acceptability of the fruits. It was carried out on strawberry samples at each monitoring time (0, 7, and 14 days). Eighteen panelists (between 25 and 64 years old) with previously experience in sensory analyses were trained before the sessions to identify the gustatory attributes to be evaluated. Each panelist was also asked to evaluate the general acceptability from a consumer point of view. The sensorial analysis was based on a 0-to-9-point hedonic scale. A score of 4.5 was considered the limit of acceptability.

4.2.4.6. Determination of Total Soluble Solids (TSS), pH, Titratable Acidity (TA), Organic Acids, and Microbiological Counts

For the determination of the chemical parameters, about 100 g of fruit was randomly taken from each sample and homogenized using an Ultra-Turrax (T 25 digital, IKA, Staufen, Germany), with the aim of obtaining a homogenate sample. The TSS was determined using a digital handheld refractometer (DBR 047 SALT), and the results were expressed in degrees Brix (°Bx). The pH value of the strawberry samples were measured

at 25 °C using a digital calibrated pH meter (pH 4, pH 7; Crison Basic 20, Spain) according to the AOAC (AOCS, 2017; AOCS, 2017; AOCS, 1989).

For the TA determination, 5 g of homogenate was diluted with 100 mL of deionized water and then titrated with 0.1M NaOH. The end-point reading was monitored using a pH meter (Crison Basic 20, Spain). The results are expressed as citric acid % values (AOCS, 1990).

The organic acid extraction process was performed according to Ikegaya et al., 2019, with some modifications. An aliquot of 5 g of strawberry homogenate and 25 mL of distilled water was blended using an Ultra-Turrax. Then, the mixture was mixed for 30 s with a vortex and centrifuged at 9000 rpm and 4 °C for 10 min in a refrigerated centrifuge (NF 1200R, Nüve, Ankara, Turkey), then the supernatant filtered with a PTFE 0.45 µm (diameter 15 mm) syringe filter. The concentration of organic acids was determined followed the method reported by Panebianco et al., 2020. The analysis was conducted using a Knauer HPLC Smartline Pump 1000, equipped with a Knauer Smartline UV Detector 2600 set at 210 nm, using an Acclaim OA5 column (4 mm i.d. × 250 mm length × 5 µm particle size). The chromatographic analysis was carried out in isocratic conditions using as the mobile phase 100 mM of Na_2SO_4 acidified to 2.65 pH with methane sulfonic acid CH_3SO_3H (30 °C; flow rate: 0.6 mL/min). For the quantification of each organic acid, external standards were used, and the results are expressed as mg 100 g^{-1} of fresh strawberries (mg 100 g^{-1}).

For the microbial analysis, each sample was serially diluted (1:10) in a Ringer solution and homogenized using a stomacher (BagMixer® 400 P, Interscience, France) for 2 min; subsequently, 1 mL of each dilution was transferred onto the surfaces of the plates used.

Dichloran Rose Bengal Chloramphenicol (DRBC) agar base plates were used for the enumeration of yeasts and molds, and the plates, after solidification, were incubated at 25 °C for 4–5 days before counting the colonies. The total bacteria count (TBC) was performed by inoculating ready-to-use chromogenic plates (Compact Dry) and incubating them at 25 ± 2 °C for 48 ± 3 h. The results are reported as Log10 colony-forming units (CFUs) g^{-1} of strawberries.

4.2.5. Antioxidant Properties of Strawberries

4.2.5.1. Extraction of Antioxidants Compounds

The extraction of antioxidant compounds was carried out according to Mustafa et al., 2021's method, with some modifications. First, 5 g of strawberry homogenate was mixed with 10 mL of an acidified (pH 3, HCl) mixture of EtOH/H_2O (70:30 *v/v*). After, the mixture was mixed for 30 s in a vortex and placed in an ultrasonic bath for one hour (40 kHz, 25 °C, 50% power). The strawberry extracts (SE) were centrifuged at 9000 rpm for 10 min at 4 °C, then the supernatants were filtered with a syringe filter (RC, 0.45 µm, diameter 15 mm) and used for the analysis.

4.2.5.2. Total Phenolic Contents (TPC)

The TPC were determined following the method reported by Letaief et al., 2016, with appropriate modifications. In a volumetric flask measuring 25 mL, 0.1 mL of AE or SE (for each sample), 9 mL of deionized water, and 0.5 mL Folin–Ciocalteau reagent were shaken vigorously and kept at room temperature. After 5 min, 5 mL of Na_2CO_3 solution (5% *w/v*) was added, brought up to volume with water, and incubated for 60 min. The absorbance was measured at 765 nm versus a blank (sample replaced by water). The total phenolic content was expressed as mg gallic acid equivalent L^{-1} of AE (mg GAE L^{-1}) and mg gallic acid equivalent 100 g^{-1} of fresh strawberry (mg GAE 100 g^{-1} F.W.).

4.2.5.3. Total Flavonoid Content (TF)

The total flavonoid content (TF) was determined following the method reported by Papoutsis et al., 2018, with slight modifications. In brief, in a volumetric flask measuring 5 mL, 300 µL of AE or SE (for each sample), 1000 µL of distilled water, and 150 µL of $NaNO_2$ (5%, *w/v*) were mixed and kept in dark conditions for 6 min. Then, 150 µL of $AlCl_3$ (10%, *w/v*) was added and incubated for 6 min at room temperature. Subsequently, 2000 µL of NaOH (1 M) was added, then at the end the water was used to complete the volume. The same solution without the sample was used as a blank and the absorbance was measured at 510 nm. The results are expressed as mg of catechin equivalents L^{-1} of AE (mg CE L^{-1}) and mg of catechin equivalents 100 g^{-1} of fresh strawberry (mg CE 100 g^{-1} F.W.).

4.2.5.4. Total Antioxidant Activity (TAA): DPPH and ABTS Assays

The antioxidant assays (DPPH and ABTS) were performed using the method reported by Imeneo et al., 2022, which was appropriately modified.

DPPH Assay

Here, 10 µL of AE or SE (for each sample) was allowed to react with 2990 µL of 6×10^{-5} M of methanol solution of DPPH under darkness at room temperature for 15 min. The absorbance was measured at 515 nm against methanol as a blank, using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV-Vis _2, Waltham, MA, USA).

ABTS Assay

Here, 10 µL of AE or SE (for each sample) was added to 2990 µL of the ethanol solution of $ABTS^{+}$. The absorbance was measured after 6 min in the dark using a spectrophotometer. The blank was prepared with EtOH.

For both the antioxidant assays (DPPH and ABTS), the quenching of the initial absorbance was plotted against the Trolox concentration (from 3 to 18 µM) and the results were expressed as mmol Trolox L^{-1} of AE (mmol TE L^{-1}) and mmol Trolox kg^{-1} of fresh strawberries (mmol TE kg^{-1} F.W.).

4.2.5.5. Total Anthocyanins Content (TAC)

The TAC extraction was performed following a different pH colourimetric method. Here, 2 g of strawberries and 10 mL of HCl-acidified methanol (99.9/0.1 *v/v*) were left in the dark overnight under refrigerated conditions (4 °C). Subsequently, the extract (TCAE) was centrifuged at 6000 rpm for 5 min and filtered through 0.45 um filters.

The TAC analysis was performed according to Tahir et al., 2018's method, with some modifications. Briefly, 0.5 mL of the TCAE (for each sample) was reacted with 4.5 mL of the two different pH buffer solutions—the first one with potassium chloride buffer (0.025 M, pH = 1.0) and the second with sodium acetate buffer (0.4 M, pH = 4.5)—then kept at room temperature for 15 min. The absorbance was recorded at wavelengths of 510 and 700 nm against a blank (HCl-acidified methanol (99.9/0.1 *v/v*) using a double-beam ultraviolet-visible spectrophotometer (Perkin-Elmer UV-Vis λ2, Waltham, MA, USA). The quantification of total anthocyanins was calculated using Equation (2) and determined as mg 100 g^{-1} of fresh weight (F.W.) of pelargonidin-3-glucoside (PGN), the anthocyanin predominant in strawberries, as reported by Sarıdaş et al., 2022:

$$TAC\ (mg/100\ g) = (A \times MW \times DF \times V \times 100)/(\varepsilon \times d \times m) \quad (2)$$

where:

MW = molecular weight of pelargonidin-3-glucoside (433.2 g/mol);

DF = dilution factor;

V = extract's volume;

ε = coefficient of molar absorptivity of pelargonidin-3-glucoside (31600 L/cm/mol);

d = path length (1 cm);

m = mass of the sample (g).

4.2.5.6. Identification and Quantification of Individual Antioxidant Compounds

For the chromatographic analysis of individual antioxidant compounds, the method reported by Romeo et al., 2018 was followed. Here, 5 µL of AE (for each sample) was injected in a UHPLC PLATINblue instrument (Knauer, Berlin, Germany) provided with a binary pump system, using a Knauer blue orchid C18 column (1.8 mm, 100 × 2 mm) coupled with a PLATINblue PDA–1 (photo diode array detector) (Knauer, Berlin, Germany) and Clarity 6.2 software. A gradient elution program was used (0–3 min, 5% B; 3–15 min, 5–40% B; 15–15.5 min, 40–100% B), where the mobile phases were (A) water acidified with acetic acid (pH 3.10) and (B) acetonitrile. For the quantification of each antioxidant compound (p-cumaric acid, ferulic acid, eriocitrin, neoeriocitrin, narirutin, naringin, neohesperidin, melitidin, and brutieridin), external standards were used, and the results are expressed as mg L^{-1} of AE (mg L^{-1}).

4.2.6. Statistical Analysis

The results obtained in this experiment are shown in the tables and figures as means ± SDs of different measurements. The statistical differences were evaluated using a one-way analysis of variance (ANOVA) with Tukey's post hoc test ($p < 0.05$), performed using SPSS software (Version 20.0, SPSS Inc., Chicago, IL, USA).

4.3. Results and Discussions

4.3.1. Characterization of Antioxidant Extract (AE) and Bergamot Essential Oil (BEO)

In this work, several edible coatings were formulated with different concentrations of antioxidant extracts obtained from bergamot pomace (AE) and bergamot essential oil (BEO), as each enrichment compound might influence the characteristics and properties of the film or of the packaged food, as reported by Ganiari et al., 2017. For this reason, it is very important to confirm that both AE and BEO show antioxidant activity.

The first step of the experimentation involved the extraction of the antioxidant compounds from bergamot pomace and their subsequent physicochemical characterization. The main results are reported in Table 4.2..

The AE showed pH values of about 3.18 and 21.1 °Brix. The low pH value can be considered a good result, as it allows the natural acidification of the substrate on which it will be applied. Regarding the evaluation of the antioxidant activity, spectrophotometric and chromatographic methods were applied, which highlighted the high TPC (7751 mg GAE L^{-1}) and TFC (2783 mg CE L^{-1}) values, while a good total antioxidant activity level was revealed through the ABTS assay (2100 mmol TE L^{-1}).

The main individual phenolic compounds identified in the AE, and in order of their determined concentrations, were neoeriocitrin, naringin, neohesperidin, brutieridin, melitidin, p-coumaric acid, eriocitrin, ferulic acid, and narirutin.

Table 4.2. Physicochemical characterization of antioxidant extract from bergamot pomace (AE).

pH	3.18 ± 0.08
TSS (°Brix)	21.1 ± 0.85
L*	49.33 ± 0.22
a*	0.57 ± 0.09
b*	2.83 ± 0.14
TPC (mg GAE L^{-1})	7751 ± 137
TFC (mg CE L^{-1})	2783 ± 30
DPPH (mmol TE L^{-1})	359 ± 2
ABTS (mmol TE L^{-1})	2100 ± 16
p-coumaric acid (mg L^{-1})	116.77 ± 5.58
Ferulic acid (mg L^{-1})	38.02 ± 1.69
Eriocitrin (mg L^{-1})	75.18 ± 5.15
Neoeriocitrin (mg L^{-1})	4194.78 ± 59.68
Narirutin (mg L^{-1})	29.72 ± 5.08
Naringin (mg L^{-1})	3544.51 ± 114.73
Neohesperidin (mg L^{-1})	2219.09 ± 4.32
Melitidin (mg L^{-1})	821.54 ± 70.66
Brutieridin (mg L^{-1})	1782.75 ± 12.74

Regarding the BEO, the total antioxidant properties and the volatile fraction were evaluated, and the data are reported in Table 4.3..

Two total antioxidant assays were tested to evaluate the properties of the BEO. The obtained results highlighted that the BEO was able to decrease the DPPH and ABTS free

radicals, showing similar results between the two assays (about 230 and 267 mmol TE L^{-1}, respectively).

An evaluation of the volatile fraction is very important to define the quality of an essential oil. The two main compounds present in the BEO were limonene (46.727%) and linalyl acetate (34.151%), which showed values similar to those reported by Gionfriddo et al., 2003. The composition of the BEO may vary according to different parameters, such as the harvest period and geographic origin (Dugo et al., 2012).

Table 4.3. Chemical composition of bergamot essential oil (BEO).

Total Antioxidants Properties		
DPPH (mmol TE L^{-1})	267.04 ± 21.21	
ABTS (mmol TE L^{-1})	230.82 ± 18.61	
Volatile fraction	**rt**	**%**
1 Tricyclene	5.65	0.001
2 α-Thujene	5.77	0.210
3 α-Pinene	6.04	0.839
4 Camphene	6.59	0.016
5 Sabinene	7.62	0.590
6 β-Pinene	7.81	3.322
7 Myrcene	8.44	1.058
8 Octanal	9.10	0.023
9 α-Phellandrene	9.20	0.024
10 δ-3-Carene	9.45	0.001
11 α-Terpinene	9.96	0.103
12 p-Cymene	10.51	0.025
13 Limonene	11.04	46.727
14 (Z)-β-Ocimene	11.39	0.013
15 (E)-β-Ocimene	12.02	0.136
16 γ-Terpinene	12.74	5.432
17 trans-Sabinene hydrate	13.20	0.025
18 Octanol	13.63	0.002
19 Terpinolene	14.48	0.235
20 Linalool	15.42	4.386

21 Nonanal	15.57	0.016
22 cis-Limonene oxide	16.17	0.003
23 trans-Limonene oxide	16.26	0.003
24 Isopulegol	17.49	0.002
25 Camphor	17.80	0.002
26 Citronellal	18.43	0.010
27 Terpinen-4-ol	19.72	0.014
28 α-Terpineol	20.49	0.035
29 Decanal	21.40	0.031
30 Octyl acetate	21.81	0.072
31 Nerol	22.63	0.042
32 Neral	23.27	0.205
33 cis-Sabinene hydrate acetate	23.92	0.051
34 Linalyl acetate	24.33	34.151
35 Geranial	24.84	0.312
36 α-Terpinyl acetate	28.62	0.119
37 Citronellyl acetate	28.87	0.029
38 Neryl acetate	29.38	0.502
39 Geranyl acetate	30.27	0.232
40 Dodecanal	31.43	0.027
41 Decyl acetate	31.57	0.013
42 (β)-Caryophyllene	31.71	0.215
43 trans-α-Bergamotene	32.42	0.247
44 α-Humulene	33.11	0.015
45 cis-β-Farnesene	33.28	0.045
46 Germacrene D	34.19	0.039
47 Sesquiterpene	34.34	0.012
48 Sesquiterpene	34.78	0.016
49 α-Farnesene	35.02	0.024
50 β-Bisabolene	35.24	0.349

4.3.2. Effect of Edible Coating Application on Physicochemical Properties of Minimally Treated Strawberries

4.3.2.1. Weight Loss

Weight loss represents an important aspect that can be used to evaluate the quality of fruit, and it is related to the transpiration and respiration of the fruit (Agapito-Ocampo et al., 2021). Strawberries are easily susceptible to water loss, which causes contraction and weakening of the fruit tissue due to their very thin skin. This has negative effects on the appearance of the fruit, causing changes in texture (softening), colour, and aroma and accelerating senescence, pathogen development, shriveling, and chilling injury (Nunes & Emond, 2012), consequently causing economic losses. The edible coatings provide a barrier function by protecting the fruit from the external atmosphere, but also by limiting transpiration by delaying dehydration, providing a qualitative improvement in weight loss.

In this study, the weight loss % increased gradually in all samples during storage (Table 4.4.), showing significant differences ($p < 0.01$) both among the treatments and among the monitoring times.

Table 4.4. Weight loss (%) values of the strawberries during storage days.

Sample/Time	1	3	7	10	14	Sign.
A	0.55 ± 0.06^{aCE}	1.51 ± 0.11^{aD}	3.12 ± 0.14^{aC}	9.27 ± 0.08^{aB}	25.38 ± 2.45^{aA}	**
B	0.52 ± 0.03^{abD}	1.02 ± 0.03^{bC}	1.26 ± 0.03^{dC}	8.33 ± 0.10^{cB}	22.09 ± 0.39^{abA}	**
C	0.42 ± 0.03^{cE}	0.95 ± 0.02^{cDE}	2.11 ± 0.08^{cC}	7.49 ± 0.06^{dB}	19.77 ± 4.48^{abA}	**
D	0.31 ± 0.04^{dD}	0.98 ± 0.03^{bcC}	0.99 ± 0.04^{eC}	4.19 ± 0.08^{gB}	12.28 ± 3.86^{bA}	**
E	0.38 ± 0.04^{cE}	0.93 ± 0.03^{cdD}	1.2 ± 0.05^{dC}	6.34 ± 0.07^{eB}	16.41 ± 1.54^{bA}	**
F	0.29 ± 0.03^{dD}	0.86 ± 0.01^{dC}	0.91 ± 0.04^{eC}	5.31 ± 0.08^{fB}	12.78 ± 2.93^{bA}	**
G	0.48 ± 0.02^{bE}	0.97 ± 0.02^{bcD}	2.37 ± 0.10^{bC}	8.78 ± 0.14^{bB}	20.46 ± 2.76^{abA}	**
Sign.	**	**	**	**	**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$; n.s., not significant.

The strawberries without coating (samples A), among all the treated samples, showed the largest decay percentage from day 3 and significantly differed at day 14 (25.38%). Meanwhile, samples D and F responded better to the coating, showing decay values of about 12% at the end of the monitoring period.

4.3.2.2. Decay Index

The decay index of coated strawberries was evaluated during the storage period, and the results are reported in Figure 4.3.. The decay index increased during the storage period

for all treatments; only the E sample at seven days of storage showed a decay percentage of 0%. At the final monitoring time, samples F (27%) and D (36%) showed lower decay index values, followed by C and E with a DP of 45% and B with 55%. Total decay was shown by sample A, namely the control. The application of the edible coating caused a decreased decomposition rate during storage, as also reported by Agapito-Ocampo et al., 2021. Even sample G showed total decay at 14 days of storage, probably due to the negative effect of the higher concentration of BEO added to the coating formulation. These results were in accordance with the expectations of the dipping solution enrichment with AE and BEO to perform the antioxidant action, coupled with the barrier effect of the coating. Therefore, for this parameter, the best result was obtained by sample D, which was the sample treated with an edible coating enriched with AE at a medium concentration (Figure 4.3.a), followed by sample F, the sample coated with gum Arabic and 0.1% BEO(Figure 4.3.b).

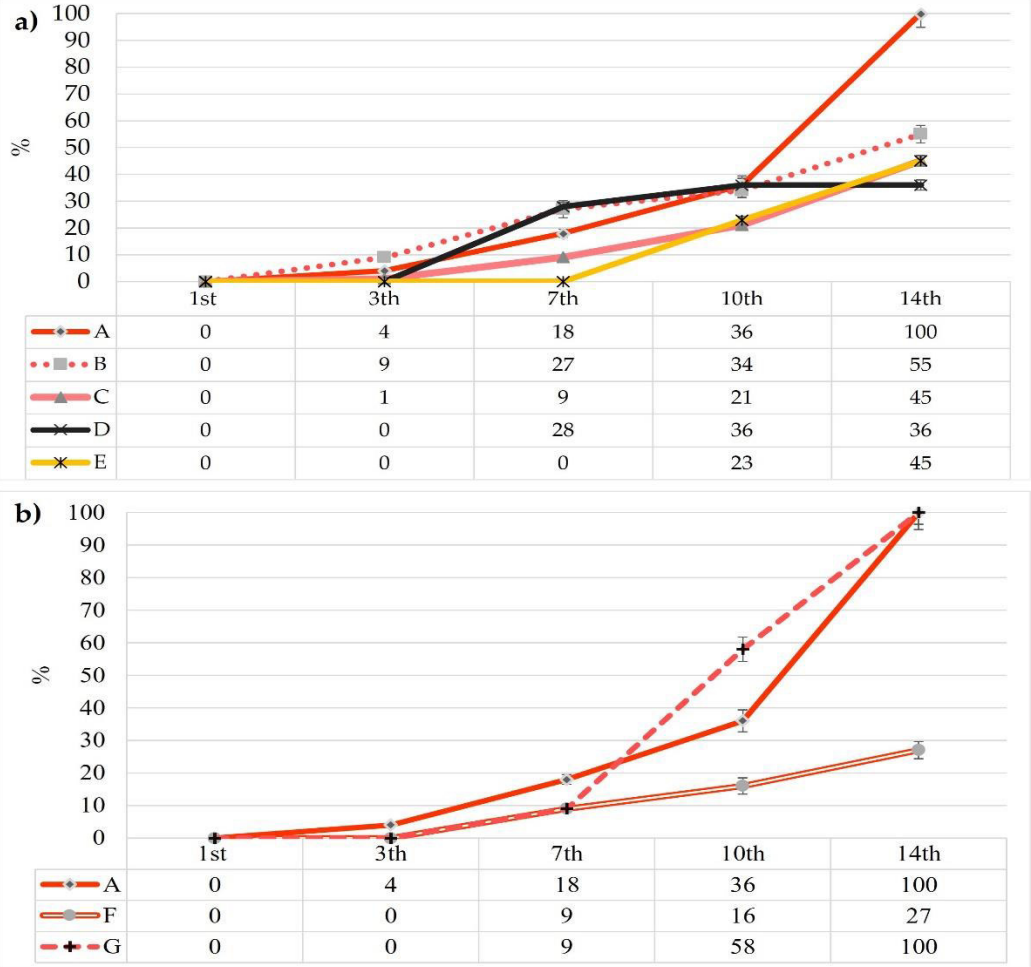


Figure 4.3. Effects of edible coatings enriched with AE (**a**) and BEO (**b**) on decay index values of strawberries during storage.

