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Department of Agronomy Food Natural Resources
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THE IMPACT OF POLYPHENOL-OXIDASE ON THE COLOR OF EDIBLE FLOWERS: THE CASE OF ANTHOCYANINS

Supervisor
Prof. Anna Lante

Co-supervisor
Dott. Peyman Ebrahimi

Student
Nicola Sterle
Matric. n.
2011612

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ABSTRACT

In recent years the use of edible flowers is a growing trend among consumers and the food industry. Edible flowers, consumed directly, as a garnish to dishes, or processed, are rich in bioactive compounds such as anthocyanins, which give flowers their typical colors, and make them a new alternative in health nutrition or as natural dyes. In addition, color is the first sensory attribute in consumers' evaluation of edible flowers, and its preservation is critical for maintaining flower quality and for subsequent applications (e.g., use of anthocyanins as a natural dye). Polyphenol oxidases (PPOs) are among the enzymes most involved in the loss of anthocyanins, and thus color, in plants and flowers. The stability of anthocyanins is severely compromised by the presence of PPO, which initially catalyzes the hydroxylation reaction from monophenol to ortho-diphenol, and then the oxidation reaction from ortho-diphenol to ortho-benzoquinone, resulting in the formation of brown compounds and loss of color. Polyphenol-oxidase is an endogenous enzyme and providing for thermal or nonthermal processes is essential to inactivate it and preserve the quality and color of flowers. There are traditional (e.g., blanching) and more innovative (e.g., ultrasound) methods for processing flowers. This thesis focuses on the action that PPO has on the color and anthocyanin content of edible flowers, with a major emphasis on technological treatments that could be applied during processing to inactivate PPO and avoid loss of color, thus ensuring better quality, shelf life, interest, and commercial value to edible flowers.

Negli ultimi anni l'impiego dei fiori edibili da parte dei consumatori e dell'industria alimentare è in crescita. I fiori commestibili possono essere consumati direttamente, utilizzati come guarnizione nei piatti, conservati o trasformati. I fiori sono ricchi di composti bioattivi come ad esempio le antocianine, che conferiscono ai fiori i loro colori tipici e li rendono una nuova alternativa nell'alimentazione salutistica o come coloranti naturali. Inoltre, il colore è il primo attributo sensoriale nella valutazione dei fiori commestibili da parte dei consumatori ed il suo mantenimento è fondamentale per preservare la qualità dei fiori stessi o per le applicazioni successive (es: uso delle antocianine come colorante naturale). Le polifenol-ossidasi (PPOs) sono tra gli enzimi maggiormente coinvolti nella perdita di antocianine, e quindi di colore, in piante e fiori. La stabilità delle antocianine è gravemente compromessa dalla presenza della PPO, che catalizza inizialmente la reazione di idrossilazione da monofenolo a orto-difenolo, e poi la reazione di ossidazione da orto-difenolo a orto-benzochinone, con conseguente formazione di composti bruni e perdita di colore. La polifenol-ossidasi è un enzima endogeno e prevedere trattamenti tecnologici, termici o non termici, è essenziale per inattivarla e preservare la qualità e il colore dei fiori. Esistono metodi tradizionali (ad esempio, la scottatura) e più innovativi (ad esempio, gli ultrasuoni) per il trattamento dei fiori. La tesi approfondisce l'attività che la PPO esercita sul colore e sul contenuto di antociani dei fiori commestibili, con particolare attenzione ai trattamenti tecnologici che potrebbero essere applicati durante la lavorazione per inattivare l’enzima ed evitare la perdita di colore, garantendo così una migliore qualità, conservabilità, interesse e valore commerciale dei fiori edibili.

1. INTRODUCTION

In numerous cultures around the world, the consumption of edible flowers has always been a popular tradition from ancient times. Flowers have been used for hundreds of years as traditional ingredients for several food preparations, aiming to improve flavor, garnish, or as alternative medicine. We cannot determine with accuracy when humans began to eat flowers, but when some of the world's ancient civilizations began to develop, a wide variety of flowers had already been used with a historic role in medicinals, food preparation, adding aroma, flavor, color, and aesthetic value.

Ancient Sumerians, Assyrians, and Egyptians included saffron (*Crocus sativus*) in their medicinals and there is documentation of the saffron flowers being grown in ancient Greece and other Mediterranean cultures for thousands of years (Kirker and Newman, 2016). In medieval France, calendula flowers (*Calendula officinalis*) were used for the preparation of various salads, and in the 17th century, there is a description of using violets (*Viola odorata L.*) as sugar source and for syrups coloring (Mlcek & Rop, 2011). In Asia, since the Neolithic time, edible flowers such as daylilies (*Hemerocallis L.*) and chrysanthemums (*Chrysanthemum morifolium*) have been popular in China, while the lotus flower (*Nelumbo nucifera*) has been an appreciated flower for its supposed ability to extend the person's longevity (Kirker and Newman, 2016). Examples of the consumption of edible flowers in various areas and eras are several.

The species of edible flowers consumed come from all continents and have different colors, characteristics, and medicinal properties (Table 1). Nowadays, the most common modality of consumption is in the fresh unprocessed form in salads, savory dishes, soups, desserts, jellies, and others, but also in a dried form such as tea, tisane, dried petals, or as spices, crystallized and even as foams in molecular gastronomy (Takahashi et al., 2020). The medicinal properties for human health are the main cause of the consumption of edible flowers (Alasalvar et al., 2013). While many potential health benefits have been reported, the consumption level of edible flowers remains low, compared to other vegetables; this may be ascribed to a common phobia of tasting novel foods (Tsimitri et al., 2022). It is common that some plants are known only for the biological, seasoning, or nutritional potential of their fruits or leaves while the flowers, also edible, are not usually part of culinary, as is the case of some flowers such as passion fruit, chive, and pumpkin (Fernandes, Saraiva, et al., 2018). Despite this, with increased public awareness about a plant-based diet's nutritional and health benefits, the cultivation and consumption of edible flowers are likely to increase further soon.