4.3.2.3. Effects of Edible Coatings on the Texture of the Strawberries

The fruit firmness was analyzed on the coated samples during the storage time (Figure 4.4. a,b), because it represents one of the essential parameters to determine fruit quality. The softening is a natural physiological effect of fruit ripening with cell wall changes and the dissolution of the middle lamella, which in turn causes loss of cell-to-cell adhesion (Oh et al., 2018; Chen et al., 2011; Villareal et al., 2016). Strawberries are more perishable and subjected to mechanical damage, pathogen attacks, and quality losses during storage.

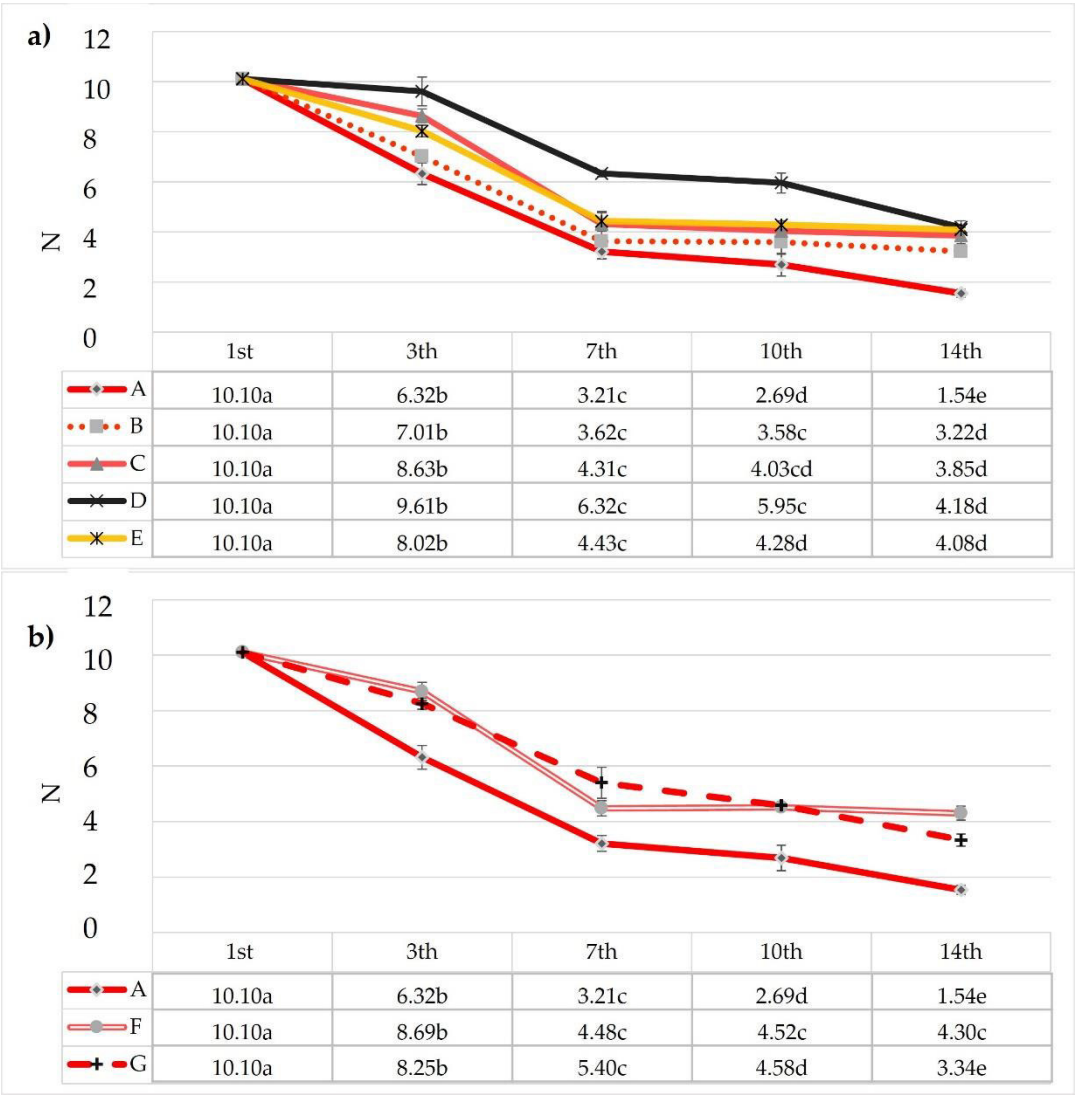


Figure 4.4. Effects of edible coatings enriched with AE (**a**) and BEO (**b**) on the firmness of strawberries during storage.

At time 0, the firmness of the fruit was 10.1 N, while after seven storage days, decreased firmness was observed, both in the uncoated and coated samples, but the lowest value was found in the control sample (3.21 N), similar to sample B (3.62 N). The highest

firmness values were recorded in samples D and G at 6.32 N and 5.4 N, respectively. Additionally, after 14 days of cold storage, samples A and B showed the lowest firmness values as compared with the other samples. The firmness results for samples A and B were 1.54 N and 3.22 N, respectively, which were very low values in terms of acceptability. The firmness is influenced by the softening of the fruit. The results were in accordance with the study by Tahir et al., 2019), where the retention of flesh firmness of blueberries was achieved by the combined effect of African baobab pulp extract and GA, while Kahramanoğlu et al., 2022 reported that during storage there was a greater decrease in firmness in untreated strawberries than in those treated with an extract incorporated in the coating. The standard deviations were high in many samples, but this is normal due to the hardness variability under the same conditions, in agreement with Doving et al., 2005. For this reason, we used ten replicates for each treatment.

4.3.2.4. Surface Colour Measurement

In Table 4.5., the results related to the surface colour are reported. The statistical analysis (ANOVA) did not show significant differences ($p > 0.05$) during the storage period (until 14 days) for the L*, a*, b*, or chroma (C*) in any of the coated samples.

Only the control (sample A), or the untreated sample, showed a decreasing trend during the storage period ($p < 0.05$), which was already apparent from the 7th day. Instead, the hue angle (H*) of the strawberries, which indicates the maintenance of the fruit's natural colour characteristics, decreased during the storage period meaningfully in some samples (A, B, E, F), particularly in the control sample (A).

The L* parameter is an indicator of fruit lightness, and the results showed that the coated samples had reduced values compared to the control. In fact, the L* value of the strawberry surfaces was highest for the untreated sample (49.89), although all dipped samples showed similar values. This reduction may have been due to the presence of gum Arabic; in fact, Tahir et al., 2018 also reported that their coating with the lowest amount of GA (10%) had the highest L* value as compared to another coating with a higher concentration (15%).

Table 4.5. Surface colour values of minimally treated strawberries.

	A	***B***	***C***	***D***	***E***	***F***	***G***	**Sign.**
				L*				
1st	49.9 ± 2.3 ^{aA}	47.2 ± 1.4 ^{B}	47.3 ± 1.5 ^{B}	46.7 ± 2.2 ^{B}	47.4 ± 1.4 ^{B}	46.7 ± 1.2 ^{B}	47.4 ± 1.5 ^{B}	**

7th	46.4 ± 1.4 [b]	47.3 ± 2.6	46.6 ± 1.0	45.8 ± 2.1	46.3 ± 0.8	47.4 ± 2.4	47.0 ± 2.2	n.s.
14th	46.6 ± 1.0 [b]	47.8 ± 1.4	47.9 ± 1.6	48.6 ± 4.4	47.2 ± 1.3	47.3 ± 1.2	47.8 ± 1.8	n.s.
Sign.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
				a*				
1st	17.6 ± 3.0 [aA]	13.0 ± 2.2 [B]	15.8 ± 2.8 [AB]	13.0 ± 3.8 [B]	14.6 ± 3.0 [AB]	13.0 ± 2.3 [B]	13.8 ± 3.1 [AB]	**
7th	13.9 ± 3.6 [b]	15.8 ± 3.8	15.0 ± 2.1	15.4 ± 4.6	15.4 ± 2.3	16.2 ± 3.7	16.1 ± 4.2	n.s.
14th	11.3 ± 2.6 [b]	14.3 ± 3.8	14.2 ± 3.4	14.6 ± 4.2	14.1 ± 3.2	15.8 ± 3.0	14.9 ± 2.0	n.s.
Sign.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
				b*				
1st	10.5 ± 3.0 [aA]	7.3 ± 2.1 [B]	8.4 ± 2.3 [AB]	7.55 ± 2.3 [AB]	8.1 ± 2.2 [AB]	6.6 ± 1.4 [B]	7.0 ± 2.1B	**
7th	7.0 ± 2.1b	8.1 ± 3.9	7.3 ± 1.0	7.4 ± 2.5	7.1 ± 1.1	7.9 ± 2.6	8.4 ± 2.9	n.s.
14th	4.8 ± 1.4c	6.6 ± 2.4	7.0 ± 2.7	7.7 ± 3.8	6.4 ± 1.8	7.3 ± 1.7	6.9 ± 2.0	n.s.
Sign.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
				C*				
1st	20.5 ± 4.1 [aA]	14.9 ± 2.8 [B]	17.9 ± 3.5 [AB]	15.1 ± 4.3 [B]	16.7 ± 3.7 [AB]	14.6 ± 2.5 [B]	15.5 ± 2.7 [B]	**
7th	15.6 ± 4.0 [b]	17.9 ± 5.2	16.7 ± 2.3	17.1 ± 5.1	16.9 ± 2.6	18.1 ± 4.4	18.2 ± 5.0	n.s.
14th	12.2 ± 2.9 [c]	15.7 ± 4.4	15.8 ± 4.2	16.6 ± 5.3	15.5 ± 3.7	17.4 ± 3.4	16.4 ± 2.6	n.s.
Sign.	**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	
				h°				
1st	30.3 ± 3.6 [a]	28.9 ± 3.9 [a]	27.8 ± 3.2	30.1 ± 3.6	28.6 ± 2.4 [a]	26.9 ± 3.0 [a]	26.7 ± 1.7	n.s.
7th	26.7 ± 2.7 [b]	26.3 ± 4.5 [ab]	26.1 ± 1.8	25.8 ± 3.5	24.7 ± 1 [b]	25.4 ± 2.5 [ab]	27.3 ± 3.3	n.s.
14th	22.7 ± 1.7 [c]	24.3 ± 2.3 [b]	25.5 ± 3.5	26.9 ± 5.6	24.3 ± 1.2 [b]	24.6 ± 2.1 [b]	24.7 ± 3.5	n.s.
Sign.	**	*	n.s.	n.s.	**	*	n.s.	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$; n.s., not significant.

The highest value of the a* parameter was found in the control sample, and in the same sample there was a big loss of red tone during the storage time, probably due to the faster perishability of the strawberries. In the sample with the lowest BE content (sample C), the value of a* decreased linearly from 15.78 at T0 to 14.99 at T7 and 14.15 at T14. This showed that the coating treatments, as compared to the control fruit, maintained their red colour over time. This may have been associated with the retarded biosynthesis in the metabolic activity of the red pigment during ripening, particularly pelargonidin-3-glucoside, which is responsible for the red colour.

Regarding parameters b* and C*, the treated samples maintained stable parameters, with both displaying non-consistent and insignificant variations during the overall prolonged storage. A change in colour is considered an indicator of ripening in fruit

(Moreno et al., 2006). It is linked to physiological processes. Considering the reductions in redness, yellowness, and chroma in the untreated samples during storage, the data suggested that the coating preserved the ripening factors correlated with changes in colour. However, postharvest colour changes do not affect the shelf life of strawberries, as reported by Ktenioudaki et al., 2019.

4.3.2.5. Sensorial Analysis

The sensorial characteristics of minimally treated strawberries were determined by a group of regular consumers of this fruit. The main aspects revealed by the panel group are shown in Table 4.6., and the results are reported as the median values of all scores. The average score of 4.5 was considered the limit of acceptability for the fruit, as also reported by Garcia et al., 2014, and in the table we report only the initial (1st day) and final (14th day) results evaluated in the samples.

Table 4.6. Sensorial characterization of minimally treated strawberries.

	Appearance		**Aroma Intensity**		**Sweetness**		**Turgidity**		**Overall Acceptability**	
	1st	***14th***	***1st***	***14th***	***1st***	***14th***	***1st***	***14th***	***1st***	***14th***
A	8.5 ^{a}	4 ^{b}	6.5 ^{a}	4 ^{ab}	4 ^{b}	3 ^{c}	7 ^{a}	3 ^{c}	6 ^{b}	3 ^{d}
B	7 ^{b}	4 ^{b}	3 ^{c}	1 ^{c}	6 ^{a}	3 ^{c}	6 ^{b}	5 ^{ab}	7 ^{a}	3 ^{d}
C	8 ^{ab}	4.5 ^{b}	4 ^{b}	3 ^{b}	5 ^{ab}	4 ^{b}	6 ^{b}	4 ^{b}	7 ^{a}	4 ^{c}
D	8 ^{ab}	6 ^{a}	5 ^{ab}	3 ^{b}	5 ^{ab}	3 ^{c}	7 ^{a}	6 ^{a}	7 ^{a}	6 ^{a}
E	7 ^{b}	6 ^{a}	4 ^{b}	3 ^{b}	5.5 ^{a}	5 ^{a}	7 ^{a}	5 ^{ab}	7 ^{a}	5.5 ^{ab}
F	7 ^{b}	6 ^{a}	3 ^{c}	4 ^{ab}	5 ^{ab}	3 ^{c}	6.5 ^{ab}	5 ^{ab}	6.5 ^{ab}	5 ^{b}
G	7 ^{b}	6 ^{a}	5 ^{ab}	5 ^{a}	4.5 ^{b}	3 ^{c}	6.5 ^{ab}	5.5 ^{a}	7 ^{a}	4 ^{c}
Sign.	*	**	**	**	**	**	*	**	*	**

Small letters within a column show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$.

At the first timepoint, the overall acceptability of the samples was good, with an average higher than 6 in all samples, including the control (A). It was possible to highlight that the application of the edible coating caused a variation in aromatic intensity compared with sample A. The film that forms on the surface of the fruit probably limits the release of aromatic components. At the end of the storage period (14 days), the coated fruit samples were subjected to significant variation, which determined the end of their shelf life. At 14 days, only some samples fell within the limit of 4.5, including samples D, E, and F, namely those formulated with the addition of AE and BEO.

The addition of higher concentrations of BEO to the coating formulation causes greater persistence of the aroma, causing an alteration of the organoleptic properties of the final product.

4.3.2.6. Total Soluble Solid (TSS), pH, Titratable Acidity (TA), and Organic Acid Levels of Coated Strawberries

The results related to the total soluble solids (TSS) during storage are reported in Figure 4.5.. At the beginning of the experiment, the strawberries showed a TSS content equal to 6.4 °Brix; during storage, the values tended to increase in all treated samples from the 3rd day onwards, reaching a peak at 7 days in all samples. From the tenth day of storage, onwards, reductions in TSS content were observed. The changes in TSS content during this time were confirmed by the ANOVA test, whereby all samples showed significant differences, although only sample F showed lower statistical differences ($p < 0.05$). The initial increase and subsequent reduction in TSS were due to the hydrolysis of carbohydrates during fruit maturity (Xin et al., 2017) and from their consumption for respiration. Sugars are accumulated during ripening and then decline during senescence.

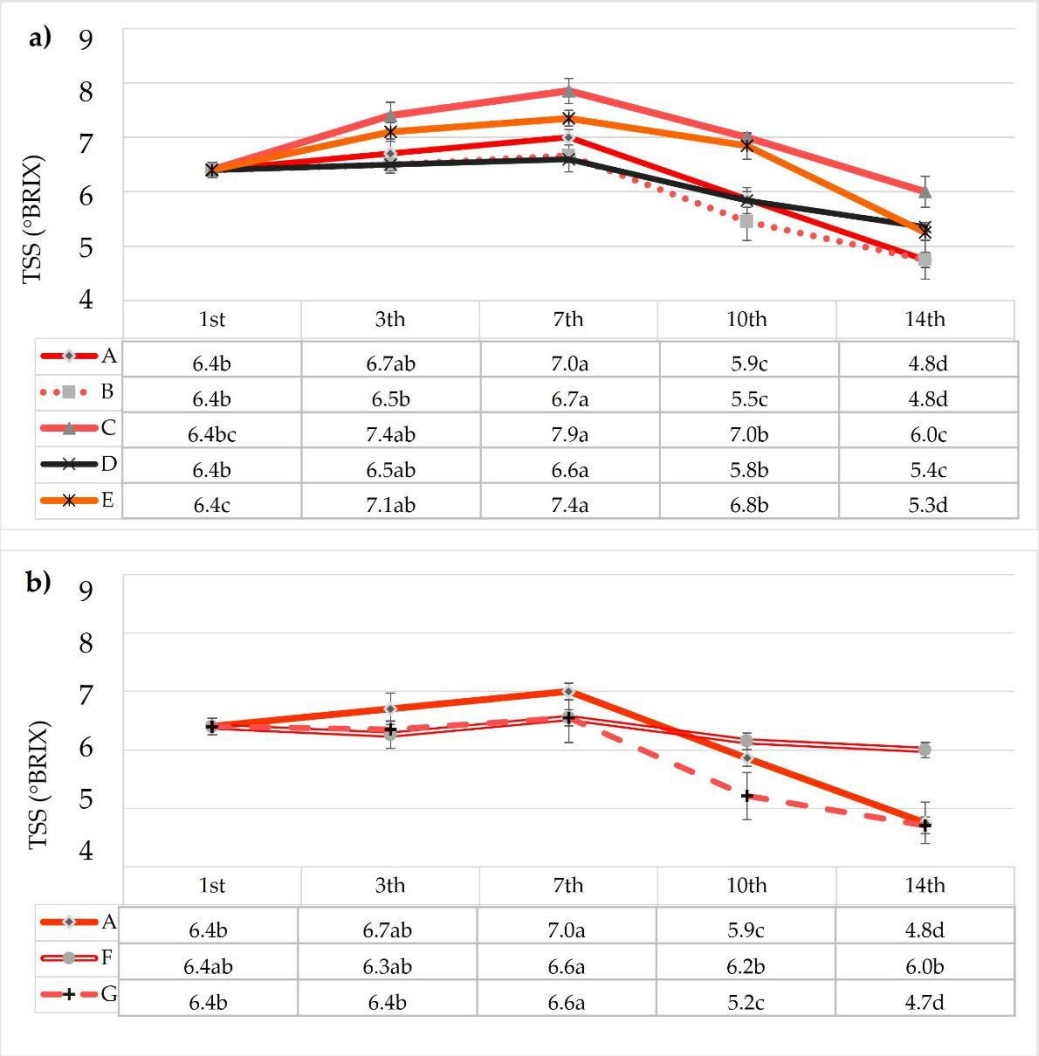


Figure 4.5. Effects of edible coatings enriched with AE (a) and BEO (b) on total soluble solids (TSS) of strawberries during storage.
Small letters within a column show a significant difference as assessed by Tukey's post hoc test.

Moreover, in accordance with Bahmani et al., 2022, the delay in the TSS could be considered an indicator of over-ripening and senescence. Among the samples, samples A and B showed faster decay.

As shown in Table 4.7., the pH values of the strawberries varied statistically during the storage period ($p > 0.05$), with the values ranging between 3.4 (1st day) and 4 (14th day). The obtained pH values were in accordance with Agapito-Ocampo et al., 2021. The pH values increased during the conservation time evenly in all test samples. Samples D and E were the samples that changed less after 14 days.

Table 4.7. The pH, TA (% citric acid), and organic acid contents (ascorbic acid, AA; mg 100 g^{-1}; citric acid, CA; mg 100 g^{-1}) of strawberries.

pH	***A***	***B***	***C***	***D***	***E***	***F***	***G***	***Sign***

1st	3.4 ± 0 ^{b}	3.4 ± 0.0 ^{c}	3.4 ± 0.0 ^{b}	3.4 ± 0.0 ^{b}	3.4 ± 0.0 ^{b}	3.4 ± 0.0 ^{c}	3.4 ± 0.0 ^{c}	ns
7th	4.0 ± 0.1 ^{aA}	3.7 ± 0.0 ^{bB}	3.6 ± 0.0 ^{aC}	3.6 ± 0.2 ^{aC}	3.6 ± 0.0 ^{aC}	3.7 ± 0.0 ^{bC}	3.6 ± 0.0 ^{bC}	**
14th	4.0 ± 0 ^{aA}	4.0 ± 0.0 ^{aA}	3.8 ± 0.0 ^{aC}	3.6 ± 0.0 ^{aD}	3.7 ± 0.0 ^{aCD}	3.9 ± 0.0 ^{aB}	3.7 ± 0.0 ^{aC}	**
Sign	**	**	**	**	**	**	**	
TA	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*Sign*
1st	0.7 ± 0.0 ^{b}	0.7 ± 0.0	0.7 ± 0.0 ^{b}	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0 ^{b}	0.7 ± 0.0 ^{b}	ns
7th	0.8 ± 0.0 ^{aA}	0.7 ± 0.1 ^{B}	0.9 ± 0.1 ^{aA}	0.7 ± 0.1 ^{B}	0.7 ± 0.1 ^{B}	0.8 ± 0.1 ^{abAB}	0.9 ± 0.1 ^{aA}	*
14th	0.5 ± 0.0 ^{cD}	0.7 ± 0.0 ^{C}	0.9 ± 0.0 ^{aA}	0.9 ± 0.1 ^{A}	0.8 ± 0.0 ^{B}	0.8 ± 0.1 ^{aB}	0.9 ± 0.0 ^{aAB}	*
Sign	**	n.s.	**	n.s.	n.s	*	**	
AA	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*Sign*
1st	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	33.0 ± 0.5 ^{a}	ns
7th	28.4 ± 0.1 ^{bCD}	27.3 ± 0.4 ^{cD}	32.6 ± 0.1 ^{aA}	29.6 ± 0.8 ^{bBC}	31.4 ± 1.02 ^{abAB}	28.5 ± 0.2 ^{bCD}	30.1 ± 0.2 ^{bBC}	**
14th	27.0 ± 0.2 ^{cB}	29.1 ± 0.3 ^{bAB}	30.3 ± 0.1 ^{bA}	31.5 ± 1.4 ^{abA}	29.8 ± 0.5 ^{bAB}	30.8 ± 1.4 ^{abA}	29.9 ± 0.4 ^{bAB}	*
Sign	**	**	**	*	*	**	**	
CA	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*Sign*
1st	692.5 ± 26.5 ^{a}	692.5 ± 26.5 ^{a}	692.5 ± 26.5	692.5 ± 26.5	692.5 ± 26.5	692.5 ± 26.5	692.50 ± 26.5 ^{a}	ns
7th	604.1 ± 42.0 ^{aC}	669.1 ± 2.2 ^{aABC}	720.5 ± 10.2 ^{A}	745.7 ± 2.1 ^{A}	698.0 ± 12.3 ^{AB}	699.5 ± 30.8 ^{AB}	636.2 ± 41.6 ^{abBC}	*
14th	402.9 ± 3.3 ^{bB}	583.5 ± 56.0 ^{bA}	711.1 ± 20.3 ^{A}	727.5 ± 32.1 ^{A}	676.8 ± 17.9 ^{A}	654.5 ± 85.42 ^{A}	558.6 ± 43.3 ^{bAB}	*
Sign	**	**	ns	ns	ns	ns	*	

Small letters within a column (among storage time) and capital letters within a row (among different treated samples) show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$; n.s., not significant.

The highest pH levels were observed in samples A and B at 14 days (3.97 and 4.04, respectively), as compared with the samples treated with coatings enriched with AE and BEO. These results may have been due to the effects of enzymatic activities and the ripening of the strawberries. These results are in agreement with those found by Gol et al., 2013, who detected a greater increase in pH in uncoated strawberries than in coated samples. Moreover, this tendency could be due to the consumption of organic acids during fruit ripening.

As is clearly visible in Table 4.7., the titratable acidity (TA) shows a different trend in the treated samples compared to the control (A). Indeed, in sample A, the TA increased at 7 days of storage and then decreased significantly at 14 days; on the contrary, in the coated strawberries, the TA values tended to statistically increase during the conservation period in samples C, F, and G.

With reference to fruit ripening, the TA values did not show changes related to the consumption of organic acids in the coated samples. This effect was more visible in sample A, in which high significant decay at the end of shelf life was shown. This effect was found in the literature according to Dìaz-Mula et al., 2011, who explained the higher acidity loss in uncoated fruits as being due to their high respiration rate during storage, which affects the organic acids' respiratory activity (Krebs cycle). The synthesis of organic acids happens during fruit maturation Fait et al., 2008), consequently causing increased acidity and decreased senescence. The obtained results highlighted how the edible coating preserved and improved the TA values; therefore, good flavor was found because of the high TA and TSS values. The data showed a low TA value in the control sample at the end of study (0.48 mg 100 g^{-1} CA), confirming the loss of quality and advance to senescence. The TA results were in accordance with Jouki & Khazaei, 2012, who observed similar average values.

Ascorbic acid (AA), or vitamin C, is one of the major components of strawberries, and its content is an indicator of quality relevant to define freshness of fruits (Cordenunsi et al., 2003). Many authors have reported that decreased AA during storage is caused by its oxidation (Atress et al., 2010). and the respiration rate of the fruit (García et al., 2012). The use of a coating promotes protection against both effects. As is possible to see in Table 4.7., the results obtained in this study promote this effect. The control sample (A) showed significant variation of the AA content values during the storage period ($p < 0.01$), with the lowest value being shown at 14 days. The initial AA content was 33.01 mg 100 g^{-1}, and after seven days it decreased to 28.35 mg 100 g^{-1}, while at 14 days it was 27.02 mg 100 g^{-1} (the lowest detected value). These results are confirmed in the literature, where Khodaei et al., 2021 reported a similar trend with delayed vitamin C deterioration over time compared with a control sample. The highest values compared with T0 were recorded in sample G, which might have been due to continued ripening. At 14 days, the AA contents decreased in all samples, particularly the control sample (A), the sample treated with BHT (B), and the samples dipped in the solutions with the highest amounts of BE and BEO (G), with values of 27.02, 29.07, 29.82, and 29.94 mg 100 g^{-1}, respectively. Samples C, D, and F had the highest amounts of AA.

Citric acid is the predominant organic acid in strawberries. Some authors have reported that citrate's synthesis is linked to fruit respiration during the different stages of

physiological growth, followed by a reduction during strawberry ripening (Etienne et al., 2013). The data detected during this experimentation process are reported in Table 4.7., where one can observe that there were great losses (highly significant, $p < 0.01$) of this organic acid, particularly in the control sample (A), from 692.5 mg 100 g^{-1} at the beginning to 402.9 mg 100 g^{-1} at the end of the shelf life (14 days).

Regarding the trend shown during the storage period, the CA contents varied significantly only in three samples, A, B, and G ($p < 0.01$); all other samples showed no significant differences ($p > 0.05$). All coatings enriched with AE highlighted the good stability of this acid during the period.

4.3.2.7. Microbiological and Sensorial Parameters of Minimally Treated Strawberries

Generally, minimally treated fresh fruit have a short shelf-life range (4–7 days), which is very important to preserve the freshness of the fruit and avoid excessive losses due to the reduction in their quality; for this reason, the evaluation the microbiological and sensorial parameters is very important.

As the minimally treated fruit are not subjected to thermal treatment, they should be processed and stored at temperatures below 5 °C, with the aim of extending their shelf life and microbiological security. Their composition makes them a favorable substrate for the growth and development of some microbial forms, such as molds and yeasts (Romeo et al., 2010).

In Table 4.8., the microbiological results are reported. The CBT, yeasts, and molds were revealed already from the 1st monitoring day in samples A (control) and B, while the other samples did not show any contamination. The use of plant extracts and essential oil in edible coatings should provide advantages to preserve their high sensibility to microbial decay. The obtained microbiological values fall within the acceptable limits set by the French regulations, which include a maximum aerobic plate count of 5×10^7 cfu/g at the end of shelf life for different fresh-cut vegetables (Zappia et al., 2019).

During the conservation period, increases in all analyzed microbiological parameters were observed, particularly at 14 days of storage. The samples more subjected to microbiological deterioration were samples A, B, and C; these values showed that the application of edible coatings is useful to improve and extend the quality of strawberries.

Table 4.8. The microbiological counts of minimally treated strawberries (Log_{10} CFU g^{-1}).

	CBT				*Yeasts*				*Molds*			
	1st	***7th***	**14th**	**Sign**	**1st**	7th	14th	**Sign.**	1st	7th	14th	**Sign**
A	1.8 aC	2.9 aB	3.4 bA	**	1.1 aC	4.9 bB	7.0 aA	**	3.0 aB	3.5 aB	5.3 aA	**
B	1.0 bC	2.0 bB	4.1 aA	**	1.1 aB	6.1 aA	6.4 bA	**	2.1 bB	2.1 bB	5.3 aA	**
C	0 cC	2.5 abB	3.7 abA	**	0 bB	5.8 aA	5.2 cA	**	0 cB	0 cB	4.4 bA	**
D	0 cB	2.9 aA	2.4 cA	**	0 bB	4.9 bA	6.4 bA	**	0 cC	2.1 bB	4.8 abA	**
E	0 cC	1.3 cB	3.1 bA	**	0 bC	1.5 dB	4.7 cA	**	0 cB	0 cB	3.2 cA	**
F	0 cB	2.3 abA	2.0 cdA	**	0 bB	2.6 cA	2.3 dA	**	0 cB	2.2 bA	1.9 dA	**
G	0 cB	1.7 bcA	1.8 dA	**	0 bB	2.7 cA	2.3 dA	**	0 cB	0 cB	1.5 dA	**
Sign	**	**	**		**	**	**		**	**	**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$.

4.3.2.8. Antioxidant Activity of Minimally Treated Strawberries

Strawberries contain approximately 390 mg of total phenols per serving and are classified in 9th place on the list of the 100 richest sources of dietary phenols, with high antioxidant activity, as reported by Mustafa et al., 2021. The more studied group of phenolic compounds in strawberries is that of anthocyanins, which are responsible for the red colour of the fruit. The other classes of phenolics that characterize the strawberries are the tannins, flavonols, and esters of hydroxycinnamic acids (Mustafa et al., 2021). Therefore, strawberries are rich in phenols and antioxidant compounds, making them a good health indicator, and the relative results obtained for their evaluation are reported in Table 4.9..

The TPC values increased during postharvest storage as a direct response to fruit ripening and depending on several factors that may influence their biosynthesis and availability (Bahamani et al., 2022; Goulas et al., 2011; Wang et al., 2006). Significant differences were noted during the storage period but no variation was noted among the coated samples.

The monitored data from the refrigerated storage period exhibited similar increments of TPC values after 7 days in all samples, which in almost all cases were maintained up to 14 days. Only samples D, E, and G decreased slowly ($p < 0.05$) after seven days. The reduction in TPC values during this period may have been due to the possible breakdown of the cellular assembly and structure as a consequence of fruit senescence (Jiang et al., 2019). Similar values were reported for sample G, which was treated with 0.2% of BEO, in accordance with Shirzad et al., 2021, who reported a similar effect of essential oil on the cell walls of fruit with ageing and an increase in the enzyme activity of the polyphenol oxidase enzyme.

Table 4.9. Total antioxidant compound levels of minimally treated strawberries.

TPC (mg GAE 100 g^{-1} FW)

	A	*B*	*C*	*D*	*E*	*F*	*G*	*Sig*
1st	101.2 ± 2.8	101.2 ± 2.8 ^{b}	101.2 ± 2.8 ^{b}	101.2 ± 2.8 ^{b}	101.2 ± 2.8 ^{b}	101.2 ± 2.8 ^{b}	101.2 ± 2.8 ^{b}	ns
7th	106.8 ± 5.7	118.1 ± 4.1 ^{a}	118.7 ± 5.7 ^{a}	122.6 ± 4.8 ^{a}	125.0 ± 8.4 ^{a}	119.7 ± 1.7 ^{a}	127.0 ± 16.0 ^{a}	ns
14th	108.2 ± 4.0	115.8 ± 3.8 ^{a}	119.5 ± 6.1 ^{a}	115.6 ± 17.0 ^{ab}	115.0 ± 16.3 ^{ab}	117.8 ± 3.4 ^{a}	109.7 ± 1.7 ^{ab}	ns
Sig.	**ns**	**	**	*	*	**	*	
TFC (mg CE 100 g^{-1} FW)								
1st	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	20.2 ± 1.1 ^{b}	ns
7th	19.9 ± 1.1 ^{bB}	18.7 ± 0.5 ^{bB}	21.2 ± 1.3 ^{bB}	19.4 ± 3.4 ^{bB}	22.0 ± 0.7 ^{bB}	20.3 ± 1.0 ^{B}	26.5 ± 1.1 ^{aA}	**
14th	32.9 ± 1.6 ^{aABC}	35.8 ± 2.1 ^{aA}	34.2 ± 1.1 ^{aAB}	32.6 ± 5.8 ^{aABC}	27.6 ± 3.9 ^{aCD}	23.7 ± 4.8 ^{D}	26.2 ± 0.8 ^{aCD}	**
Sig.	**	**	**	**	**	**n.s.**	**	
TAC (mg PGN 100 g^{-1} FW)								
1st	20.34 ± 0.43 ^{b}	20.34 ± 0.43 ^{b}	20.34 ± 0.43	20.34 ± 0.43 ^{b}	20.34 ± 0.43 ^{b}	20.34 ± 0.43 ^{c}	20.34 ± 0.43	ns
7th	33.74 ± 1.36 ^{aA}	24.70 ± 0.73 ^{aC}	21.84 ± 0.76 ^{D}	24.75 ± 1.06 ^{aBC}	24.20 ± 1.17 ^{aC}	26.90 ± 0.87 ^{aB}	18.09 ± 0.24 ^{E}	**
14th	17.52 ± 3.68 ^{bC}	21.16 ± 2.55 ^{bABC}	22.74 ± 2.68 ^{ABC}	23.94 ± 1.04 ^{aAB}	24.96 ± 2.95 ^{aA}	24.85 ± 0.93 ^{bA}	18.44 ± 2.79 ^{BC}	**
Sig.	**	**	**ns**	**	*	**	**n.s.**	
DPPH (mmol TE kg^{-1} FW)								
1st	145.9 ± 9.8	145.9 ± 9.8 ^{ab}	145.9 ± 9.8 ^{b}	145.9 ± 9.8	145.9 ± 9.8 ^{b}	145.9 ± 9.8 ^{b}	145.9 ± 9.8	ns
7th	156.8 ± 15.1 ^{BC}	129.9 ± 5.0 ^{bC}	189.4 ± 19.8 ^{bAB}	164.6 ± 13.6 ^{BC}	205.1 ± 14.4 ^{aA}	215.5 ± 12.1 ^{aA}	153.5 ± 19.3 ^{BC}	**
14th	161.1 ± 8.5	160.1 ± 13.5 ^{a}	149.9 ± 20.3 ^{a}	145.9 ± 19.2	131.6 ± 10.7 ^{b}	153.7 ± 5.9 ^{b}	153.7 ± 7.1	ns
Sig.	**n.s.**	**	*	**n.s.**	**	**	**n.s.**	
ABTS (mmol TE kg^{-1} FW)								
1st	382.4 ± 7.8 ^{b}	382.4 ± 7.8 ^{b}	382.4 ± 7.8 ^{b}	382.4 ± 7.8	382.4 ± 7.8 ^{b}	382.4 ± 7.8	382.4 ± 7.8	ns
7th	436.9 ± 36.4 ^{aBC}	504.1 ± 37.0 ^{aB}	448.8 ± 31.7 ^{abBC}	364.5 ± 54.9 ^{C}	615.4 ± 42.5 ^{aA}	386.2 ± 35.3 ^{C}	438.5 ± 44.8 ^{BC}	**
14th	449.6 ± 14.8 ^{aAB}	511.7 ± 51.5 ^{aA}	511.3 ± 73.5 ^{aA}	412.8 ± 58.9 ^{AB}	425.7 ± 39.2 ^{bAB}	391.3 ± 18.4 ^{B}	419.1 ± 36.4 ^{AB}	**
Sig.	**	**	*	**n.s.**	**	**n.s.**	**n.s.**	

Small letters within a column and capital letters within a row show significant differences as assessed by Tukey's post hoc test. Abbreviations: **, significance at $p < 0.01$; *, significance at $p < 0.05$; n.s., not significant.

Moreover, the TPC values for all other samples were higher than the control, which may have been due to the protective barrier formed by the coatings on strawberries' surfaces reducing the enzymatic effect on the oxidation of phytochemicals, with a consequent loss of quality (Bonilla et al., 2012; Dong & Wang 2017).

The effects of the edible coating treatments on the total flavonoid content (TF) of the strawberries are illustrated in Table 4.9.. In all samples, the TF increased over time, except for the sample coated with the lowest quantity of BEO dipping solution (F), in which no statistical differences were found over time. Significant differences were revealed among the different coated samples, with high values found in sample B at the 14th day (35.8 mg CE 100 g^{-1}). Similar values were recorded in the samples with the highest amounts of AE and BEO (samples E and G), with 27.59 and 26.19 mg CE 100 g^{-1}, respectively. The obtained results are in accordance with Chen et al., 2019.

The results related to the anthocyanin concentrations determined on the tested samples are presented in Table 4.9.. During the storage period, the samples showed a different trend, with significant differences ($p < 0.01$), while only samples C and G preserved the same contents of anthocyanin during the monitoring period (14 days).

The control sample (A) showed the lowest TAC concentration of 20.34 mg 100 g^{-1}, in agreement with Zheng et al, 2007 and Tahir et al., 2019, as compared with the coated samples. All tested samples showed an increase in the TAC at 7 days of storage, particularly sample A (33.74 mg 100 g^{-1}), but the same sample underwent a faster decrease during the following storage days (17.52 mg 100 g^{-1}). The obtained data highlight that the application of an edible coating on the strawberries preserves the TAC. For sample G, even though it presented the lowest TAC, it maintained constant levels of anthocyanins during the time period, with no statistical differences, and with the lowest TAC after sample A. This was probably due possible to an excess of BEO, which has negative effects on metabolic activity and on cell membranes (Perdones et al., 2012), promoting senescence. Samples B and C at the end of the shelf life displayed similar values compared to the beginning, while samples D, E, and F showed the highest TAC values (23.94; 24.96; 24.85 mg 100 g^{-1}). At 14 days of storage, the samples showed a reduction in TAC, which was probably associated with fruit senescence, leading to the inhibition of anthocyanin biosynthesis, as also reported by Wang et al., 2013 and Khodaei et al., 2021.

Different values were highlighted between the two antioxidant tests, each of which evaluated different reaction mechanisms; in fact, the results were different, with a higher total antioxidant activity (TAA) revealed by the ABTS assay (Table 4.9.).

The DPPH radical scavenging capacity assay showed a different trend among the samples, with the highest values being obtained after 7 days, in particular in samples E and F, which showed values of 205.06 and 215.53 mmol TE kg^{-1} in strawberries, respectively, which slightly decreased during storage. Indeed, at 14 days, the assay showed similar values to the 1st day. No significant differences ($p > 0.05$) were evidenced for samples A, D, and G, for which the total antioxidant activity levels determined with the DPPH assay remained stable over time (for several days).

Applying the ABTS assay, the antioxidant capacity showed higher values than the DPPH assay, with values that ranged between 382.4 (1st day) and 615.4 mmol TE kg^{-1} FW (7 days, in E sample). The TAA tended to increase during the storage of the fruit, particularly in samples A, B, C, and E, while the other three samples did not show significant differences during the time period ($p > 0.05$).

4.4. Conclusions

The application of enriched gum Arabic coating could be a valid alternative during fruit storage, providing beneficial effects by retarding the ripening process. The results obtained in this experimentation process confirmed the positive actions of the coatings, creating good conditions such as an increase in storage time as compared with the control sample and the samples coated with a synthetic antioxidant. In fact, the control sample deteriorated rapidly. The application of edible coatings provided a useful barrier to preserve the antioxidant parameters, delaying ripening and senescence. The enriched coatings can retain the quality parameters in strawberries after prolonged refrigerated storage.