The color of the food can affect the quality and consumer acceptance (Y. Wang et al., 2012). Nowadays, consumer health attention is steadily increasing, and interest in the development of natural dyes as a substitute for synthetic dyes in the food industry has grown. The importance of color acceptability and the need to meet and attract more consumers to the market have led to the development of new pigments for industrial products (Barani et al., 2022). Plants and flowers are excellent sources of bioactive compounds, which are also natural pigments, such as flavonoids and anthocyanins. Anthocyanins are typical of flowers in red and blue color and can be extracted by different methods, such as solvent extraction, and used as natural dyes in the food industry.

The aesthetics, color, and characteristic taste of edible flowers and their phytochemical properties promote their consumption worldwide (Rodrigues, Cielo, Goméz-Corona, et al., 2017). Many flowers can be eaten, but there are also many poisonous or toxic ones as well, and proper identification is rigorously required. Some flower species have toxic or antinutritional substances, such as alkaloids (Pires et al., 2019). Beyond the toxicity of certain flowers, nowadays, the main problems are related to the use of unauthorized compounds such as dimethoate and sulfites; this fact highlights the need for appropriate standards for safe cultivation and preservation, thereby increasing awareness and further utilization of these foods in future (Barani et al., 2022).

Scientific name	Common name	Origin	Medicinal uses	Pictures	References
Antirrhinum majus	Dog flower	Europe, Central America	Diuretic, treatment for scurvy and liver disorders		(Al-snafi, 2015)
Calendula officinalis	Pot marigold	South Europe	Hepatoprotective, anti-inflammatory, anti-bacterial and anti-fungal, antidiabetic, anti-HIV and anti-cancerous		(Benvenuti et al., 2016; Jan et al., 2017)
Rosa spp.	Rose	Northern Hemisphere	Cancer, inflammation, aging, heart diseases		(Kumari et al., 2018)
Viola × wittrockiana	Pansy	Europe	Laxative, expectorant, emetic, anti-inflammatory, diuretic, sedative, and antiseptic		(Fernandes et al., 2019; Gonçalves et al., 2019)
Echium amoenum	Borage	Europe, Mediterranean basin, northern Iran	Anti-inflammatory, antiviral, antioxidant, antibacterial, sedative, cardiovascular, and pulmonary diseases		(Zannou et al., 2022)
Hibiscus rosa-sinensis	Hawaiian Hibiscus/Chinese Hibiscus	Probable tropical Asia, widely cultivated	Anti-tumor, spasmolytic, hypoglycaemic, anti-inflammatory, analgesic		(Al-snafi, 2018; Bahuguna et al., 2018)

Table 1. *Common edible flowers consumed worldwide.*

2. EDIBLE FLOWERS AS A SOURCE OF BIOACTIVE COMPOUNDS

Worldwide, there are more than 180 species of edible flowers, belonging to over 100 genera and 97 families (Mounir et al., 2017). Simultaneous with the growing interest in edible flowers, research has deeply evaluated plenty of species of them, from all over the world, such as centaurea (*Centaurea cyanus L.*), dog flower (*Antirrhinum majus*), pot marigold (*Calendula officinalis*), hibiscus (*Hibiscus rosa-sinensis L.*), chrysanthemum (*Dendranthema grandiflora*), lavender (*Lavandula pedunculata Cav.*), peony (*Paeonia suffruticosa Andr.*), rose (*Rosa spp.*), Dandelion (*Taraxacum officinale L.*), chamomile (*Matricaria chamomilla L.*), pansy (*Viola × wittrockiana*), and others. Chamomile is rich in phytochemicals such as flavonoids and phenolics compounds and is associated with attenuation of carbohydrate digestion and sugar absorption which can bring about new approaches for dealing with gastrointestinal tract problems (Villa-Rodriguez et al., 2018). Pot marigold possesses a wide variety of phytochemicals and pharmacological activities and many reports have shown highly effective anti-bacterial, anti-fungal, anti-helminthic, anti-inflammatory, anti-HIV, and other properties with no toxicity (Jan et al., 2017). There are also a variety of flowers or inflorescences such as broccoli (*Brassica oleracea L. var. italica Plenck*), cauliflower (*B. oleracea L. var. botrytis*), and artichoke (*Cynara scolymus L.*), rich in bioactive compounds, incorporated in human daily nutrition worldwide, but the consumers rarely correlate these vegetables to flowers (Takahashi et al., 2020). It is widely recognized that edible flowers are a source of a high variety of biologically active phytochemicals that mainly belong to the class of phenolic compounds, carotenoids, and tocols (Skrajda-Brdak et al., 2020).

Understanding the impact of bioactive compounds on health, the sources from which they are derived, and their mechanism of action are opening a new era in the prevention of many diseases.

Phytochemicals are non-nutritionally bioactive ingredients in plant-based foods that can decrease the risk of chronic diseases such as cardiovascular disease, diabetes, cancer, and obesity (Kumari et al., 2021) (Figure 1). These compounds are not essential in our diet, but it’s widely known that they are enrolled in the protection of human health when their dietary intake is significant. Phytochemicals are widely studied in fruit, vegetables, coffee, tea, and other products, and research in recent years is also studying their presence in flowers as they are an alternative source where these molecules can be found in abundance. Bioactive compounds shield plants from illness and environmental harm such as pollution, stress, drought, UV exposure, and pathogenic attack and add to the plant's color, aroma, and flavor (Koche et al., 2018). There is therefore a relationship between color and phytochemical properties. Some phytochemicals, especially those belonging to the flavonoid group, such as anthocyanins, accumulate in the outer layers such as leaves, flowers, and fruit, giving them attractive color and appearance (Koche et al., 2018). In edible flowers, the increased anthocyanin content is decisive in obtaining flower pigmentation but is also one of the key factors for their high phytochemical properties, such as antioxidant activity.

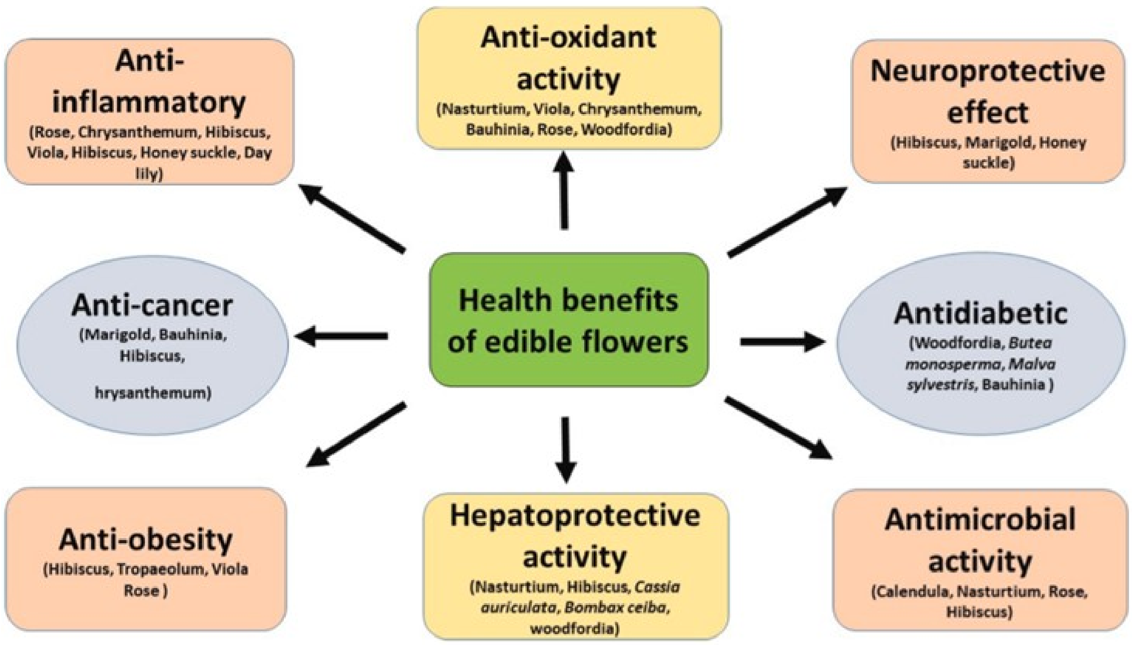

Figure 1. *Main health benefits of edible flowers (Kumari et al., 2021)*

3. IMPORTANCE OF ANTHOCYANINS IN EDIBLE FLOWERS: THE CHEMISTRY BEHIND

As described above, carotenoids and flavonoids are among the most representative bioactive compounds found in edible flowers. Within the family of flavonoids, one of the major classes is anthocyanins (Figure 2). Beyond their nutraceutical properties, anthocyanins, are the most frequently studied natural dyes because they are the largest class of water-soluble compounds present in the plant kingdom and are responsible for a great variety of colors, involving practically all visible spectra, from orange and red to purple and blue.

These types of natural pigments play an important role in plants and are abundant in flowers. They protect plants from several biotic and abiotic stresses such as excess light, drought, or pests, and promote reproduction because they attract pollinators and seed dispersers such as herbivores by presenting attractive colors (Cruz et al., 2022). Peony, pansy, chrysanthemum, hibiscus, and rose are typical edible flowers that contain anthocyanins mostly (Barani et al., 2022). Nowadays, the consumption of edible flowers is increasing, the assessment of their quality will be crucial and will necessarily pass through their color and the factors that can compromise it, whether the flowers are consumed as products or to be used to produce natural dyes.