After 14 days of storage, the samples that showed the best qualitative characteristics were those coated with the antioxidant extract at 2.5% (D) and with bergamot essential oil at 0.1% (F). For these samples, lower decay rates were observed (36% for sample D and 27% for sample F), with better acceptability from sensory and textural points of view, with scores above 4.5, which represents the limit of acceptability (appearance scores of 6, turgidity scores of 6 and 5, and overall acceptability scores of 6 and 5, respectively, for samples D and F), as well as showing good maintenance of the organic acids, especially ascorbic acid (31.47 for sample D and 30.80 mg 100 g^{-1} for sample F), an indicator of quality. This experimentation process revealed good results compared to the normal shelf life of strawberries.

The use of a natural antioxidant extract and bergamot essential oil in the coating formulation revealed the good possibility to achieve the double effect of barrier resistance to respiration and transpiration and an antimicrobial effect against microbial growth during shelf life, preserving fruit quality.

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5. MICROENCAPSULATION OF ANTIOXIDANT EXTRACT RECOVERED BY BERGAMOT POMACE THROUGH FREEZE-DRYING METHODOLOGY AND APPLICATION IN HYDROPHYLIC AND LIPOPHILIC FOOD SYSTEMS

This work is being revised by the authors and will be submitted to a journal for publication.

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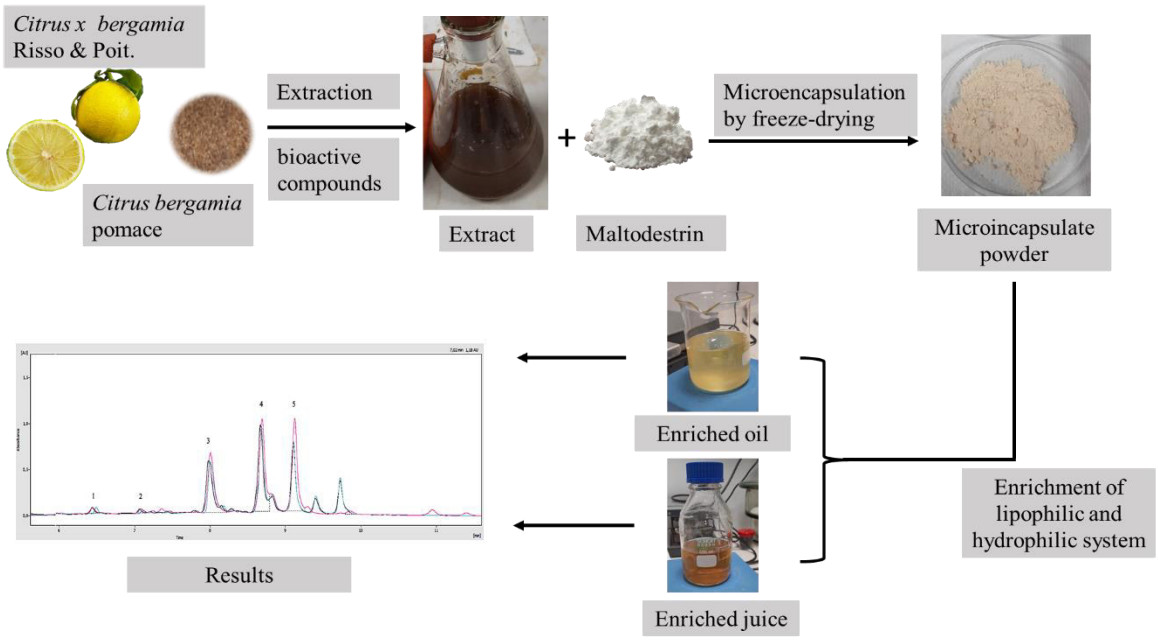
Abstract

The interest for the use of microencapsulated antioxidant compounds is becoming increasingly important in scientific and industrial thanks to their functionality. For this reason, in aim of this work was to apply a freeze-drying procedure for encapsulation of an antioxidant extract recovered by bergamot pomace. Maltodextrin was used as coating agent and was added at concentration of 20%. The microencapsulated was applied for the enrichment of two different matrices, apple juice and sunflower oil, with the purpose to evaluate the efficiency as natural antioxidant. The antioxidant extract, the microencapsulated and the enriched products were evaluated in their physicochemical and antioxidant activity characteristics. Moreover, the main properties of the enriched products were assessed during storage at 25°C for 90 days. The addition of the microencapsulated allowed the enrichment of a vegetable oil and a juice enriched increasing the content of polyphenols and antioxidant properties for up to 90 days of storage.

Keywords: antioxidant; bergamot pomace; microencapsulate; enriched lipophilic system, enriched hydrophilic system.



Chapter 5. Graphical abstract

5.1. Introduction

In recent years, consumers' needs regarding nutrition have changed profoundly. In fact, consumers awareness of food-related health problems is growing, along with the growing incidence of chronic diseases related to the lifestyle and eating habits of society. In this context, the consumer is increasingly attentive to nutrition which directly affects their health. Furthermore, the world population, which is growing exponentially, entails a high demand for food and energy to satisfy his needs. Thus, there has been a significant increase in the number of food processing industries resulting in an increment in the production of food waste and for this reason increased the need not only to prevent their formation, but above all, to manage the existing ones. Among the innovations carried out by the industry there are the production of new food ingredients and materials, new process technologies, innovations in quality or new packaging methods.

However, the research has focused on the possibility of adding natural ingredients to foods that can bring health benefits. These natural ingredients avoid the use of potentially harmful synthetic additives, making room for bioactive natural products considered safe substitutes for a market looking for new products (Marcillo-Parra et al., 2021). In this regard, functional foods arise as a compromise between nutrition, health and environment. They are able to incorporate nutritional and functional properties as well as sustainability thanks to the possibility of using active ingredients recovered from agro-

food by-products for new foods enrichment or fortification (Merlino et al., 2022; De Bruno et al., 2022; Cerdá-Bernad et al., 2023).

The functional food market is growing globally, supported by a continuous launch of new products approved by current socio-demographic trends and generally accepted by consumers. The trend seems to have important potential for which research and development on this issue remain constantly updated (Bigliardi and Galati, 2013; Dias et al., 2015).

Natural phenolic compounds such as polyphenols are of particular relevance in this context, which are commonly extracted from plant matrices and agro-food waste and used in food productions. The limits in their use concern aspects related to organoleptic characteristics, instability and low shelf life. For instance, the exposition to light, alkaline environment, high temperatures affect their stability because of their structure with many phenolic hydroxyl groups (de Oliveira et al., 2016).

A proposed solution to overcome these limitations refers to the use of microencapsulation techniques that allow the enhancement of raw phenolic extracts derived from agro-food waste through the protection of bioactive compounds from external environmental agents. The protection takes place through a coating capsule that acts as a barrier due to the non-covalent/covalent binding of polyphenols to biopolymers with the achievement of a better stability, bioavailability and antioxidant activity of the active ingredient considered (Fang & Bhandari, 2010). The interesting, for the use of microencapsulated biological compounds is scientific and industrial (Donsì et al., 2016), and their functionality has been demonstrated by several studies (Costa et al., 2020; Yazdi et al., 2020).

Hence, the valorization of natural extract through its use as novel natural ingredient, is also ethical due to its high added value when recovered by agro-food waste. This type of application would lead to reduce the environmental impact, guaranteeing optimization of natural resources and improving food products.

The main aim of the present research is to find an alternative use of an antioxidant extract recovered by bergamot (*Citrus Bergamia Risso*) pomace. The extract was used to obtain a microencapsulated ingredient added to two different liquid system, hydrophilic (apple juice) and lipophilic (sunflower oil). The main properties of the enriched apple

juice and sunflower oil were assessed during storage at 25°C for 90 days. For sunflower oil oxidative stability was also evaluated.

Therefore, this work contributes to the valorization of bergamot pomace, so far not utilized in this way, to develop a new ingredient (antioxidant extract) providing information on its use in liquid food systems.

5.2. Materials and methods

5.2.1. Materials

Bergamot pomace (*Citrus Bergamia Risso*, BP) was supplied by an agro-processing company located in the province of Reggio Calabria (Italy). After the transport at the laboratory the BP was subjected to drying process (50 °C) to reduce the moisture content (12%) and then subjected to extraction process.

The preparation of the antioxidant extract from bergamot pomace (AE) was carried out according to Gattuso et al., (2023) with slight modifications to optimize the microencapsulation process. Briefly, 100 g of BP were added to 400 mL of an ethanol:water solution (1:1, v:v) and stirred for 30 min at 70°C. After, the mixture was centrifuged (8000 rpm, 8 min, 4°C, in a refrigerated centrifuge, NF 1200R, Nüve, Ankara, Turkey) and the liquid extract recovered was concentrated with a rotavapor to remove the ethanol and the removed portion of ethanol was replaced with distilled water.

The preparation of the microencapsulated antioxidant extract (MBE) was carried out as reported by Ballesteros et al., 2017, with slight modifications. Maltodextrin (MD) was used as coating agent and was added to AE at concentration of 20%. The mixture was homogenized using an Ultra-turrax (T 25 digital, IKA, Staufen, Germany) at 7000 rpm until obtaining a good homogenization.

The prepared samples were frozen and then freeze-dried (VirTis lyophilizer - SPscientific, Gardiner, NY, USA) chamber at -65°C under vacuum of 550 mtorr (for 48 h). The dried samples were grinded obtaining a powder (called MD20- maltodextrin 20%) and stored at room temperature in darkened and sealed containers until further analyses.

The obtained MD20 was used to enrich two different matrices: apple juice and sunflower oil. After samples preparation (Table 5.1), enriched (with 2% of MD20) and control samples (without MD20) were stored in darkened containers (filled with little head space and sealed) for each product and each monitoring time and stored at 25°C until further analysis.

Table 5.1. Composition of tested samples (%)

Sample	Apple Juice	Sunflower Oil	MD20
Control Juice (CJ)	100	-	-
Enriched Juice (EJ)	98	-	2
Control sunflower oil (CO)	-	100	-
Enriched sunflower oil (EO)	-	98	2

5.2.2. Methods

5.2.2.1. Physicochemical characteristics of AE

Colour parameters was determined using a Minolta CM-700d Spectrophotometer (Perkin-Elmer UV-Vis λ2, Waltham, MA, USA). The coordinates L*, a*, b* were analyzed and used to calculated Chroma (C*) and hue angle (h°) following the equations:

$$C^* = (a^2 + b^2)^{1/2}$$

$$h° = \arctan (b^*/a^*)$$

The pH was measured with a Crison pH-meter, basic model 20; while the total soluble solids (TSS) with a digital refractometer (PR-201a Atago).

The total flavonoid content (TFC) was determined according to Papoutsis et al., 2018, as follow: 500 µL of AE were added to 1000 µL of distilled water and 150 µL of $NaNO_2$ (5%, w/v) solution (in a total of 5 mL). The reaction mixture was left 6 min in dark and 150 µL of $AlCl_3$ (10%) solution were added. After 6 min, 2000 µL NaOH solution were added, and the volume was adjusted with distilled water. The absorbance was measured at 510 nm and the results were expressed as mg of catechin equivalents 100 mL^{-1} of AE.

The total antioxidant activity (TAA): was measured through ABTS and DPPH assays. For DPPH assay, 50 µL of AE and 2950 µL of DPPH (6 X 10^{-5} M) were mixed and after 30 min the absorbance was measured at 515 nm (Brand-Williams et al., 1995). For the ABTS assay, 25 µL of AE and 2975 µL of $ABTS^+$ solution (7 mM) was mixed and after 6 min, the absorbance was measured at 734 nm.

In order to calculate the TAA, for both assays results were expressed as mM Trolox equivalents L^{-1} for of AE (mM TE L^{-1}).

The identification and quantification of individual phenolic compounds (IPC) was carried out following De Bruno et al., 2023.. 5 µL of each sample was injected in a UHPLC system (PLATINblue, Knauer, Berlin, Germany), using a Knauer blue orchid C18 column (1.8 mm, 100 × 2 mm) and as mobile phases: water acidified with acetic acid

(pH 3.10) and (B) acetonitrile. For the quantification of each phenolic compound, external standards were used, and the results are expressed as mg L^{-1} of AE (mg L^{-1}).

5.2.2.2. Physicochemical characterization of MD20

a_w and moisture content (MC%) were evaluated respectively using a hygrometer (Aqualab LITE, Decagon, Nelson Court, Pullman, Washington), and an Electronic Moisture Analyser MA37 (Sartorius, Goettingen, Germany).

For the determination of bioactive compounds and to evaluate the antioxidant activity of MD20, was followed the procedure described by Zhang et al., 2007. In order to evaluate the microencapsulation efficiency (M.E.%), total compounds in the microparticles (TCM) and the compounds on the microparticles surface (CMS) were extracted. For the CMS was used a mixture ethanol/water (46.5/53.5, v/v) acidified with hydrochloric acid 2N (pH 2.00) with a solvent-residue ratio of 8.7 mL g^{-1}. The mixture was homogenized in a vortex system and sonicated in an ultrasound bath for 50 min at 30 °C. Then, the mixture was centrifugated (8000 rpm, 10 min, 4°C) and filtered. For the TCM was performed as described above for CMS, using for the extraction exclusively water as solvent.

The Folin–Ciocalteu method reported by Azarpazhooh et al., 2019 was used to determine the TPC of TCM and CSM. Briefly, 0.1 mL of extract were mixed with 6 mL of water and 0.5 mL of Folin-Ciocâlteu reagent. After 8 min, 1.5 mL of Na_2CO_3 (20% w/v) was added and the mixture was kept at room temperature in dark conditions for 30 min. The absorbance was measured at 765 nm and results were expressed as mg GAE 100 g^{-1} of microencapsulate.

TPC results of TCM and CSM were used to calculate the M.E. applying the formula below described by Rezende et al. (2018):

$$\text{M.E. \%} = [1-(\text{CSM}/\text{TCM})] \times 100$$

The TFC was determined as reported by Papoutsis et al. (2018), with some modifications (as described for AE). The results were expressed as mg catechin equivalent per 100 g^{-1} of microencapsulated. The ABTS and DPPH assays was determined according to procedure described by Sarabandi et al. (2019). Results were reported as mmol TE per kg^{-1} of microencapsulated.

The IPC in MD20 was conducted on an aliquot (5µL) of the TCM extract with UHPLC-DAD system as previously reported for AE. The results were expressed as mg per 100 g^{-1} of microencapsulated.

5.2.2.3. Physicochemical characteristics of enrichment of apple Juice with MD20

Colour parameters and pH of juices were evaluated follow the methods reported for AE. TPC was analyzed using Mafrica et al., 2023 method, and the results were expressed as milligrams of gallic acid equivalents per 100 mL of juice.

TFC, TAA and IPC were conducted as described for AE.

5.2.2.4. Physicochemical characteristics of enrichment of sunflower oil with MD20

Sunflower oil samples colour was evaluated following the methods described for AE.

Total acidity (TA) was calculated according to Official and standards methods (AOCS 2017a; 2017b; 1989) as % of oleic acid.

Peroxide value (PV) was performed according to the standard method of AOCS (2000).

Oxidative stability (OXITEST), following the method reported by Gattuso et al., 2023. In order to determine the oxidative stability, samples were treated under conditions of accelerated oxidation, monitoring the oxygen uptake of the reactive constituent of food samples in an oxidation Test Reactor (VELP Scientifica, Usmate Velate, MB, Italy). The OXITEST allows to measure the modification of absolute pressure inside the two chambers and automatically generates the IP (induction period) expressed as hours (AOCS International Standard Procedure (Cd 12c–16); AOCS,2017c).

Regarding the antioxidant fraction of the sunflower oils, an extraction according to the method of Baiano et al., 2009, was performed. 5 g of oil were mixed with 2 mL of methanol:water (70:30) and 2 mL of hexane. The hydroalcoholic fraction was recovered and filtered.

The spectrophotometric analysis of TPC was carried out following De Bruno et al., 2022. The results were expressed as mg gallic acid equivalents per 100 g of oil. TFC, TAA and IPC were evaluate as reported for AE.

5.2.3. Statistical analyses

After calculating the mean and standard deviation of three measurements, the data were subjected to analysis of variance (one-way ANOVA), that was carried out by SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) by applying the Tukey post hoc test at $p<0.05$.

5.3. Results and Discussions

5.3.1. Bergamot antioxidant extract (AE) characterization

The analyses carried out on AE allowed its characterization in terms of colour, pH, TSS, TFC and TAA, and the results were reported in Table 5.2.. The pH analysis confirmed the bergamot pomace acidity with an average value of 3.3, index of microbiological stability as it creates an unfavourable environment for microbial growth.

The evaluation of TFC and TAA in AE proved the good content of these compounds in bergamot as already reported in literature (Postorino et al.,2002; Mandalari et al., 2006).

The main IPC quantified by UHPLC in AE were shown in Table 5.2.. The results showed that AE is a source of phenolic acids, in particular, p-coumaric acid and ferulic acid (5.77±0.23 and 1.64±0.19 mg 100 mL^{-1}). According to previous works (Postorino et al. 2002; Castaldo et al. 2003), bergamot pomace is a good source of flavonoids, and the main compounds identified in the extract were eriocitrin, neoeriocitrin, narirutin, naringin, neohesperidin, melitidin and brutieridin.

Table 5.2. Physicochemical and antioxidant properties of AE

Colour	L*: 42.39 ± 0.26
	a*: 2.3 ± 0.02
	b*: 3.63 ± 0.09
	C*: 9.55 ± 0.37
	h°: 56.18 ± 0.62
pH	3.3 ± 0.05
°Brix	6.05 ± 0.07
TFC (mg CE 100 mL^{-1})	64.72 ± 1.01
TAA (ABTS, mmol TE L^{-1})	452.98 ± 27.02
TAA (DPPH, mmol TE L^{-1})	115.95 ± 9.10
p-coumaric acid[1]	5.77 ± 0.23
Ferulic acid[1]	1.64 ± 0.19
Eriocitrin[1]	6.31 ± 0.26
Neoeriocitrin[1]	232.42 ± 5.26
Narirutin[1]	3.1 ± 0.2
Naringin[1]	280.05 ± 16.2
Neohesperidin[1]	148.52 ± 2.39
Melitidin[1]	52.16 ± 3.23
Brutieridin[1]	107.45 ± 2.59

[1] *mg 100 mL^{-1}*

5.3.2. Characterization of the microcapsules

The physicochemical analyses conducted on the microencapsulated extract point out the results in term of colour, a_w, MC and antioxidant activity, these parameters were shown in Table 5.3..

The colour analysis showed values of 87.36±0.17 (L*), 1.99±0.03 (a*), 13.37±0.08 (b*), 81.56±0.11 (C*) and the h° exhibited a value to red and yellow, settling to 91.37±1.11.

a_w and MC affect the storage time of the microencapsulated powders. The percentage of MC 3.90±0.21 was like those reported by Sharifi et al. (2015); while data of a_w (0.18±0.01) were lower than those reported by Dìaz et al. (2015), who reported a higher a_w for microcapsules of blackberry juice obtained with maltodextrin as a coating agent. These values found in MD20, subsequently used for enrichment, were lower than 0.3 a_w and 5% of MC so it can be considered potentially microbiologically and chemically stable over time (Tontul et al. 2017).

The TPC on microencapsulated sample TCM and SCM were evaluated. TCM and SCM were also considered to calculate the encapsulation efficiency and results are reported in Table 5.3.. M.E. % was higher than 90% due to the low TPC detected in the SCM (28.99±14.89 mg 100 g^{-1}) and the higher content in TCM (947.35±39.96 mg 100 g^{-1}). This value was similar to those reported by Saikia et al., 2015 on microencapsulated extract of carom pomace (*Averrhoa carambola*) with maltodextrin through lyophilization between to 78% and 97%. Moreover, in the same Table 5.3. were shown MD20 results of TFC (190.68±14.89 mg 100 g^{-1}) and of ABTS and DPPH assays (64.93±0.09 and 21.19±0.17 mmol kg^{-1} respectively).

The chromatography analysis carried out by UHPLC system was conducted on the TCM extract, in order to identify and quantify the main phenolic compounds in MD20 (Table 5.3.). Results showed that compounds with the highest concentration were flavonoids (naringin, neoeriocitrin and neohesperidin) in accordance with previously results reported for the extract (Table 5.2.).

Table 5.3. Physicochemical findings of MD20

L*	87.36±0.17
a*	1.99±0.03
b*	13.37±0.08
C*	81.56±0.11
h°	91.37±1.11

a_w	0.18±0.01
MC (%)	3.90±0.21
TCM[1]	947.35±39.96
SCM[1]	28.99±14.89
TFC[2]	190.68±14.89
ABTS[3]	64.93±0.09
DPPH[3]	21.19±0.17
E.E. (%)	94.28±4.56
p-coumaric acid[4]	0.16±0.00
Ferulic acid[4]	0.03±0.00
Eriocitrin[4]	0.2±0.00
Neoeriocitrin[4]	7.32±0.09
Narirutin[4]	0.11±0.01
Naringin[4]	8.55±0.1
Neohesperidin[4]	4.75±0.19
Melitidin[4]	1.27±0.08
Brutieridin[4]	3.2±0.11

[1;]mg GAE 100 g^{-1}; [2;]mg CE 100 g^{-1}; [3;]mmol Trolox kg^{-1}; [4;]mg g^{-1}

5.3.3. Characterization of enriched Apple Juice with MD20

pH is a considerable parameter in foods, acts influencing the microorganism growth and can also influence the stability of many compounds in juices (Chia et al., 2012). In the analysed samples, pH results (Table 5.4.) showed significance change for CJ stored at 25°C, but in EJ did not detect significance differences. The variations between samples at the same temperature were not significant until the 90th day. In particular, for CJ an increase in pH from 3.54 to 3.65 was observed during the storage period while EJ showed a stable pH throughout the storage period with values around 3.50-3.55.

Colour is one of the first quality attributes perceives by consumers and which contributes to the determination of a qualitative judgment of the product. Colour changes during juice samples' storage are reported in Table 5.4.. The brightness of CJ and EJ showed statistical differences over time for each sample. There were no differences between the samples until time 90, when there was a slight decrease. CJ did not show statistical differences during storage time in a* value. Different was the trend highlighted for EJ resulting in a positive value of a* at T90 probably due to a higher redness due to the presence of phenolic compounds. Both samples showed an increase towards yellowness (b*) showing high statistically differences over time and between the samples, probably due to a browning effect. This value was lower in EJ at T90 (1.75±0.17) compared with CJ (1.90±0.11). Storage time also affected the C*, showing a greater

increase in CJ during conservation compared to EJ, in which the change was less. The h° trend was different for the compared samples, with a higher redness in EJ at T90.

Table 5.4. Physicochemical characteristics of enriched apple juice

Analysis		*Times*	***CJ***	***EJ***	***Sign.***
pH		T0	3.54±0.01^{c}	3.52±0.01	*ns*
		T15	3.58±0.02^{abc}	3.55±0.00	*ns*
		T30	3.57±0.01^{bc}	3.52±0.04	*ns*
		T45	3.55±0.01^{bc}	3.50±0.01	*ns*
		T60	3.61±0.00^{ab}	3.55±0.00	*ns*
		T90	3.65±0.04^{a}	3.52± .01	*
		Sign.	**	*ns*	
Color parameters	*L**	T0	49.5±0.08^{a}	49.5±0.06^{a}	ns
		T45	48.55±0.56^{b}	49.31±1.34^{a}	ns
		T90	47.4±0.10^{c}	47.02±0.03^{b}	**
		Sign.	**	**	
	*a**	T0	-0.18±0.01	-0.24±0.02^{c}	**
		T45	-0.18±0.03	-0.52±0.19^{b}	**
		T90	-0.20±0.07	0.07±0.02^{a}	**
		Sign.	ns	**	
	*b**	T0	1.25±0.06^{c}	1.41±0.03^{b}	**
		T45	1.63±0.14^{b}	1.52±0.19^{b}	**
		T90	1.90±0.11^{a}	1.75±0.17^{a}	**
		Sign.	**	**	
	*C**	T0	0.80±0.08^{c}	1.02±0.04^{b}	**
		T45	1.06±0.29^{b}	0.88±0.06^{b}	ns
		T90	1.83±0.20^{a}	1.55±0.33^{a}	*
		Sign.	**	**	
	h°	T0	98.34±0.84^{a}	99.61±0.92^{a}	**
		T45	98.78±1.26^{a}	98.07±0.6^{b}	ns
		T90	96.20±2.38^{b}	87.84±0.79^{c}	**
		Sign.	**	**	

Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations: n.s: not significant;**:significance at p<0.01; *:significance at p<0.05

The study of the antioxidant activity and of the bioactive compounds allowed to verify the release of phenolic compound from the microencapsulated to the apple juice (Figure 5.1.). The statistical analysis carried out on the different formulated samples (CJ and EJ) has allowed to show statistically significant differences, both between control and enriched sample, and in the individual samples evaluated during the storage period (90 days). The TPC of CJ remained stable over time, starting from a value equal to 15.87±0.60 mg 100 mL^{-1} at time 0 and reaching 18.73±1.24 mg 100 mL^{-1} at time 90. EJ sample

showed significant differences ($p<0.01$), . In particular, at time 0 highlighted values equal to 34.07±0.68 mg 100 mL^{-1}, whereas at time 60 the maximum value of 40.59±0.78 mg 100 mL^{-1} was reached. The increase in the phenolic content of EJ over time could be due to the storage temperature and the release of bioactive compounds from the microencapsulated powder. The results obtained were in agreement with what reported by Hamid et al., 2020 and Zokti et al., 2016 who observed an increase in the total polyphenol content on beverages enriched respectively with microencapsulated phenolic extract of pomegranate flavedo and green tea extracts encapsulated in maltodextrin, gum arabic and chitosan.

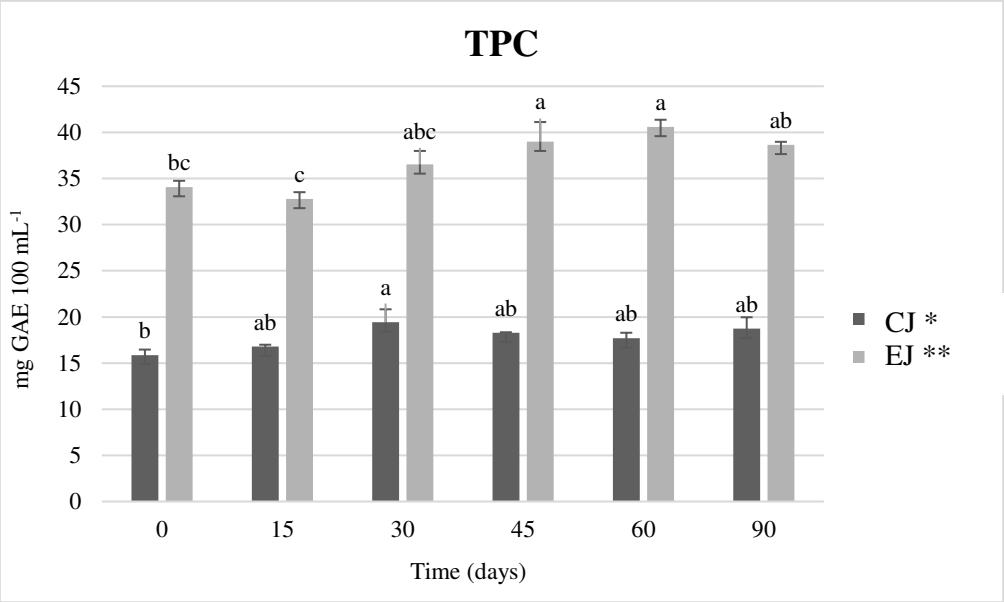


Figure 5.1. TPC values of apples juice samples during storage time.
Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations:**:significance at $p<0.01$; *:significance at $p<0.05$.

Figure 5.2. showed the changes of flavonoids in apple juice samples during the storage period Also for TFC as for TPC, statistical differences were found, particularly for sample EJ, that showed significant higher values than CJ, and an increase of the same TFC during the storage period. In particular, was highlighted an increasing trend of TFC in EJ with an initial value equal to 127.54±2.96 mg 100 mL^{-1}, reaching maximum value at T60 equal to 214.32±6.46 mg 100 mL^{-1} which was stable also after 90 days with a value of 213.76±2.74 mg100 mL^{-1}. The flavonoid content in the enriched juice as it is, showed a increasing trend probably due to the release of bioactive compounds present in microencapsulated extract, that tend to spread in the hydrophilic matrix (apple juice) over time.

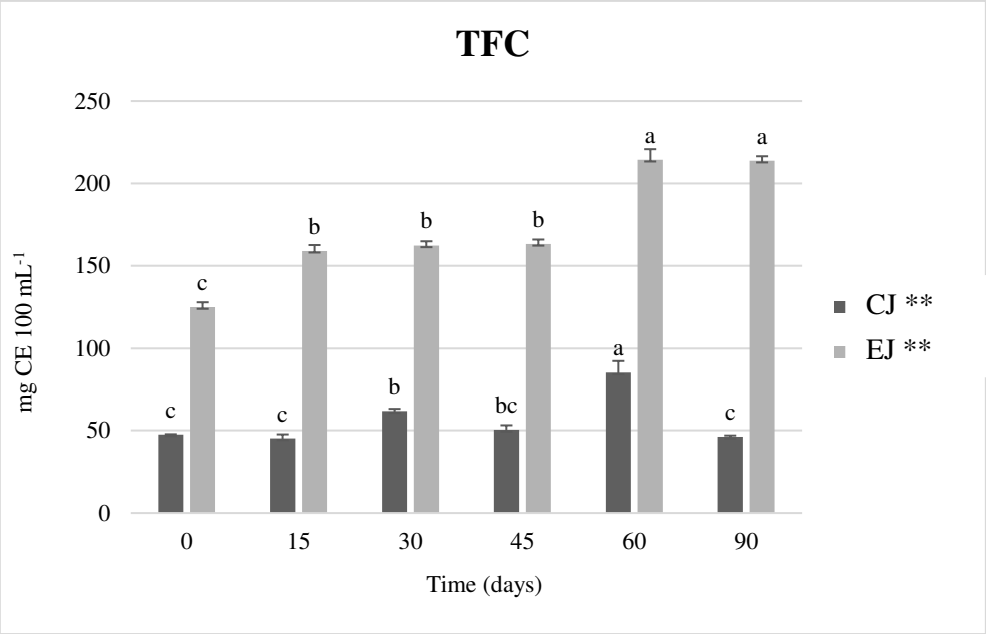


Figure 5.2: TFC values of apples juice samples during storage time.
Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations:**:significance at $p<0.01$

TAA measured by ABTS and DPPH assays was illustrated in Figures 5.3. (a,b). As expected, EJ showed a higher antioxidant activity than CJ for both the ABTS and the DPPH assay.

The ABTS values (Figure 3a) displayed a greater antioxidant activity for EJ compared to CJ. EJ showed a growing trend of antioxidant activity, related to the increase in phenolic compounds over time observed previously. In particular, from the initial value reported an increase was observed up to 90 days where the maximum value of 71.59 ± 0.64 mmol TE L^{-1} was reached. For CJ sample the trend was instable following a decreasing trend.

DPPH values (Figure 3b) displayed statistically differences for CJ ($p<0.01$) and for EJ ($p<0.05$). EJ maintained stable values over time with similar values at time 0 (19.49 ± 0.01 mmol TE L^{-1}) and time 90 (19.46±0.04 mmol TE L^{-1}). In CJ the assay exhibited a decreasing trend of about 50% after 30 days remaining constant until the end of storage.

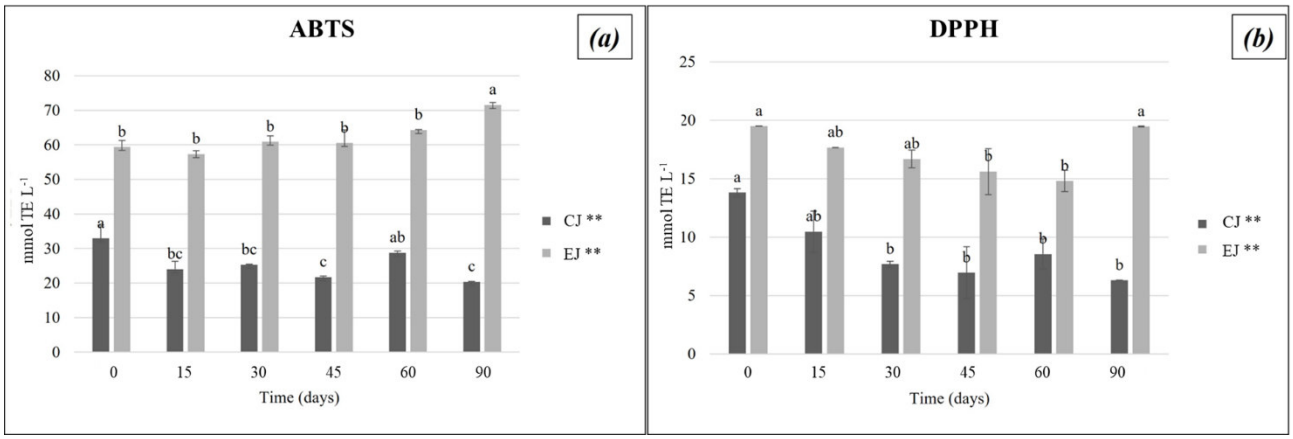


Figure 5.3: TAA values of apples juice samples (a: ABTS; b: DPPH) during storage time.
Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations:**:significance at p<0.01; *:significance at p<0.05

The chromatographic analysis of the main IPC detected in EJ by UHPLC is reported at three monitoring times, 0, 45 and 90 days tracking the behavior of microencapsulated compounds in juice sample. Table 5.5. showed that no statistically significant variations were highlighted for the phenolic acids (p-coumaric acid and ferulic acid). Flavonoid concentrations in EJ during monitoring times displayed statistical differences (Table 5.5) for all of them. In EJ the major flavonoids, neoeriocitrin, naringin and neohesperidin followed the same trend with an increment after 90 days. The other compounds followed a decrease at the 90th day of monitoring. Specifically, eriocitrin, narirutin and brutieridin exhibited a stable trend after 45 days with values comparable to time 0, and a significative ($p<0.01$) reduction at the last monitoring time. Melitidin presented a small decrement after 45 days passing from 19.94 mg L^{-1} (T0) to 17.18 L^{-1} (T45) reaching 5.37 mg L^{-1} at T90. These different trends could be due to a gradual and different release of single compounds. However, the overall content increased over time, showing a gradually release of phenolic compounds. This effect was also observed by Wyspiańska et al., 2019, who found a gradually increasing of isoflavonones after a degradation over time of maltodextrin capsules with a release of phenolic molecules in the solution.

Table 5.5. Individual phenolic compounds (IPC) determined in enriched apple juice (mg L^{-1}).

EJ	T0	T45	T90	Sign.
p-coumaric acid	1.68 ± 0.08	1.75 ± 0.3	1.6 ± 0.46	ns
Ferulic acid	n.d.	0.19 ± 0	0.42 ± 0.27	ns
Eriocitrin	2.29 ± 0.1^{a}	2.31 ± 0.1^{a}	1.23 ± 0.29^{b}	*
Neoeriocitrin	88.18 ± 1.45^{b}	90.41 ± 2.08^{b}	107.16 ± 1.99^{a}	**

Narirutin	0.85 ± 0.04[a]	0.87 ± 0.03[a]	0.11 ± 0.01[b]	**
Naringin	104.38 ± 1.47[b]	111.54 ± 3.02[b]	129.81 ± 0.63[a]	**
Neohesperidin	57.44 ± 1.21[b]	63.18 ± 1.93[b]	94.41 ± 0.71[a]	**
Melitidin	19.94 ± 0.56[a]	17.18 ± 0.2[b]	5.37 ± 0.31[c]	**
Brutieridin	41.12 ± 0.42[a]	41.23 ± 1.79[a]	4.68 ± 0.08[b]	**

Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations: n.s: not significant;**:significance at p<0.01; *:significance at p<0.05

5.3.4. Characterization of enriched "Sunflower Oil" with MD20

The following results were obtained from the qualitative-quantitative analysis carried out on the enriched and control oil samples. Colour results of sunflower oil samples (CO and EO) are reported in Table 5.6.. For all colour parameters considered (L*, a*, b*, C* and h°), both samples showed high statistical differences (p<0.01) over time. L* values of EO decreased with values at time 0 of 51.03±0.12 and of 46.78±0.07 at the end of the storage. In contrast to L*, a* value increased, while b* and C*increased over time reaching values of 1.72±0.04 (b*) and 1.49±0.07 (C*) at the 90th day indicating a greater tendency towards yellow. h° findings showed an increment after 15 days and gradually a slight decreased over time with the lowest value at the end of storage indicating a slight tendency towards green. L* results in CO highlighted a decrement on the 90th day. a* coordinate decreased up to 60th day with a slight increase at time 90. As possible to see in Table 5.6., b* and C* parameters followed the same trend, with a decrease until time 30 with values respectively of 0.50±0.04 and 0.17±0.02, index of a trend towards blue. However, in the two last monitoring times, these values increased indicating a tendency towards yellow. Moreover, h° values of CO changed during storage period with high statistical differences increasing over time up to the highest value after 30 days (121.75±3.30), followed by a continuous reduction until time 90.

Table 5.6. Colour parameters of Sunflower oil" samples during storage time

Colour parameters	Time (days)	CO	EO	Sign.
L*	T0	51.06±0.26[a]	51.03±0.12[a]	ns
	T30	51.09±0.03[a]	49.80±0.08[b]	**
	T60	48.70±0.02[b]	46.60±0.08[d]	**
	T90	47.31±0.03[c]	46.78±0.07[c]	**
	Sign.	**	**	
a*	T0	-0.31±0.03[b]	-0.31±0.02[c]	ns
	T30	-0.31±0.03[b]	-0.28±0.02[b]	ns
	T60	-0.61±0.02[a]	-0.38±0.01[d]	**

	T90	-0.27±0.02[a]	-0.19±0.02[a]	**
	Sign.	**	**	
	T0	0.68±0.08[c]	0.79±0.10[c]	ns
	T30	0.50±0.04[d]	1.18±0.02[b]	**
b*	T60	1.72±0.02[b]	1.70±0.03[a]	ns
	T90	1.93±0.03[a]	1.72±0.04[a]	**
	Sign.	**	**	
	T0	0.28±0.05[c]	0.36±0.08[c]	**
	T30	0.17±0.02[d]	0.73±0.03[b]	**
C*	T60	1.66±0.04[b]	1.52±0.06[a]	**
	T90	1.90±0.06[a]	1.49±0.07[a]	**
	Sign.	**	**	
	T0	114.80±2.43[b]	111.78±2.27[a]	ns
	T30	121.75±3.30[a]	103.19±0.72[b]	**
h°	T60	109.65±0.53[c]	102.76±0.35[b]	**
	T90	97.88±0.59[d]	96.42±0.43[c]	**
	Sign.	**	**	

Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations: n.s:not significant;**:significance at $p<0.01$; *:significance at $p<0.05$

In edible oil the oxidation control is a parameter that defines its quality and safety for human health. It depends on several factors intrinsic and extrinsic, and the enrichment with natural antioxidants is a good strategy for its preservation (Fadda et al., 2022). Total acidity value is a great indicator of oil quality. As clearly visible in Figure 4a, EO sample did not show significant changes during the experimentation, while CO exhibited differences ($p<0.05$) with a steady increase in the two last stages (T69 and T90). Considering the same storage conditions for both samples (CO and EO) its increase could be partially ascribed to the autoxidative process (Sayyari et al., 2016). These results highlighted that the addition of antioxidants in the form of microencapsulate is more efficient than the addition of liquid extract as demonstrated by several studies (Kiritsakis et al., 2017; Arfoui et al., 2021).

The graph reported in Figure 4b displayed the increasing trend in the peroxide values in both oil samples with high statistical significance ($p<0.01$). The two samples values remained stable until time 30 with a slight increasing trend settling around 2.20±0.42 meq O_2 kg^{-1} for CO and 1.85±0.08 meq O_2 kg^{-1} for EO, subsequently the increasing trend became more evident at time 60 and up to time 90 where the samples reached values of 9.99±0.58 meq O_2 kg^{-1} for CO and 11.80±0.42 meq O_2 kg^{-1} for EO with a higher increase for the samples enriched with microencapsulate. The results agree with what reported by

Taghvaei et al., 2014 which showed an increase in peroxide values during the storage of 20 days at 55 °C of soybean oil enriched with phenolic extract from olive leaves encapsulated in maltodextrin, gum arabic and a blend of the two.

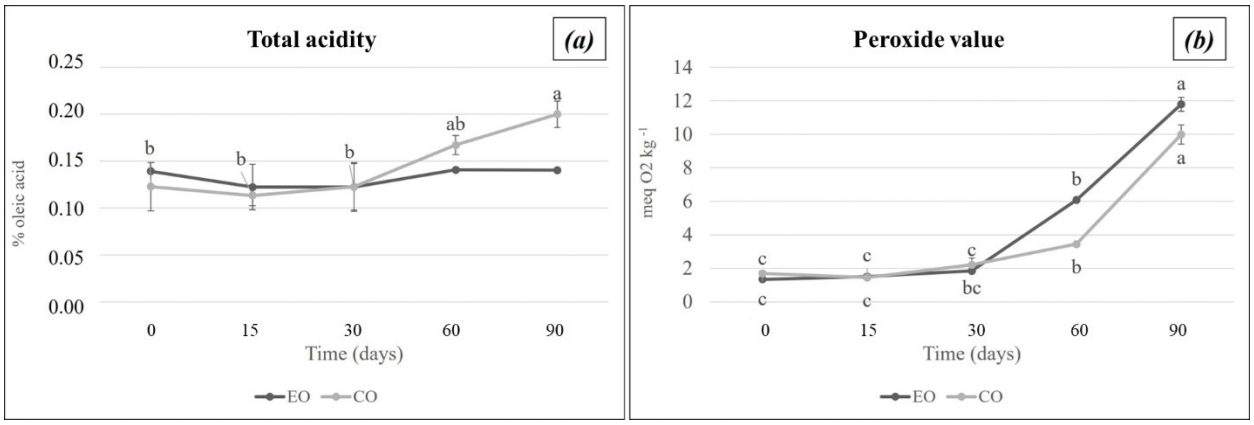


Figure 5.4. Total acidity and peroxide value of sunflower oil samples.
Letters show significant differences as assessed by Tukey's post hoc test.