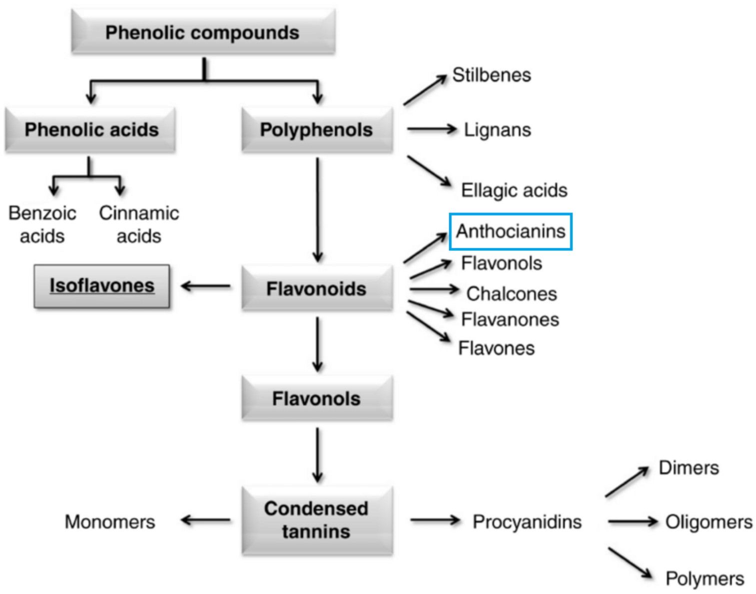

***Figure 2**. Subdivision of phenolic compounds.*

Anthocyanins are water-soluble glycosidic dyes. Aglycone is defined as the chromophore group of anthocyanins, is also called anthocyanidin, and has the structure of the flavylium ion variously methoxylated and hydroxylated. The structure consists of an aromatic ring [A] bonded to a heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring [B] (Konczak and Zhang, 2004) (Figure 3). When the anthocyanidins are in glycosylated form (bonded to a sugar) they are known as anthocyanins (Castañeda-Ovando et al., 2009). Anthocyanins always have a glycosidic residue at position 3, sometimes at position 5, rarely at position 7, and even more rarely at positions 3' and 4' (Cabras and Martelli, 2004). Since the discovery of the first anthocyanin, more than 700 related chemical structures have been identified in nature (Andersen et. al, 2006). Most of the described anthocyanins are derived from the six basic anthocyanidin structures (cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin) which differ only in the number of hydroxyls and/or methoxy substituents in the phenyl or B-ring (Ananga et al., 2016; Silva et al., 2016) (Figure 4). Different types of monosaccharides (e.g., glucose, galactose, rhamnose, and arabinose) and disaccharides (e.g., rutinose, sambubiose, and sophorose) can be attached to the flavylium core, and these sugars can also be acylated with hydroxycinnamic acids (e.g., caffeic, coumaric, ferulic, and sinapic acids) or aliphatic acids (e.g., acetic, malic, oxalic, and succinic acids) (Clifford, 2000). An innumerable number of glycosylation combinations occur in nature, leading to an undefined number of different anthocyanins. In accord with Silva et al. (2016) the most abundant is cyanidin derivates (50 % of the total) which are also the most common anthocyanins found in edible flowers, cyanidin-3-O-glucoside (Barani et al., 2022). There are also other major anthocyanins in edible flowers such as delphinidin-3-O-sambubioside (Grajeda-iglesias et al., 2016), peonidin 3-O-sophoroside and glucoside (Cendrowski et al., 2017), malvidin-3-O-glucoside and delphinidin-3-O-glucoside (Deng et al., 2013). Anthocyanins in general can bind to many compounds resulting in the formation of various anthocyanins derivates that have been described in food matrixes, from monomers to more complex structures, adducts conjugated with other polyphenols, proanthocyanins, and other compounds (Cruz et al., 2022).

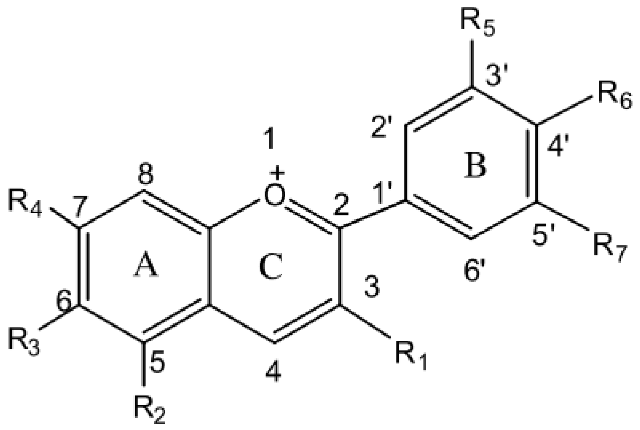

Figure 3. *General structure of anthocyanins (Castañeda-Ovando et al., 2009)*

4. ANTHOCYANINS IN EDIBLE FLOWERS: COLOR-RELATED ASPECTS

The trend in recent decades is to pay more attention to the food we eat. This increasing focus is not only on taste or health aspects but also on other sensory aspects such as color. The color of food is used as the first index to assess the quality of what we eat. Sight is the most developed sense in humans, the evaluation of color is immediate and allows us to anticipate other sensory factors such as taste. The visual aspect influences us enormously on a psychological level and in sensory analyses sight is considered an anticipator of other perceptions ('we buy' with our eyes and with the color of food we imagine the other senses). Color can make food more attractive and increase consumer acceptability. The food industry often makes use of colorants as additives to remedy color losses caused by certain technological processes such as heat treatments or simply to improve the color of certain foods or beverages. Traditionally, synthetic dyes have been widely used for their stability, but nowadays, people have become increasingly suspicious of the use of these synthetic additives, mainly for security issues. For this reason, in many cases, food manufacturers are trying to replace these colorings with natural substitutes. Understanding how to preserve the color of flavylium compounds such as anthocyanins is crucial if we want to maintain edible flowers at a higher quality level or if we want to use them as a natural coloring agent effectively.

Polyphenol-oxidases can have a strong impact on anthocyanins in edible flowers. These enzymes are naturally present in plant tissues, causing the degradation of anthocyanins, so keeping the action of polyphenol-oxidases under control is essential to control their activity during the storage or processing period of edible flowers, or when anthocyanins are used as a natural dye.

The term 'anthocyanin' is derived from the union of two words, 'anthos' and 'kianos', meaning 'blue flower' in Greek. The color exhibited by these molecules was first explained by Linus Pauling in 1939, who proposed that the resonant structure of the flavylium ion caused the intensity of their color (Wrolstad et al., 2005). As described above, anthocyanins can have different forms and this structural diversity is responsible for a wide range of wonderful colors found in many flowers and plants, ranging from yellow-orange to blue-violet. The color of edible flowers depends on the type of anthocyanidins they contain (Figure 4), but above all on the pH. The pH is not the only factor influencing the color of anthocyanins, but it is certainly the most influential factor, changing it even completely. For example, cyanidin is mainly responsible for the color of flowers such as cornflower or poppy, but in cornflower the lymph is alkaline and the color tends towards blue, whereas in poppy the lymph is acid, and the color tends towards red (Cabras and Martelli, 2004). Color changes related to pH variation are reversible but may be a limiting factor in the use of anthocyanins as colorants in the food industry. At $pH < 2.5$, the principal form of the anthocyanins is the colored flavylium cation form (red color); above pH 3, most anthocyanins undergo nucleophilic attack by water with subsequent loss of a proton to give the colorless; with increasing pH, additional colors such as violet, blue or yellow can be observed because of additional deprotonations of the hydroxyl groups (Silva et al., 2016) (Figure 5).