The oxidative stability of oil samples was studied by mean of the IP measured through the Oxitest. In Figure 5 was displayed the trend of the IP values over time through the time-pressure curve at different monitoring times: 0, 45 and 90 days of EO. As expected, the presence of phenolic compounds added with MD20, resulted in a significantly grown in IP (Figure 5) after 45 days at the storage temperature of 25°C, reaching 14:12 (h:m) starting from 13:16 (h:m) with a consequently enhancement due to the antioxidant effect of bioactive compounds as observed by De Bruno et al. (2022). After 90 days, the IP of EO showed a lower value compared to IP of T0 probably due to different mechanism reactions given to the natural irreversible oxidation combined with the storage temperature (25°C).

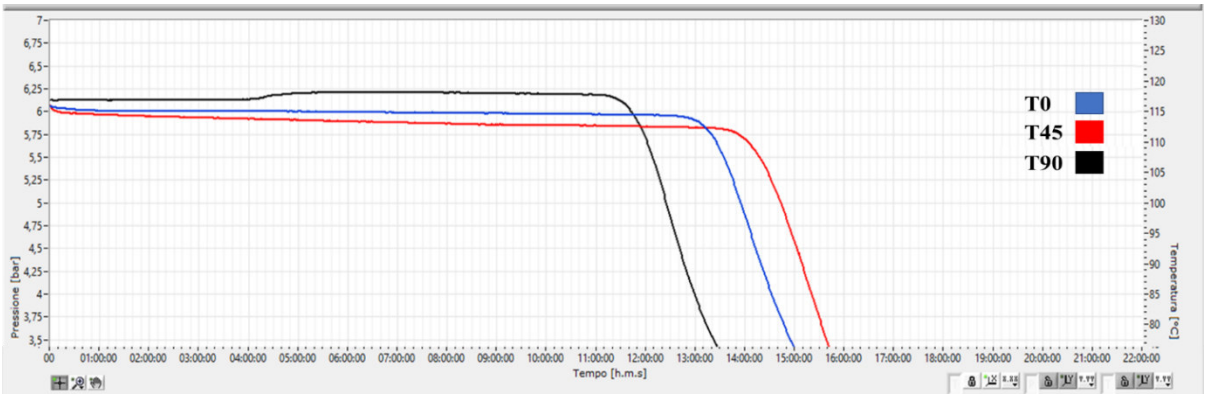


Figure 5.5. Oxidation curves of EO during storage.

In order to evaluate changes and release of bioactive compounds in the sunflower oil from MD20 the antioxidant activity and capacity was detected analysing TPC, TFC, TAA and IPC.

TPC of EO are displayed in Figure 6. Data confirmed that the enrichment with microencapsulated powder provided a quantity of phenolic compounds to oil with an increasing release over time and a high statistical significance. At time 60 was reached the maximum value of 207.91±2.94 mg GAE 100 g^{-1} which was stable at time 90.

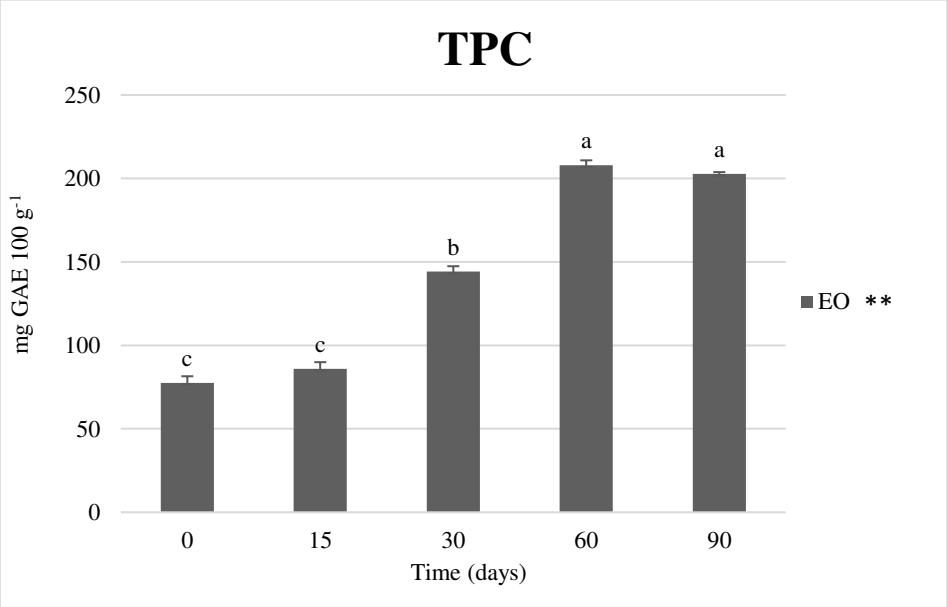


Figure 5.6. TPC values of enriched Sunflower oil samples during storage time.
Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations:**:significance at $p<0.01$.

The chart illustrated in Figure 7 showed an increasing trend up to 30th day where was reached the highest value of 2.97±0.04 mg CE 100 g^{-1}. Subsequently, the content decreased and remained stable at time 60 and 90 with values of around 2.50 mg CE 100 g^{-1}. The trend reported for TPC and TFC is in agreement with Taghvaei et al., 2014 who also reported an increasing release of both class compounds over time.

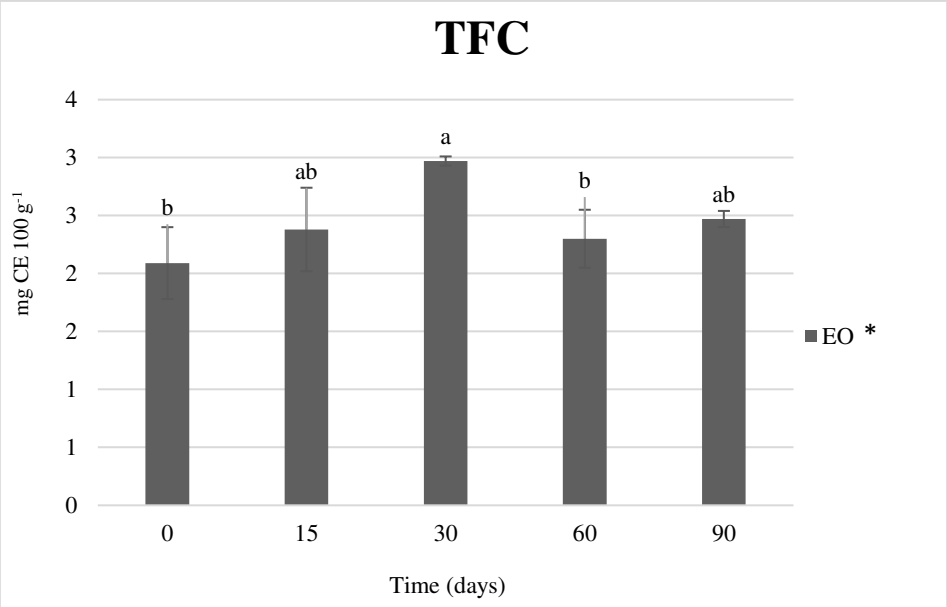


Figure 5.7. TFC values of enriched Sunflower oil samples during storage time.
Letters show significant differences as assessed by Tukey's post hoc test. Abbreviations:*:significance at $p<0.05$.

The antioxidant assays confirmed the antioxidant activity of enriched oil thanks to the content in antioxidant phenolic compounds provided by the microencapsulated powder (Figure8). In particular, the ABTS assay showed a great antioxidant activity over time with a minimum value of 15.70±0.22 mmol TE kg^{-1} at time 0 and a maximum value of 20.43±1.97 mmol TE kg^{-1} at time 90 with high statistical significance ($p<0.01$) between the different analysed times.

The DPPH values followed an increasing trend up to time 30 where the maximum value of 5.07 ± 0.99 mmol TE kg^{-1} was reached and subsequently showed a slight decrease at time 60 and 90. This trend is similar to what observed in the analysis of TFC indicating that the antioxidant activity expressed by this in vitro assay is probably influenced by these compounds.

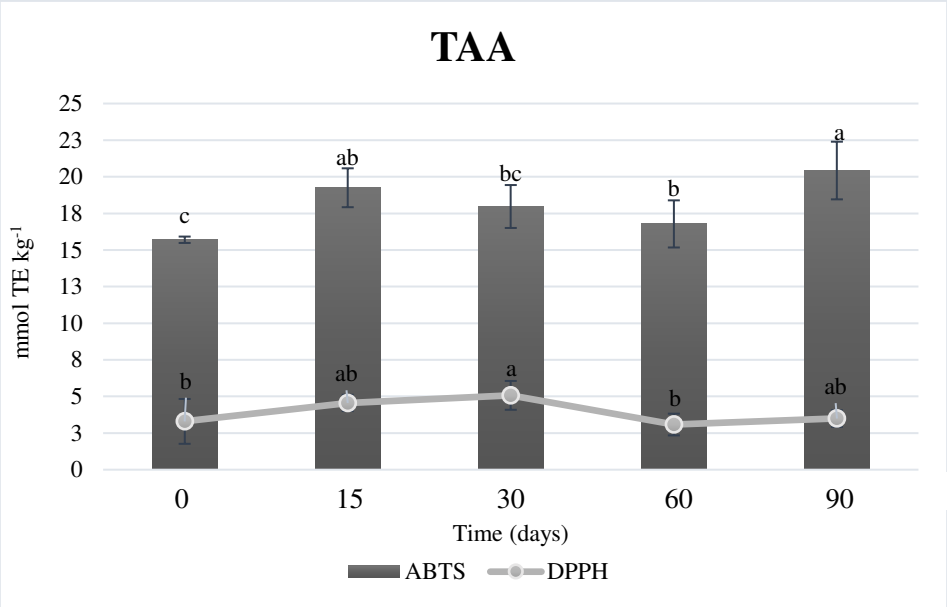


Figure 5.8. TAA values of enriched Sunflower oil samples during storage time. Letters show significant differences as assessed by Tukey's post hoc test.

MD20 in sunflower oil showed a different behaviour that in juice. as reported in Table 5.7., except for melitidin, differences of flavonoids content were found during the storage of EO. Eriocitrin, neoeriocitrin and brutieridin evidenced the same trend with a maximum level at 45th day and a subsequently slightly decrease ($p<0.05$). Narirutin increased in the second monitoring time to decrease below the initial value at the end. High statistical differences ($p<0.01$) were also found in naringin content which revealed an initial value of 47.1 mg L^{-1}, and higher values at time 45 (58.89 mg L^{-1}) and time 90 (56.26 mg L^{-1}). Moreover, p-coumaric acid and neohesperidin, after an initial increase maintained constant concentrations. The gradually release of encapsulated antioxidants in oil phase was also observed by Mohammadi et al. 2016. The different behaviour of the microencapsulate in EJ and EO could be due to the nature of maltodextrin, which is hydrophilic and high soluble in water, permitting an easy release of compounds from the capsules (Hermanto et al., 2016). This could also explain a faster solubilization of the microencapsulate in EJ founding a higher amount of phenolic compounds just from the beginning of the experimentation.

Table 5.7. Individual phenolic compounds (IPC) determined in enriched Sunflower oil (mg L^{-1}).

EO	T0	T45	T90	Sign.
p-coumaric acid	0.7±0,02^{b}	1.01±0.01^{a}	0.98±0.01^{a}	**
Eriocitrin	1.03±0.02^{b}	1.27±0.03^{a}	1.13±0.05^{ab}	*
Neoeriocitrin	37.77±1.25^{b}	46.71±2.04^{a}	43.31±1.13^{ab}	*
Narirutin	0.97±0.04^{b}	1.28±0.08^{a}	0.39±0.02^{c}	**
Naringin	47.1±0.66^{c}	58.89±0.4^{a}	56.26±0.65^{b}	**
Neohesperidin	25.37±0.62^{b}	32.4±1.61^{a}	30.32±0.14^{a}	*
Melitidin	8.84±0.03	10.42±0	9.53±1.41	n.s.
Brutieridin	18.01±0.79^{b}	22.87±0.77^{a}	20.4±0.48^{ab}	*

5.5. Conclusions

The chemical composition of the bergamot pomace extract has shown that it can be a good source of polyphenols and the positive results obtained have highlighted the possibility of enhancing a citrus industry by-product by converting it from waste to resource through the extraction of bioactive compounds. The microencapsulation of the extract has been useful to preserve its characteristics and has made it possible to apply it to hydrophilic and lipophilic systems to give functional and nutraceutical properties. In particular, microencapsulation with 20% of maltodextrin preserved a high polyphenol content, a great antioxidant activity and an excellent encapsulation yield. The addition of the microencapsulated allowed the enrichment toa vegetable oil and a juice increasing the content of polyphenols and antioxidant properties for up to 90 days of storage. The storage test at a controlled temperature of 25°C conducted on the enriched oil showed good stability over time highlighted by the results of the oxidative stability analysis. Moreover, it was pointed out the different release of phenolic compounds in the two products performing the expected function that is the preservation of the antioxidant properties. Enrichment with microencapsulated powder has shown the desired results, that is, a significant improvement in the antioxidant properties of the two food systems compared to nor enriched samples. This research can be considered a good result in the functionalization of oil and beverage with natural microencapsulated ingredients derived from by-products of the food industry.

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6. BERGAMOT POMACE FLOUR: A FUNCTIONAL INGREDIENT FOR PASTA PRODUCTION

This work is in a preparing phase by the author and will be submitted to a journal for publication.

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Abstract

Modern consumers are increasingly interested in the consumption of food formulated with sustainable products and with beneficial effects. In this work both requests were combined through the formulation of pasta with the replacement of wheat flour with flour obtained from bergamot paste (BF) and bergamot paste flour recovered after the extraction of phenolic compounds (BFE) with two concentrations: 2.5% and 5%. From the results obtained from the study it emerged that bergamot paste is a source of different phytochemical compounds such as minerals, fibers, polyphenols, which make it a good ally of human health. The paste enriched with both flours showed a significant increase in antioxidant properties, measured as a total polyphenol and flavonoid content, from the DPPH assay and from chromatographic analysis. From the latter, it emerged that phenolic compounds, in particular flavonoids, were resistant to cooking, except in sample D for melitidin and E for eriocitrin, melitidin and brutieridin, the two samples formulated with 5 and 2.5% BFE respectively. Hence, BP can be considered as a functional ingredient for the formulation of pasta increasing the functionality of the product or in consideration of the addition as flour in gluten-free products.

Keywords: citrus pomace fibre; citrus pomace flour; zero waste, functional food; pasta.

Chapter 6. Graphical abstract.

6.1. Introduction

Agri-food waste is represented by wastewater and solid residue derived from the industrial processing. The sector counts a significant amount of cellulosic by-products (Torres-Leon et al., 2018), and the fruit and vegetable pomace consist of a great quote of raw and fresh products. For instance, in the case of citrus, pomace weight represents about 40-50% of the total processed fruits (Yadav et al., 2022). As reported in Chapter 1 (Introduction), citrus pomace (CP) represents a potential source of high added value compounds such as fibre, pectin, polyphenols, organic acids, cellulose, hemicellulose, macro and micro minerals and more other products (Nieto et al., 2021; Zannini et al., 2021). In recent years, the change in environmental policy has oriented industry and research in the search for alternative solutions, combining recovery, circular economy and resource optimisation, highlighting the importance added values of these by-products. This new challenge is at the heart of the 2030 agenda (Smol et al., 2020).

As a means of reducing the environmental impact that the disposal of such residue causes, some researchers proposed the use of citrus residue as biomass to produce biogas, bioethanol, and bioenergy (Yu et al., 2023; Uçkun Kıran et al., 2015; Choi et al., 2015). In the way of the sustainable development goals of the agenda 2030, the extraction of natural elements from fruit processing waste is assumed as a key research line (Ademosun

2022). Citrus fruits are rich in fibre, with polysaccharides and polyphenol-like components, and the main component of which is insoluble dietary fibre (Hua et al., 2019). Citrus fibre has a better quality compared to cereal, presenting a highest content of total dietary fibre, and better features like water holding capacity and water swelling capacity due to the presence of hydroxyl groups cellulose creating stable hydrogen bonds with water (Jiang et al., 2022). Citrus fibre can be divided in two groups: insoluble and soluble dietary fibre. The insoluble fraction is constituted by lignin, hemicellulose, and cellulose, while soluble fraction by non-cellulosic and hemicellulose polysaccharides, and pectin. In recent years several innovative and green fibre extraction techniques such as enzyme-assisted extractions (Chen et al., 2023; Fuso et al., 2023), steam explosion (Wang et al., 2023), extrusion and high hydrostatic pressures (Perez-Pirotto et al., 2022) have been investigated. The European Food Safety Authority (EFSA) and the Food and Agriculture Organization of the United Nations (FAO) recommend a fibre daily intake of 25 g per day (Santos et al., 2022) due to its importance in human health, for instance in controlling hypertension, cardiovascular and glycaemic disease (Reynolds et al., 2022; Hojsak et al., 2022), lower cholesterol levels and blood pressure (Kesbiç et al., 2022). Moreover, the assumption of fibre promotes the intestinal microbiota activity (Thomson et al., 2021) acting as prebiotic elements (Tang et al., 2020). Fibre furthermore increases the bioaccessibility of polyphenols binding phenols protecting them during digestion (Fernández-Fernández et al., 2021).

Polyphenols, dietary phytochemical compounds, are present as individual or complex molecules based on the chain length and can be found conjugated, connected to sugars, or in combination with lipids, phenols, acids or amines (Gasmi et al., 2022). The effect of these compounds on human health is well known and studied. Indeed, numerous effects have been studied and reported in the literature such as neuro and cardio protective, antidiabetic, antiaging, anticancer and many others (Rana et al., 2022).

Because of this, citrus by-products are considered a potential natural source of high added value compounds with potential high biological activity. For these reasons, recently consumers interest in eating foods rich in these compounds has grown. Polyphenols and fibre have been studied as ingredients in the formulation of functional foods.

In this context, research on the use of natural compounds and components with functional properties in various food types has significantly increased. This is of great interest to the food industry as it allows them to produce unique products with high added value (Granato et al., 2020; Aziz et al., 2022; Sarkar et al., 2023). In addition, fibre have been considered as functional ingredient, for e.g. have been used due to their capacity to bind water in low-fat meat products, including citrus fibre, have been suggested as fat alternatives (Magalhães et al., 2023; Jiang et al., 2023), in ice-cream and cookies being assessed for technological and physicochemical properties (Mohammed et al., 2022; Ali Redha et al., 2023) or in gluten free bread increasing water holding capacity (WHC) with a subsequential reduction of firmness (Bugarín & Gómez, 2023).

The objective of this work is to create value from bergamot pomace (BP), a by-product derived from processing bergamot (*Citrus Bergamia* Risso) fruit. The growing interest in this tree crop has led to an increase in the production of these fruits, which are mainly destined for the processing industry, resulting in large quantity of waste. In this research a perspective of total recovery and circular economy was carried out. BP was turned into functional ingredient exploiting the functional and technological potential of fibre and antioxidant compounds in the production of pasta.

Grinded and dried BP (BF) and BF recovered after a previous extraction of phenolic compounds (Bergamot Pomace Exhausted – BFE) were considered as ingredient and physico-chemical and sensorial properties were analysed.

6.2. Materials and methods

BP were collected from a local citrus processing industry, located in Reggio Calabria (Calabria, Italy), where juice and essential oil has been extracted. BP was transferred to the University "Mediterranea" of Reggio Calabria, Agricultural Department at the Food technology laboratory and immediately dried up to 13% of moisture content in a tangential air-flow cabinet (a modified version of the "Scirocco" model, produced by Società Italiana Essicatoi, Milan, Italy) equipped with an automatic air moisture and temperature control system. Dried BP was ground and bergamot pomace flour (BF) was obtained, which was then sieved with 0.8 mm sieves.