It is widely known that color alteration has much important nutritional, economic and technological cost implications, and despite the great potential application of edible flowers in the food industry, the loss of color due to the instability of anthocyanins is a problem to be solved. Anthocyanins also represent a good source of natural dyes but their use has been limited because of their relative instability and low extraction percentages (Castañeda-Ovando et al., 2009). Therefore, studying the effect of PPO, the stability of anthocyanins, the effects on color, and how to technologically control these issues will be very useful in the coming years.

The chemical stability of anthocyanins and consequently their color can be influenced by several factors such as pH (as described above), enzymes, oxygen, temperature, light, or the presence of various substances such as SO_2, metal ions, co-pigments, carbohydrates, and proteins. In addition, the development of various plant organs also affects the degradation of anthocyanins. Studies have shown that such degradation is likely caused by the turnover of pigments in the vacuoles and the activity of related enzymes, which has laid the foundation for a further study of anthocyanin degradation in plants (Y. W. Zhao et al., 2021). A better understanding of the reasons that lead to the loss/color change of anthocyanins will be crucial for the development of new food products and strategies that will allow a greater application of these pigments. However, in accord with Cruz et al. (2022), understanding the variety of colors of flavylium compounds is much more complex than the scientific community generally acknowledges, therefore, it is necessary to maintain a cautious approach when assessing these aspects. A thorough understanding of the variety and color variation in flavylium pigments requires a holistic literature-based approach that integrates all the multidisciplinary sciences underlying the organic and physical chemistry of flavylium compounds (Cruz et al., 2022). Knowing these aspects when discussing the color of anthocyanins is fundamental but considering all the factors that can influence their color is not the aim of this work.

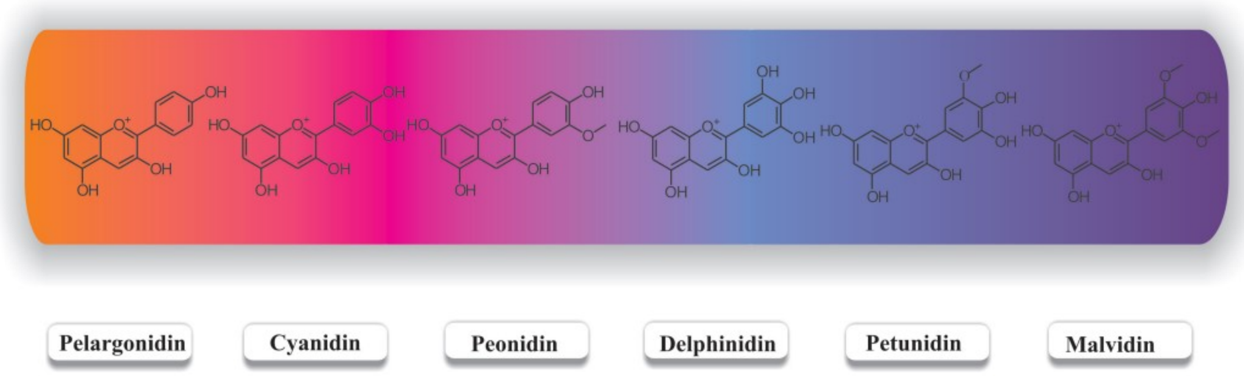

Figure 4. *The visible color range of common anthocyanidins (Ananga et. al, 2016).*

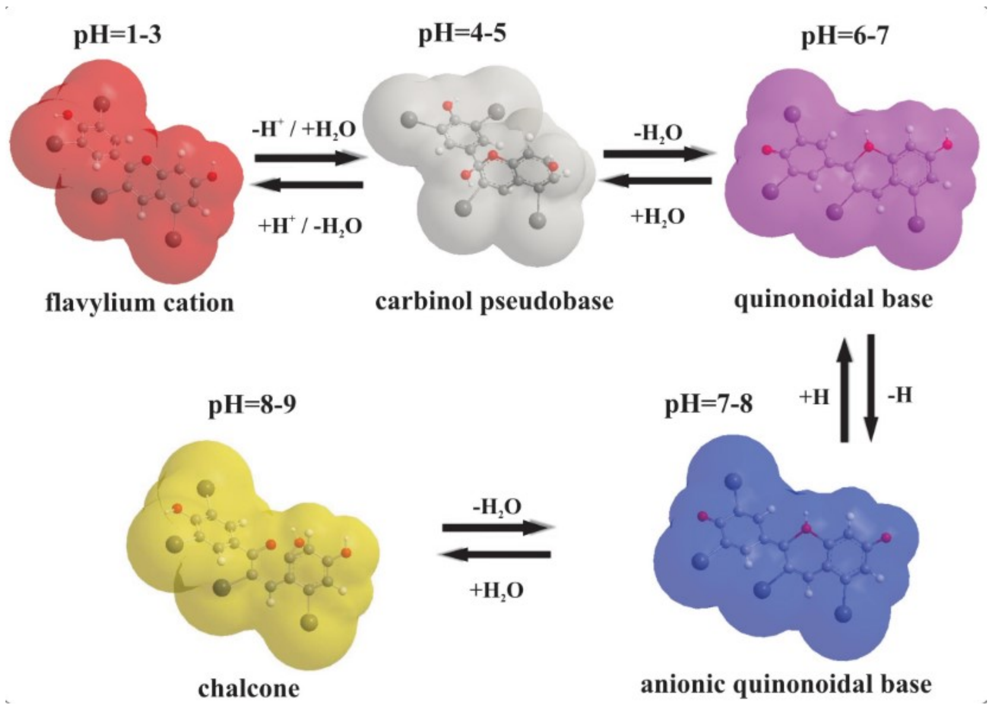

Figure 5. *Structural transformations of anthocyanins in aqueous medium with different pH (Ananga et. al, 2016)*

5. EDIBLE FLOWERS: CONSUMPTION AND FACTORS RELATED TO THEIR CULTIVATION, PROCESSING AND USE

5.1. Consumption and factors affecting consumer's preference

According to the studies reviewed, the main quality attributes that drive consumers when choosing edible flowers are appearance, aroma, and color. Color is a critical factor in the choice of ornamental flowers and some authors suggest the same behavior for the consumption of edible flowers (Pires, Dias, Barros, Calhelha, et al., 2018). Simoni et al. (2018) conducted a sensory analysis to test the acceptance of products containing irradiated edible flowers as an ingredient. The authors tested tree salads containing *B. variegata* flowers regarding flavor, color, texture, and overall aspects. The results showed that the untreated flowers had better characteristics in terms of acceptability, with better color and texture. The color of the flowers is closely related to the presence of anthocyanins, which provide an advantage both in nutraceutical terms (as a source of bioactive compounds) and in terms of color and appearance. According to Takahashi et al. (2020), there are two main factors driving consumers to consume flowers as food. The first one is visual perception, with color being the first sensory attribute observed by the consumer. As flowers are much pigmented, colors arise curiosity, the second deciding factor. Only after eating the consumer adhere to other sensory aspects such as taste, aroma, and texture. In addition, Kelley et al. (2001) suggests that potential consumers are often concerned about the quality of the flowers and the treatments they have undergone during cultivation.

Edible flowers are part of a niche market and from a strategic point of view, the inclusion of edible flowers in our diets could be a way to differentiate food products while giving color, aroma, and phytochemicals. Some consumers may not be attracted to certain products because of some sort of barrier to new foods, while in others curiosity about new foods may be one of the main reasons for the choice. Edible flowers, although they have been consumed since ancient times, are considered new foods, and analysing the factors that influence consumer attitudes can be helpful in better understanding their market, consumption and application. Chen and Wei (2017) test a model of predictors of attitudes toward the consumption of edible flowers by sampling 357 consumers in Taiwan. The researchers confirm what has been found in the literature regarding choices related to the consumption of novel foods. The study shows that in the case of edible flowers, specific curiosity has the greatest influence, followed by the floral aroma. Besides that, consumer health consciousness (through a healthy and caring lifestyle) has important effects on attitudes toward the consumption of edible flowers, and it is possible that those who are more informed about floral food enjoy floral food more than the less informed. This study also points out that the taste of edible flowers is often bland, in contrast to their aroma, so it is uncertain whether consumers will continue to appreciate edible flowers continuously once they have tasted them. In addition to this, since the reasons for purchase may be different, it would be useful to understand whether consumers who prefer edible flowers for health reasons are less likely to be disappointed than consumers who prefer edible flowers for their aroma or curiosity.

Rodrigues et al. (2017) explore attitudes and consumers' representation of edible flowers, investigating how foods made from flowers were perceived by consumers. The study was conducted in Brazil and involved 549 consumers. The test was conducted through a free word association task, asking participants to give their opinions about foods made with flowers and, more specifically, about yogurts made with flowers. For the former, the use of flowers as food reflects a condition of "good for health" while for the specific product it is perceived as a novelty and unexpected. In addition, the rejection toward food products containing edible flowers was negligible showing a remarkable propensity toward consumption of these innovative products. In general, most of the recent work shows a promising expansion of flower consumption culture, and moreover, greater knowledge of their health benefits will increase the use of flowers beyond decorative purposes.

5.2. Market overview

Given the growing popularity, the market for these products is expected to grow in the coming years and many companies are turning to this new market possibility. Many nursery companies are beginning to see flowers as a food product and not just an ornamental one, thus diversifying their productions in order to respond effectively to the competitiveness of the sector and the increased demands of consumers (Falla et al., 2020). Unfortunately, this thesis work, in agreement with Takahashi et al. (2020) found no statistical data in the literature regarding the market of edible flowers. Despite this, the global market of floriculture, comprising the cultivation of ornamental plants, is expected to garner USD 3.65 billion by the end of 2027 (ResearchNester, 2019), and in accordance with this, given the growing interest, the market for edible flowers is also expected to grow. According to Market Research Future's (MRFR) research, due to the strong increase in the consumption of edible flowers for their medicinal properties, the EU is expected to dominate this market in the future (Barani et al., 2022). So far, there is no authoritative list of flowers suitable for safe human consumption, and European Regulation n. 258/97 (EC) deals only with the safety of edible flowers for new foods and ingredients. In addition to this, it is important to set global standards to certify organically produced edible flowers for safe consumption (Tuladhar, 2021). The new possibilities for scaling up the production of edible flowers requires dealing with the short lifetimes through the development of new processing and preservation technologies to bring new impulse to this market (Takahashi et al., 2020).