6.2.1. Pasta preparation

Five formulations of pasta were made (Table 6.1.). The simple preparation (Control sample – A) was prepared kneading durum wheat flour (DWF) and water in a kneading

machine (Sigma, model CHEF 20). In the other four samples an aliquot of DWF was replaced as follow: 5% and 2.5% of BF for samples B and C, meanwhile the same quantity (5% and 2.5%) of BFE flour for samples D and E. The dough was kept in refrigerator for 30 min and then was rolled out in 2 mm thickness using an electric dough sheeter (Sigma, vertical dough sheeter T50) and cut in 7 cm length and 3 cm width. The pasta was cooked in a pot in boiling water with a ratio dough/water of 1:10 (w/w).

Table 6.1. Pasta samples formulation.

*g	DWF	H_20	BF	BFE
A	500	200	-	-
B	475	210	25	-
C	487.5	210	12.5	-
D	475	210	-	25
E	487.5	210	-	12.5

6.2.2. Characterization of BF

6.2.2.1. Colour, pH, moisture content (MC) and water activity (a_w) determination

Colour was measured using a spectrophotometer (Minolta CM-700d), considering the CIE L* a* b* coordinates (L*= brightness; a*= positive values point out redness while negative values greenness; b*= positive values point out yellow and negative values blue). The measurement was detected directly on the flour. The coordinates were used to calculate Chroma (C*) and hue angle (h°). C* represents the degree of colour saturation or fullness; and h° is defined as 0°/360° for red/magenta, 90° for yellow, 180° for green, and 270° for blue or purple. It details how red and yellow hues are proportionately distributed. C* and h° were calculated as follow:

$$C^* = (a^2 + b^2)^{1/2}$$

$$h^\circ = \arctan (b^*/a^*)$$

pH was analysed following the AACC International Method 02-52.01 mixing for 30 min 15 g of BF with 10 mL of distilled water and then left for 10 min at room temperature. The pH was detected on the supernatant using a pHmeter (Crison Basic 20, Barcelona, Spain).

The MC was determined on 5 g of BF in a Sartorius Moisture Analyzer MA37 thermal balance at 105 °C. The results were expressed as % of MC.

a_w was determined at room temperature (25°C) with a hygrometer (Aqualab LITE, Decagon, Nelson Court, Pullman, Washington) placing the sample into a container, therefore in the cell of the instrument for the analysis.

6.2.2.2. Determination of nutritional profile and oxidative properties of BF

The determination of fibre fraction was conducted analysing neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content following the method reported by Van Soest et al., 1991. Neutral detergent solubles (NDS) was estimated as difference between 100 and NDF. Hemicellulose (HE) content was determined by subtracting the NDF from the ADF, whereas cellulose (CE) content was calculated by taking the difference between ADF and the residue remaining after digestion according to Trujillo et al., 2010. Crude protein (CP) was also determined following the AOAC method 984.13 (AOAC, 1995).

Mineral content analysis was performed with inductively coupled plasma mass spectrometry (ICP-MS), model Shimadzu MS-2030 (Shimadzu, Kyoto, Japan) as described by Botella-Martínez et al., 2023. Briefly, the calibration of single elements in the ICP-MS was conducted for mineral analysis in BF. The operating conditions were 0.70 L min^{-1} of carrier gas, 9.0 L min^{-1} of plasma gas, 1.10 L min^{-1} of auxiliary gas using a radio frequency of 1.2 kW and an energy filter of 7.0 V.

The oxidative stability of BF was studied with a OXITEST system (Accelerated Storage Test) following the method reported by Gattuso et al., 2023. 45 g of BF were submitted to oxidation test (oxygen at 6 bar pressure; reactor temperature at 90 °C) in the OXITEST reactor to detect the Induction Period reported as IP. IP measures the time to attain an oxidation endpoint associated with an identifiable rancidity or change in oxidation rate. The procedure was performed as described by the AOCS International Standard Procedure (Cd 12c–16) for the determination of oxidation stability of food, fats, and oils (AOAC).

6.2.3. Characterization of the physicochemical properties of Pasta

Except for the sensory analysis, all the analyses in this section were conducted both on samples before (raw) and after cooking (cooked).

6.2.3.1. Physicochemical evaluation

Colour parameters (L*, a*, b*, C* and h°), MC, pH and a_w were performed as reported as reported in section 2.3.1. Colour measurements were carried out directly on the surface

of the pasta in twenty casual points. For pH determination 15 g of the product was homogenised with 100 mL of deionised water and stirred for 30 minutes at room temperature. Subsequently, the suspension was allowed to stand for fifteen minutes until a visible phase separation occurred.

6.2.3.2. Sensorial analysis of Pasta samples

The sensory analysis was conducted in accordance with ISO 13299:2003 in order to assess differences among the different functionalized samples compared to the control (A sample). The test was carried out in a sensory laboratory according to ISO 8589:2007 by 18 judges composed by 10 females and 8 males with age ranging between 25–55 years, recruited among researchers and workers of the Agricultural Department of the University of Reggio Calabria. Panelists were previously trained for a month to know the products and create a shared language to clearly express their perceptions. Moreover, they all agreed to the principles of the Declaration of Helsinki, refraining from smoking, and ingesting food and drink, excluding water, prior to the test. Pasta samples were served in white polyethylene dishes with a secret code to identify the sample in different order and time. Pasta was cooked in salt water and was scored considering different descriptors: four appearances (colour intensity, presence of stains, surface integrity and internal homogeneity after section), five aromatics (citrus, cooked, anomalous odours, alcohol and oil), four flavours (vegetable, pasty, bitter, salty), four texture (stickiness, nerve, tooth/alto adhesiveness, granularity). In addition, the judges were asked to make a general hedonistic judgement (attractiveness, harmony, general acceptability). The descriptors were evaluated using a nine-point intensity scale, considering 1 as barely perceptible and 9 as strongly perceptible. Data were collected and elaborated by calculating the median of results.

6.2.3.3. Antioxidant properties and phenolic composition (UHPLC-DAD) of Pasta

The phenolic extraction method for raw and cooked samples was performed as described by Imeneo et al., 2021. In brief, 5 g of sample were mixed with methanol (20 mL), distilled water (2.5 mL), and hydrochloric acid (0.25 mL). The prepared mixtures were sonicated at 30°C with 20 kHz ± 500 Hz of frequency in a Sonoplus ultrasonic bath (Series 2000.2, HD 2200.2 - BANDELIN, Berlin, Germania). After 60 minutes of sonification the extract was centrifuged for 8 min at 7000 rpm in a refrigerated centrifuge

(Sigma 3-16KL, Germany). The supernatant was recovered, filtered (Whatman n. 4 filter), and made up to volume in a 25 mL flask with a methanol/water mixture (1:10).

The total polyphenols content (TPC) was detected using the technique described by De Bruno et al., 2023. In short, in a flask (5 mL) 150 µL of $NaNO_2$ (5%, w/v) solution, 1000 µL of water and 300 µL of $AlCl_3$ (10%, w/v) were mixed. After 6 minutes, 2000 µL of NaOH (1N) was added e kept for 6 minutes. Thus, the solution was made up to volume with deionised water. At the same time, a blank without sample was prepared and the absorbance at 510 nm was detected. Data were expressed as milligrams of catechin equivalents per 100 g^{-1} of dry weight (mg CE 100 g^{-1} DW).

The total antioxidant activity was performed with DPPH assay as described by Boninsegna et al., 2023. The assay is founded on the reaction between the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and antioxidant compounds of the extract. The result is a discolouration of solution caused by the extinction of the radical. The analysis was carried out placing 30 µL of extract and 2970 µL of DPPH solution (6 x 10^{-5} M of methanol DPPH solution) in a cuvette and left in the darkness to react for 30 minutes. Methanol was used as blank and the absorbance was measured at 515 nm in a double-beam ultraviolet visible spectrophotometer Agilent 8453 diode-array UV–Visible spectrophotometer (Agilent Technologies). Results were expressed as mM Trolox kg^{-1} dry weight of pasta (mM TE kg^{-1} DW).

The extract was also analysed with liquid chromatographic technique in a UHPLC-DAD system following the method reported by De Bruno et al., 2022 identifying and quantifying the main phenolic compounds. A UPLC PLATINblue (Knauer, Berlin, Germany) equipped with a Photo Diode Array Detector PLATINblue (Knauer, Berlin, Germany) and column C18 (1.8 µm, 100 × 2 mm) at 30 °C, was used to evaluate the extract (2 µL) phenolic composition. The flow rate was set up at 0.4 mL min^{-1}. The eluents were water (UHPLC grade) acidified with formic acid (pH 3.10) A) and acetonitrile (B). The applied elution gradient is reported in the following Table (Table 6.2.):

Table 6.2. Elution gradient in chromatographic analysis.

	Time (min)	A (%)	B (%)	Flow (mL min^{-1})
1	0.00	95.00	5.00	0.400
2	3.00	95.00	5.00	0.400
3	15.00	60.00	40.00	0.400
4	15.50	0.00	100.00	0.400
5	20.00	95.00	5.00	0.400
6	22.00	95.00	5.00	0.400

External standards (1–100 mg L^{-1}) were used for the quantification of phenolic compounds. The method was validated evaluating the limit of quantification (LOQ = SD x 3.3) and the limit of detection (LOD = SD x 10), defined as the lowest concentration in the standard solution with the percentage of the relative standard deviation (% RSD) ≤ 10%. The results were expressed as milligrams per 100 g^{-1} of dry weight (mg $100g^{-1}$ DW).

6.2.4. Data statistical analysis

Data are reported as mean value ± standard deviation of data. The statistical analysis to assess the variance was one-way ANOVA, conducted by SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) applying the Tukey post hoc test at $p < 0.05$.

6.3. Results and discussion

6.3.1. BF characterization

In Table 6.3. were showed the main physicochemical features of BF. Colour data reported values of 76.53 for L*, 4.49 for a*, 27.66 for b*, 28.03 for C*. h° showed a value of 80.84 which indicates a colour approximately yellow. The pH value was 3.46, MC% was 13% and a_w 0.407. These results were similar to those reported by Belluco et al., 2022, in albedo orange [*Citrus sinensis* (L.) Osbeck] flour used as functional ingredient.

Table 6.3. Physical-chemical characteristics of BF.

Bergamot Pomace Flour (BF)		
COLOUR	L*	76.53 ± 2.12
	a*	4.49 ± 0.68
	b*	27.66 ± 1.64
	C*	28.03 ± 1.69
	h°	80.84 ± 1.12
	pH	3.46 ± 0.08
	MC%	13 ± 0.23
	a_w	0.407 ± 0.03

The results of BF fibre characterization are reported in Table 6.4. and are in accordance with values similar to other citrus fruit studied in literature (Olowu & Yaman Firincioglu, 2023; Vastolo et al., 2022; Lashkari & Taghizadeh, 2013). Fibre's fraction expressed as % dw highlighted percentages of 17.79 for NDF, 10.68 for ADF, 2.27 for ADL, 82.21 for NDS, 7.11 for HE, 8.41 for CE and 7.79 for CP 7.79.

The mineral composition of BF (Table 6.4.) showed the highest concentration in K (10.8 mg g^{-1} dw) and Ca (7.11 mg g^{-1} dw). It is worth to note the absence of microelements such as As, Cd, Pb, Hg that are considered toxic (Rahman et al., 2019). Minerals are essential elements for human organism, and in fruits its content is dependent on several factors like soil composition, ripening period and agronomic cultivation practices. However, the main relevant minerals presented values within the ranges reported in other citrus species (Lubinska-Szczygeł et al., 2023; Neshovska, 2023; Silva et al., 2017; Xu et al., 2008).

Table 6.4. Fibre and mineral composition of BF.

	BF	
Fibre composition		
% dw	NDF	17.79 ± 1.55
	ADF	10.68 ± 0.83
	ADL	2.27 ± 0.03
	NDS	82.21 ± 7.64
	HE	7.11 ± 0.54
	CE	8.41 ± 0.61
	CP	7.79 ± 0.89
Mineral composition		
mg g^{-1} dw	Ca	7.11 ± 0.09
	Cu	nd
	Fe	0.02 ± 0
	K	10.8 ± 0.13
	Mg	1.05 ± 0.01
	Na	0.65 ± 0.03
µg g^{-1} dw	As	nd
	Cd	nd
	Cr	0.14 ± 0.06
	Hg	nd
	Ni	nd
	Pb	nd
	Se	nd

6.3.2. Pasta characterization

6.3.2.1. Physicochemical characteristics of pasta samples

Table 6.5. provided colour coordinates values of pasta samples. In raw samples, L* showed the lowest value in control sample (A) and the lowest in E. Instead, A showed the highest value after cooking and the lowest in sample B, enriched with the highest content of BF. Statistical change was registered in all samples, indicating a darkening effect after the cooking, especially for enriched samples. This could be possible due to Maillard browning and other enzymatic reaction during cooking. Sample A showed a decrease in a* value, with a reduction in redness after cooking, and sample E, enriched with the lowest quantity of BFE did not show statistical differences after cooking, while the other (B, C and D) enriched samples showed increased redness. Regarding yellowness coordinate (b*) the trend was the same for all, indicating a reduction after cooking process with sample A showed the highest value. This resulting in a less yellow colour. Results suggested that the cooking process affected the colour intensity of pasta reported as C*, with a reduction in the colour intensity. The type of colour of pasta samples (h°) changed after cooking except for E sample, which was stable. The samples with the highest concentrations of BF and BFE revealed the lowest value of h°. In general, findings demonstrated that the cooking process and the addition of BF and BFE changed colour parameters.

Pasta samples, as expected, increased their MC when cooked (Table 6.6.). However, sample B, absorbed the highest quantity of water, due to the water holding capacity of citrus fibre that allow to retain more water (Figuerola et al., 2005). BF and BFE affected the pH value of pasta with a significant acidification of samples related to the quantity and type of bergamot flour added in the recipe. Raw sample A displayed the highest value (6.45) following the order to A>E>D>C>B. The a_w was not influenced by the different flour. Statical differences were detected in samples D ($p<0.05$) and E ($p<0.01$) after cooking.

Table 6.5. Colour characteristics of pasta samples.

	L*			a*			b*			C*			h°		
	Raw	Cooked	Sign	Raw	Cooked	Sign	Raw	Cooked	Sign	Raw	Cooked	Sign	Raw	Cooked	Sign
A	78.77±0.29^{c}	76.57±0.4^{a}	**	2.01±0.07^{a}	1.54±0.07^{c}	**	24.04±0.66^{a}	19.88±0.81^{a}	**	24.13±0.66^{a}	19.94±0.81^{a}	**	85.27±0.12^{b}	85.62±0.24^{a}	**
B	81.03±0.67^{a}	72.45±0.67^{c}	**	1.5±0.14^{bc}	1.82±0.1^{a}	ns	19.55±1.21^{c}	17.67±0.65^{b}	**	19.6±1.22^{c}	17.77±0.65^{b}	**	85.64±0.37^{a}	84.15±0.23^{c}	**
C	81.43±0.99^{a}	74.15±0.73^{b}	**	1.4±0.11^{d}	1.53±0.14^{c}	**	19.56±1.77^{c}	16.67±0.42^{c}	**	19.61±1.77^{c}	16.74±0.43^{c}	**	85.93±0.36^{a}	84.8±0.43^{b}	**
D	81.06±0.48^{a}	74.64±0.84^{b}	**	1.55±0.14^{c}	1.68±0.21^{b}	ns	18.68±0.67^{c}	15.84±0.5^{d}	**	18.74±0.67^{c}	15.93±0.5^{d}	**	85.31±0.35^{b}	84.01±0.68^{c}	**
E	80.28±0.69^{b}	74.49±0.4^{b}	**	1.89±0.1^{b}	1.52±0.11^{c}	ns	21.55±0.69^{b}	16.51±0.74^{cd}	**	21.63±0.7^{b}	16.58±0.74^{cd}	**	85.04±0.19^{b}	84.78±0.44^{b}	ns
Sign	**	**		**	**		**	**		**	**		**	**	

Table 6.6. MC, pH and a_w of pasta samples.

	MC %			pH			a_w		
	Raw	Cooked	Sign	Raw	Cooked	Sign	Raw	Cooked	Sign
A	35.41±0.38^{b}	53.84±0.4^{bc}	**	6.45±0.04^{a}	6.24±0.11^{a}	ns	0.963±0.011	0.975±0.003	ns
B	36.18±0.22^{ab}	57.23±0.29^{a}	**	4.66±0^{d}	4.57±0.09^{d}	ns	0.956±0.009	0.973±0.003	ns
C	36.31±0.23^{ab}	54.38±0.3^{b}	**	5.15±0.11^{c}	5.04±0.01^{c}	ns	0.956±0.007	0.970±0.004	ns
D	36.67±0.18^{a}	53.21±0.18^{c}	**	5.09±0.03^{c}	4.95±0^{c}	*	0.953±0.004	0.975±0.002	*
E	36.15±0.1^{ab}	53.56±0.23^{bc}	**	5.73±0.04^{b}	5.5±0.03^{b}	*	0.953±0.001	0.971±0.001	**
Sign	*	**		**	**		ns	ns	

6.3.2.2. Sensorial characteristics of pasta samples

The sensorial characteristics of pasta were determined by a group of trained panelists and the sensory attributes evaluated are presented in Figure 6. 1.. Fortified pasta cooked differed from sample A. Appearance attributes showed the higher scores for colour intensity in samples enriched with BF and the lower in sample D. The surface integrity was higher in A due to the homogeneity of the used flour, confirmed also by the score of the internal homogeneity. The presence of stains was significantly higher in D and E. Regarding the aromatic evaluations (Figure 6.1.b), the scent of citrus was recorded with scalar values in B (5), C (4), D (3), E (2) and A (1), which covered in part the scent of baked, equal for all enriched samples. The oil aroma score was lower in the samples indicating the highest score in B (2), and no anomalous odour were perceived. Flavour attributes data indicated the most vegetable, pasty and bitterness perception in B, containing the highest amount of BF. Except for vegetable, for all the other characteristics sample C and D showed the equal values. No differences in salty were found among the samples. Among the textural attributes, scores of nerves, stickiness and tooth/alto adhesiveness were higher in enriched sample. Granularity was slight perceived in B (1), and more in sample containing BFE, and was not perceived in A and C.

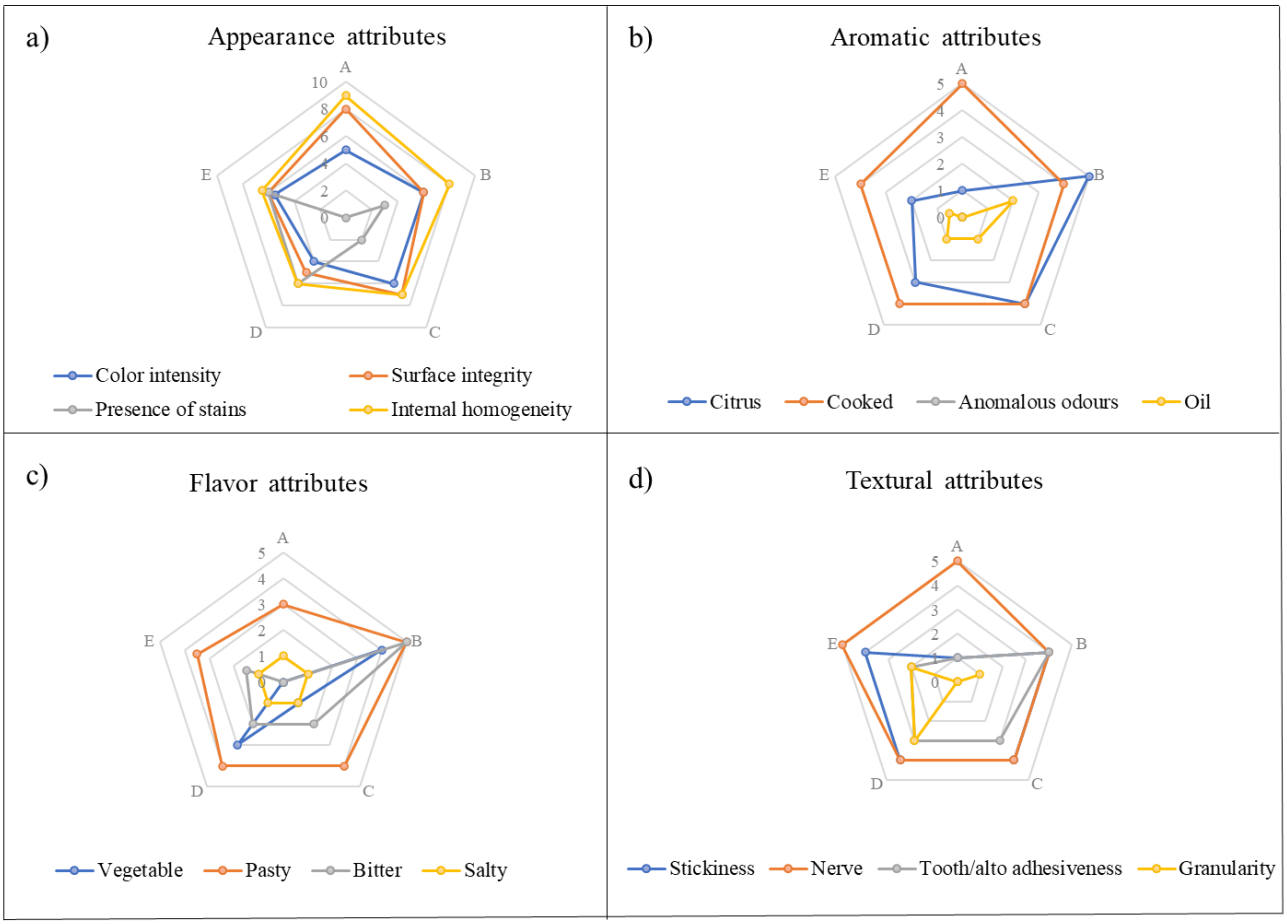

Figure 6.1. Sensorial attributes.