5.3. Growing conditions of edible flowers

From an agronomic and health point of view, organic cultivation is the only way to control the traceability, safety and quality of edible flowers, and this type of produced flowers is the most suitable for consumption (Johansson et al., 2014). Organic cultivation systems are recommended for the growing of edible flowers (Harris et al., 2000) because these species mainly have a natural resistance to pests and therefore do not require significant use of pest control products or pesticides, which also makes them more suitable for consumers. In addition, the cultivation of flowering plants can have a positive impact on agricultural crops, which is important for the preservation of biodiversity and has numerous advantages for organic farming in general (Dujmović et al., 2022). Some flowers such as African marigold (*Tagetes erecta L.*), nasturtium (*Tropaeolum majus L.*), pyrethrum daisy (*Tanacetum cinerariaefolium L.*), tansy (*Tanacetum vulgare L.*) and chamomile (*Matricaria chamomilla L.*) could be useful for other plants. They can significantly increase soil fertility and act as weed suppressants, repellents (natural insecticides, so there is no need for use of chemical insecticides), or attractants for beneficial insects such as bees (Wang et al., 2007). An additional advantage of edible flowers could be that they can be easily grown even in limited urban areas, such as private gardens, terraces and balconies, giving consumers easy access to a source of phytochemicals (Łysiak, 2022). The cultivation methods used to grow edible flowers can vary depending on the species and the practices used by growers, but in general the methods are similar and can often be used across species. Falla et al. (2020) studied the production cycle of begonia (*Begonia x semperflorens*) and viola flowers (*V. cornuta*). In this case, the production timeline consists of a total cultivation period of 20 weeks for begonia and 10 weeks for viola flowers, divided into several stages. Initially, the mother plants of begonia are pruned, and the cuttings are planted in trays filled with a peat-based substrate. At this point, the cuttings are waited for rooting (in a greenhouse and under artificial lighting) and then propagation is carried out (again in a greenhouse). In the case of violet flowers, seeds were taken from the mother plant, sown in peat, watered, and waited for seedlings to propagate in the greenhouse. When propagation is finished, the seedlings are transferred to larger pots. The rooting rate is 80-90% so the seedlings are always less than the starting number. Here the seedlings grow to adulthood, after which they can follow two different paths: finish the production cycle and be sold as potted plants or be transplanted into the ground for direct sale as edible flowers. Upon reaching bloom, the flowers are harvested manually and transferred to plastic containers with an absorbent microfiber cloth to control moisture. The containers are then labeled to ensure traceability and stored at refrigeration temperature until consumption.

5.4. Edible flowers as a fresh-cut preparation or as a dried product

Edible flowers are used by consumers mainly in two ways: dried or consumed directly as fresh products. When consumed as a fresh product, packages of edible flowers should have a reasonably long shelf life, and the package should contain the necessary information on how to store and use the product (Kelley et al., 2001). Usually, the flowers are marketed using rigid plastic trays, similar to those used to store and protect perishable items such as strawberries and raspberries. Despite this, edible flowers are highly perishable products (e.g., due to loss of color and texture) and cannot be stored for long periods without processing. Presently, edible flowers must be used within 2–5 days after harvest, so they need to be transported via air transport to other areas before the expiration date (Kou et al., 2012). Therefore, it is essential to develop advanced technologies, such as MAP or edible coating, to preserve fresh flower quality and extend the shelf life of post-harvest flowers in order to reduce transportation costs. Modified atmosphere packaging (MAP) is a technology widely used by the food industry to preserve food, and in fresh fruits and vegetables, it is often used to retain respiration products within the packaging to ensure better freshness, quality, and shelf life of the product. For fresh products such as cut flowers, it is a common practice to decrease the metabolic activity of the product, which is reducing O_2 concentration and increasing CO_2 concentration (L. Zhao et al., 2019b). Although MAP is not recommended for packaging long-stemmed cut flowers, it is very suitable for packaging other edible flowers. Evaluation of this technology in carnation, lily and rose has shown promising results in extending shelf life (Aros et al., 2017). During MAP, flowers can produce ethylene that causes abscission and aging of petals, so it might be useful to combine MAP with 1-MCP. 1-MCP is a non-toxic ethylene antagonist that can bind to ethylene receptors for long periods, delaying flower senescence (Kou et al., 2012).

An additional alternative to extend the shelf life of these products is edible coating technology. Edible coatings can be used in most plant products to improve product quality and reduce economic production costs through the use of edible materials from renewable sources, such as lipids, proteins, and polysaccharides (L. Zhao et al., 2019c). The coating allows for the prevention of color loss but also moisture and gas loss, helping to better retain volatile flavor substances in the product. There are numerous studies on the coating of post-harvest and fresh-cut fruits and vegetables, but the application of this technology to edible flowers is rarely reported. Xie (2015) studied the application of a composite coating composed of soy protein isolate, sodium alginate and chitosan in Night-Fragrant flowers. The results were promising and showed that the coating could effectively maintain the sensory quality of flowers, inhibit shedding rate, decrease weight and vitamin C losses, and limit the impact of polyphenol oxidase. Fernandes et al. (2018) studied the effect of alginate coating on microbiological and physico-chemical quality of pansy flower stored at 4 °C in a refrigerated environment for up to 21 days. The shelf life of the product doubled from 7 to 14 days, and the content of tannins, flavonoids and anthocyanins was higher in coated flowers than in uncoated flowers. In addition to this, the visual appearance and color of the flowers were studied, and it was noted that the alginate coating significantly preserved the flowers compared with the control group. From the data available, it can be seen that this technology can be useful and should be taken into consideration in the development of innovative techniques for the preservation of fresh edible flowers.