Moreover, was asked to the judges an evaluation based on hedonistic descriptors (attractiveness, harmony, general acceptability) (Figure 6.2.). Even if the scores were acceptable for all samples, results clearly indicated that sample with 2.5% of BF (C) gained the same results of A.

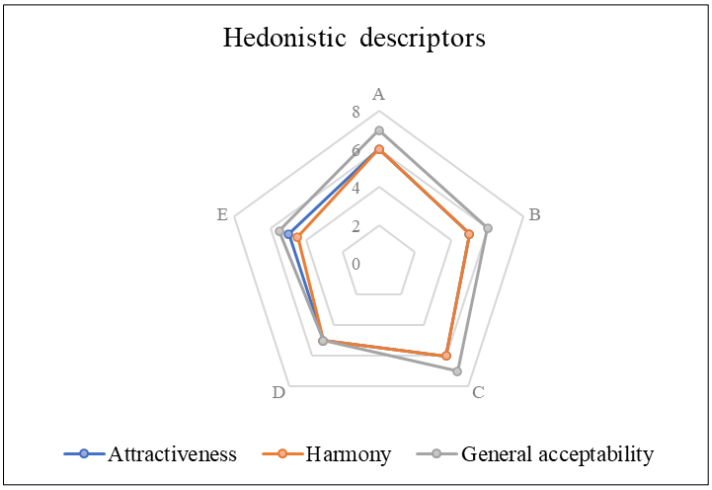

Figure 6.2. Hedonistic descriptors.

6.3.2.3. Antioxidant properties and individual phenolic compounds

TPC reported in Table 6.7. showed statistical differences ($p<0.01$) both among the samples in raw and cooked pasta, and between the same sample before and after cooking. Results suggested similar values among samples formulated with the low amount of BF (C) and with BFE (D and E) in raw pasta followed by a major loss after cooking by sample E. For all samples the cooking led to a reduction of TPC. This could be due to main factors as the solubility of some phenolic compounds in water, especially at high temperatures, thermal degradation, oxidation reactions or leaching into water (Simonato et al., 2019; Gull et al., 2018).

As regard of TFC (Table 6.7.), were found the highest content in enriched samples, with similar values among them. After cooking, except for E sample, were not detected any change in TFC showing a good stability.

Table 6.7. TPC and TFC of pasta samples.

	TPC (mg GAE 100 g^{-1} dw)			**TFC** (mg CE 100 g^{-1} dw)		
	Raw	Cooked	Sign	Raw	Cooked	Sign
A	36.58 ± 1.34^{c}	30.6 ± 0.55^{d}	**	12.52 ± 1.75^{b}	15.13 ± 2.99^{c}	ns
B	62.79 ± 2.92^{a}	50.03 ± 0.81^{a}	**	21.73 ± 4.96^{a}	25.24 ± 1.66^{a}	ns
C	49.09 ± 3.44^{b}	39.54 ± 1.12^{b}	**	19.31 ± 2.34^{ab}	19.95 ± 1.58^{b}	ns
D	48.62 ± 2.1^{b}	41.82 ± 2.24^{b}	**	21.77 ± 4.43^{a}	20.85 ± 1.43^{b}	ns
E	44.02 ± 1.54^{b}	35.08 ± 1^{c}	**	26.49 ± 3.52^{a}	17.06 ± 1.51^{bc}	**
Sign	**	**		**	**	

The antioxidant capacity analysed in vitro with DPPH assay (Figure 6.3.). On the contrary of TPC and TFC, DPPH results presented significant increasing ($p>0.01$) after heat treatment for all analysed samples. In particular, the most relevant increasing rate was detected in A, and in both samples containing BF.

A similar trend, in which after cooking TPC decrease and DPPH values increase was registered in pasta made with banana powder by Biernacka et al., 2020.

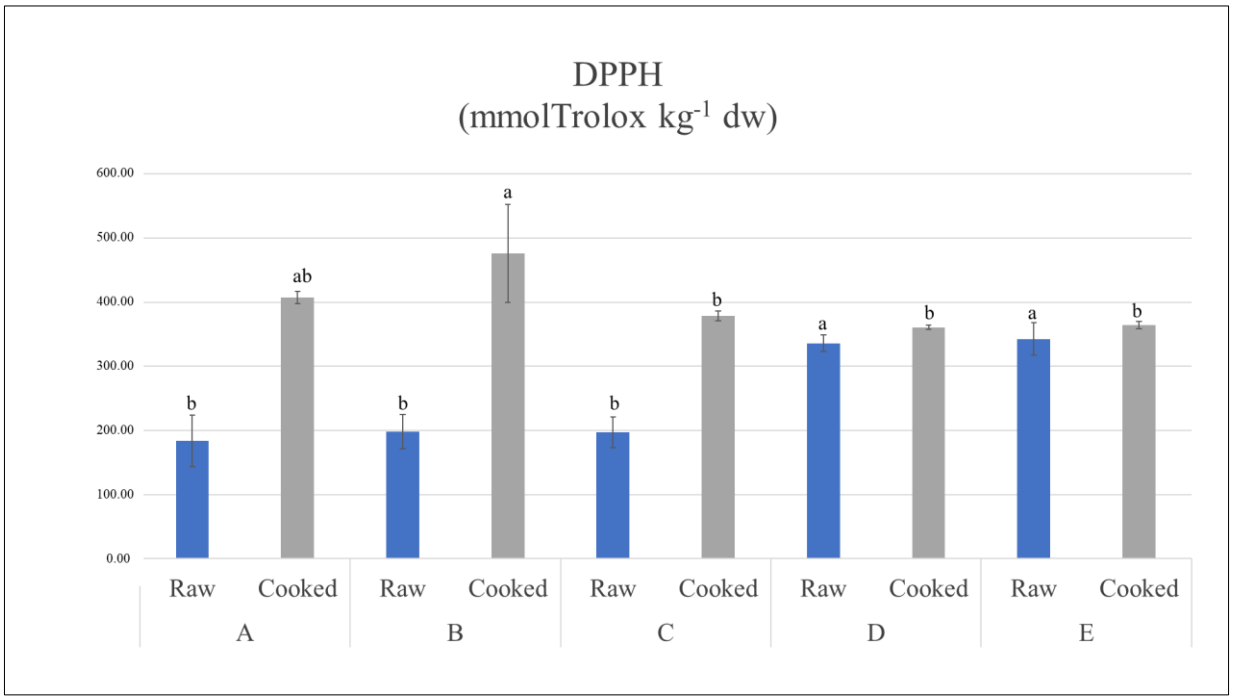

Figure 6.3. DPPH assay results.

The main flavonoids detected were eriocitrin, neoeriocitrin, naringin, neohesperidin, melitidin and brutieridin (Table 6.8), in accordance with those reported by Gattuso et al., 2023. The chromatographic analysis revealed the same trend reported for TFC. Specifically, after cooking there were not detected significant changes among the flavonoids detected, except in sample D for melitidin and in E for eriocitrin, melitidin and brutieridin, probably due to a better bioavailability due to the previous extraction process in which BFE was involved. Among the samples eriocitrin did not show statistical differences, whereas the other flavonoids showed high statistical differences among raw and cooked samples recording values maximum in B, similar between sample C and D, and minimum in E. Even if in the major cases it was not significant, after cooking there was a slight increase in individual flavonoid concentrations, probably favoured by a better extractability.

Table 6.8. Individual flavonoids detected with UHPLC-DAD**.**

mg 100 g^{-1} dw	**Eriocitrin**			**Neoeriocitrin**			**Naringin**		
	Raw	Cooked	Sign	Raw	Cooked	Sign	Raw	Cooked	Sign
B	0.62±0.17	0.83±0.14	Ns	28.86±3.93a	35.66±3.88a	ns	28.09±3.29a	33.84±3a	ns
C	0.44±0	0.52±0.03	Ns	12.08±2.95b	18.51±0.25b	ns	12.98±2.98b	19.06±0.54b	ns
D	0.45±0.08	0.62±0.17	Ns	14.18±0.55b	16.01±0.34b	ns	14.93±0.4b	15.92±0.97b	ns
E	0.29±0	0.45±0.02	**	7.04±0.22b	7.13±0.28c	ns	7.74±0.02b	8.19±0.27c	ns
Sign	ns	ns		**	**		**	**	
	Neohesperidin			**Melitidin**			**Brutieridin**		
B	13.66±1.69a	16.48±1.49a	Ns	6.33±0.31a	7.06± 0.45a	ns	13.21±2.84a	16.06±0.39a	ns
C	6.29±1.70b	8.65±0.66b	ns	3.05±0.71b	4.43± 0.26b	ns	5.70±1.14b	9.01±0.43b	ns
D	6.15±0.01b	6.49±0.22b	ns	2.99±0.13b	3.53± 0.02b	*	5.70±0.23b	6.35±0.23c	ns
E	3.14±0.02b	3.1±0.03c	ns	1.49±0.04b	1.8±0.04c	*	2.62±0.18b	3.47±0.01d	*
Sign	**	**		**	**		**	**	

6.4. Conclusions

In this research, the effect of the addition of BF and BFE, in quality characteristics of pasta was studied. The physicochemical characteristics (fibre, minerals) of BF highlighted in this work, coupled to other health-promoting compounds (polyphenols, essential oil, organic acids) allow to use BP in food industry improving the nutritional and nutraceutical goods. Despite the enriched samples presented an external colour different compared to control sample (A). BF and BFE as replacers of durum wheat flour acidified pasta lowering the pH and increasing the moisture content after cooking. This affected the perception of the nerve and tooth/alto adhesiveness. Regarding the general acceptability preference of panelists, the results that emerged from the sensory analysis was that the dough formulated with 2.5% BF showed the same level of acceptability as the control (A). Moreover, also the antioxidant properties were improved in all the theses considered in the experimentation. The exhausted flour also showed good values of TPC, TFC and antioxidant activity against the DPPH radical. The contribution of BF and BFE flours to the dough was confirmed by chromatographic analysis (UHPLC-DAD), whereby the major flavonoids were identified and quantified.

Hence, BP can be considered as a functional ingredient for the formulation of pasta increasing the functionality of the product or in consideration of the addition as flour in gluten-free products.

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7. CONCLUSIONS AND FUTURE PERSPECTIVES

The thesis work carried out during this three-year PhD project focused on the valorisation of Bergamot Pomace (BP) through the recovery of bioactive compounds and their use to produce new functional foods. It became apparent that BP serves as a crucial and promising source of high biological value compounds, including fibre, antioxidants, and minerals. The natural compounds extracted through food-grade extraction techniques and incorporated into various food products, lead not only to improve the antioxidant properties but also to preserve the shelf life of the food itself. During the research, it was demonstrated that the extracted compounds, used as ingredients, enhanced the functional and oxidative characteristics of a vegetable fat, leading to notable improvements in a resultant bakery product (biscuits) produced with it.

Moreover, the antimicrobial and preservative characteristics of the extract were evidence when applied as an edible coating for strawberries, resulting in an increased shelf life and improved quality of the packaged product.

In addition, experimentation results suggested that microencapsulation of antioxidant extract (AE) can overcame stability and degradation problems. Tests conducted in both hydrophilic and lipophilic food systems allowed to study the effects on these matrices and the release of antioxidant compounds over time.

The replacement of a part of durum wheat flour with Bergamot Pomace Flour (BF) in the pasta production emerged as a sustainable alternative, yielding zero waste. The formulation of pasta with the incorporation of BF and Bergamot Pomace Flour after phenolic compounds extraction (Bergamot Pomace Exhausted - BFE) improved antioxidant characteristics preserving them even after cooking and gaining positive consumer acceptability.

In conclusion, BP has demonstrated substantial potential as a source of valuable compounds suitable to various industries (pharmaceutical, nutraceutical, cosmetic and food and beverage). The transformation of bergamot pomace from waste to a valuable resource signifies a crucial step towards sustainability and resource optimization.

Despite the positive results outcomes of this PhD thesis, it is necessary to continue research into innovative extraction and large-scale systems to enhance the recovery of bioactive compounds from BP. Additionally, there is a critical requirement for further purification of the extracts to separate individual compounds or compound classes.

Further research is needed on refining techniques for the inclusion of bioactive compounds in complex food matrices, studying their interactions and ensuring prolonged shelf life. In this regard, new microencapsulation techniques wort to be explored and the bioavailability and absorption of phenolic compounds in the human body necessitate to be studied, in vitro and/or in vivo. The factors that influenced the absorption and the impact of the 'transport' medium, liquid or microencapsulated, on bioavailability should be analysed.

Based on the obtained results, the formulation of predictive models to predict the behaviour of phenolic compounds in different food systems, using modelling approaches to optimise formulations and predict release kinetics in foods and during digestion is necessary.

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SCIENTIFIC ACTIVITIES CONDUCTED DURING PHD COURSE

Publications on international peer reviewed journals during PhD course

1. Imeneo, V.; Romeo, R.; Gattuso, A.; De Bruno, A.; Piscopo, A. (2021). Functionalized Biscuits with Bioactive Ingredients Obtained by Citrus Lemon Pomace. *Foods*, 10, 2460. https://doi.org/10.3390/foods10102460

2. De Bruno, A., Romeo, R., Gattuso, A., Piscopo, A., Poiana, M. (2021). Functionalization of a vegan mayonnaise with high value ingredient derived from the agro-industrial sector. *Foods*, 10(11), 2684

3. Merlino, M.; Tripodi, G.; Cincotta, F.; Prestia, O.; Miller, A.; Gattuso, A.; Verzera, A.; Condurso, C. (2022). Technological, Nutritional, and Sensory Characteristics of *Gnocchi* Enriched with Hemp Seed Flour. *Foods*, *11*, 2783. https://doi.org/10.3390/foods11182783

4. De Bruno, A.; Gattuso, A.; Romeo, R.; Santacaterina, S.; Piscopo, A. (2022). Functional and Sustainable Application of Natural Antioxidant Extract Recovered from Olive Mill Wastewater on Shelf-Life Extension of "Basil Pesto". *Appl. Sci.*, *12*, 10965. https://doi.org/10.3390/app122110965

5. De Bruno, A.; Gattuso, A.; Ritorto, D.; Piscopo, A.; Poiana, M. (2023). Effect of Edible Coating Enriched with Natural Antioxidant Extract and Bergamot Essential Oil on the Shelf Life of Strawberries. *Foods*, *12*,488. https://doi.org/10.3390/foods12030488

6. Gattuso, A.; Piscopo, A.; Romeo, R.; De Bruno, A.; Poiana, M. (2023) Recovery of Bioactive Compounds from Calabrian Bergamot Citrus Waste: Selection of Best Green Extraction. *Agriculture*, *13*, 1095. https://doi.org/10.3390/agriculture13051095

7. Gattuso, A.; Piscopo, A.; Santacaterina, S.; Imeneo, E.; De Bruno, A.; Poiana, M. (2023). Fortification of Vegetable Fat with natural antioxidants recovered by Bergamot Pomace to use as ingredient for the production of biscuits. *Sustainable Food Technology*. 2023 DOI: 10.1039/d3fb00125c

In progress manuscripts

Gattuso, A., De Bruno, A., Giacondino, C., Piscopo, A., Poiana, M. **Microencapsulation of antioxidant extract recovered by bergamot pomace through freeze-drying methodology and application in hydrophilic and lipophilic food systems.**

Gattuso, A., De Bruno, A., Poiana, M. **Bergamot pomace flour: a functional ingredient for pasta production.**

Gattuso, A., De Bruno, A., Cerdá Bernad, D., Poiana, M., Frutos, M.J. **Bergamot antioxidant extract as novel active ingredient for beverage industry.**

Gattuso, A., De Bruno, A., Cerdá Bernad, D., Poiana, M., Frutos, M.J. **Functional drinks enriched with bergamot pomace antioxidants: in vitro digestibility assessment.**

Gattuso, A., De Bruno, A., Poiana, M. **Physicochemical properties of biscuits made with bergamot by-product flour.**

Mafrica, R., Gattuso, A., De Bruno, A., Poiana, M. **Effects of harvest time on the main characteristics of Fantastico and Femminello bergamot fruits.**

Mafrica, R., Gattuso, A., De Bruno, A., Poiana, M **Influence of cultivar, harvest time and agronomical practices on the maturity index of bergamot juice and essential oil.**

Mafrica, R., Gattuso, A., De Bruno, A., Poiana, M. **Evaluation of different rootstocks on the quality of bergamot fruits (Fantastico and Femminello cv).**

Mafrica, R., Gattuso, A., De Bruno, A., Poiana, M. **Quality assessment of "Lemon of Rocca Imperiale" and its clones.**

Mafrica, R., Gattuso, A., De Bruno, A., Poiana, M. **Advancement in cultivation of "Lemon of Rocca Imperiale" through the study of different rootstocks.**

Contributions to national and international conferences

Gattuso, A. (2023). *Oral communication*: "Application of functional molecules recovered from bergamot by-products: development and improvement of food systems". In: 27th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, September 13th-15st, 2023

Gattuso, A. (2023). *Oral communication*: "Selection of bioactive compounds extraction method to valorise Bergamot (Citrus bergamia Risso) waste" at 4th International Virtual Conference on Food Science & Nutrition, 07th-08th July, 2023

Gattuso, A. (2023). *Oral communication*: "Bergamot Pomace Extract: A Potential Source of Antioxidants for Enhancing Vegetable Fat and Baked Goods". In "I Congreso de CyTAV- Congreso de innovación alimentaria", Universitat de Valencia, Facultad de Farmacia, March 13rd, 2023

Gattuso, A., De Bruno, A., Piscopo, A., Poiana, P. (2023). *Poster presentation*: "Bergamot Pomace Extract: A Potential Source of Antioxidants for Enhancing Vegetable Fat and Baked Goods". In "I Congreso de CyTAV- Congreso de innovación alimentaria", Universitat de Valencia, Facultad de Farmacia, March 13rd, 2023

Gattuso, A. (2022). *Poster presentation*: "Application of functional molecules recovered from bergamot by-products: development and improvement of food systems". In: 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Asti, September 19th-21st, 2022

Gattuso, A. (2021). *Poster presentation*: "Application of functional molecules recovered from bergamot by-products: development and improvement of food systems". 1st telematic Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Palermo, September 14th-15th, 2021.

Organisation of Seminar and Conference

Seminar held on: "Quality and innovation in agro-food industry"; at CEIP ELS Garrofers de Algoda-Matola (Elche, Spain), May 4th, 2023;

Seminar held on "Valorisation of agro-food industry by products: application of bioactive compounds in food systems"; at the University Residence Miguel Hernández (Orihuela, Alicante, Spain), May 16th 2023.

Participation in International Competition and Training School

Gattuso, A., Cerdá Bernad, D., Clemente-Villalba J. (2023). Team participation in the "FoodFactory-4-Us-Sustainable Supply Chain International Student Competition on Increasing innovation in existing SFSCs to make them stronger and more competitive (ISEKI-Food Association) with the project: "smarTMaRkETS: Towards an e-local Market based on Resilience, Efficiency, Technology and Sustainability".

COST action participation. 3rd Yeast4Bio Training School (2023): "Yarrowia lipolytica as a cell factory for lipids from short-chain fatty acids considering molecular and engineering techniques"; at "Faculty of Biotechnology and Food Science, Wroclaw University of Environmental and Life Sciences", Chełmońskiego, Wrocław, Poland.

Other academic activies

Subject Expert and Teaching Assistant in the academic year 2022/2023 in "Packaging and distribution technology of agri-food products'" at 1st Level Degree Course; and in "Quality assessment and management in gastronomy and food service" at 2nd Level Degree Course in Food Science and Technology course at Agriculture Department, University Mediterranean of Reggio Calabria (Italy);

PhD visiting student (January-June 2023) at "Agro-Food Technology Department of the Miguel Hernández University of Elche (Spain)";

Subject Expert and Teaching Assistant in the academic year 2023/2024 in "Chemical Analysis and Control of Food Products" at 1st Level Degree Course; "Gastronomy" at 1st Level Degree Course; "Food service technology" at 1st Level Degree Course and in "Food technology processes" at 2nd Level Degree Course in Food Science and Technology course at Agriculture Department, University Mediterranean of Reggio Calabria (Italy).