The flowers maintain high vital activity after harvest, which causes rapid quality deterioration of the petals and consequent loss of the most interesting attributes such as color, appearance, and aroma. The extremely short shelf-life limits the commercial use of edible flowers, so it is possible to dry them in a more stable form through different drying technologies. Drying can inhibit enzymatic and microbial degradation activity, and compared with fresh flowers, dried flowers have the advantage of being easier to handle and store. It also helps to reduce transportation and storage volumes and reduces packaging costs (Jin et al., 2018). In addition, it is safer to consume dried flowers than to consume the fresh petals directly, because the latter have a high-water content and microorganisms proliferate easily. More conventional methods include natural drying and hot air drying, both of which are characterized by their simplicity and low production costs. However, these are methods with some major critical issues. Natural drying can be conducted at the place of origin but is strictly dependent on the climate, takes too long and can cause contamination (Zhang et al., 2017). Hot air drying, on the other hand, has the problem of subjecting the product to high temperatures for long times, causing loss of aroma and a significant reduction in total flavonoid content and consequent loss of color and nutritional value (Bae & Lee, 2008; Reyes et al., 2010; Sirithon et al., 2012). Modern drying technology adopts new drying equipment and more advanced drying technology, especially freeze, vacuum, microwave and infrared drying which greatly improves the quality of flowers (Zheng et al., 2015; Siresha et al., 2016; Jangam and Mujumdar, 2017). These techniques have higher costs but due to lower temperatures cause a lower loss of aroma and nutrients, greatly improving the quality of treated flowers (L. Zhao et al., 2019b). Although the various new techniques available are generally better than traditional ones, it is difficult to match the quality characteristics that only freeze-drying can provide to dry products, such as rehydration, color, and texture (Barani et al., 2022). The advantages in quality, storage, transportation costs, and shelf life of flowers are obvious, but the operational costs and initial investment of freeze-drying are high and cannot always be balanced. To reduce operating expenses and improve overall efficiency, techniques involving the combination of two or more processing operations or drying systems have been developed in recent years (Atuonwu et al., 2013). Combining drying techniques with other drying or pre-treatment techniques, both thermal and nonthermal (e.g., ultrasound treatments) allows for improved quality and shelf life of flowers, reducing treatment time, costs, and the environmental impact of the processes. The flowers, once dried, can be used for the preparation of drinks, herbal teas, recipes, or alternative food preparation. From a commercial point of view, dried flowers are fragile and must be handled with care in order to ensure their quality. It should also be considered that dried flowers are highly hygroscopic and in the presence of water can be a suitable medium for the growth of molds and microorganisms. For this reason, appropriate packaging should be provided that can inhibit or minimize the passage of water through the packaging (Barani et al., 2022).

5.5. Use of anthocyanin extracted from edible flowers as food colorant

Beyond their more common possible uses as food or beverages (e.g., salads, soups, teas, tisane, cocktails, bakery products, etc), edible flowers usually have a relevant anthocyanin content that could make them an alternative source to use as a natural dye during food development. As early as 2004, Sowbhagya et al., studied the application of pigments extracted from *Calendula officinalis* to be applied as natural dyes, emphasizing that with the growing legislative restrictions on the use of synthetic colors, a reappraisal of natural plant pigments is taking place intending to use them as possible colorants in foods (Sowbhagya et al., 2004). During the processing or storage of many foods, color loss often occurs, so food industries often use of both natural and synthetic dyes. Synthetic dyes are commonly used by the food industry because, in general, they are more physically and chemically stable, and have greater coloring power, but most importantly, they cost less than natural dyes. However, the recent concerns about the safety of artificial colorants in food products has encouraged the development and application of natural colorants, which are generally considered safer than artificial ones (Pop et al., 2010). In addition, natural additives are the current trend in the food industry because of the health benefits of many of these compounds (Faustino et al., 2019; Isabel et al., 2019). The natural dyes market is a growing industry, and the annual growth rate between 2018 and 2019 was 8.5% (Jędrusek-golińska et al., 2022). Anthocyanins extracted from edible flowers could be an excellent natural dye giving food products colorings such as red, blue, and purple color. As described earlier, there are multiple types of anthocyanins (e.g., pelargonidin, malvinidin, etc.), but beyond the type, anthocyanins in general are reactive and therefore unstable compounds. This reactivity is because the flavylium cation nucleus experiences a lack of electrons and its color is easily degraded (Nurtiana, 2019). Anthocyanin is stable under conditions of acidity, low temperature, absence of oxygen, light, and enzymes such as polyphenol oxidase. The extraction of anthocyanins from plant products can be done by several techniques, and the most popular is the solvent extraction method. It is noteworthy that the purity and quality of these color compounds are strongly linked to the extraction technique employed and the process conditions thereof (Shantamma, Monica, et al., 2021). From a regulatory point of view, anthocyanins are authorized food colorants (E163 in the EU) and have previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1982 and by the EU Scientific Committee for Food (SCF) in 1975 and 1997 (Rodriguez-amaya, 2016). Pires et al. (2018) studied the incorporation of natural dyes obtained from edible flowers in yogurts. In this study, the hydrophilic extracts of rose, cornflower, and dahlia were tested as potential substitutes for E163 (anthocyanin extract). Yogurt is usually stored in the dark, at low temperatures, and typically has an acidic pH (around 4.5), so this is an interesting matrix where there are prerequisites for the stability of anthocyanins. This work aimed to develop a new coloring strategy in yogurt products using natural anthocyanin-rich extracts obtained from edible flower petals of *Dalia mignon, Centaurea cyanus L.,* and *Rosa damascena "Alexandria"* mixed with *Rosa gallica "French"* draft in *Rosa canina*. The study found that rose extract was the most suitable alternative to E163 because, in addition to having similar nutritional characteristics, it has similar values in measured color parameters. The extraction of anthocyanins to use them as a natural dye has also been conducted in other flowers such as *Crocus sativus* (Ana et al., 2021), *Hibiscus sabdariffa* (Pinela et al., 2019), *Clitoria ternatea L.* (Salacheep et al., 2020), *Akebia trifoliata* (Jiang et al., 2020). In addition, some anthocyanins could be a possible source of natural blue dyes that can be used by the industry to replace synthetic ones. All blue synthetic colorants (e.g. E131, E132, E133) present a risk to the consumer's health if consumed above the established dosages (Landim Neves et al., 2021). Blue is the natural dye that probably poses the greatest challenge because it is very rare in nature and the sources from which to obtain it are limited. There is no natural blue colorant with the same physical and chemical stability, coloring power, and easy scale-up production in the market yet, compared to the synthetics (Landim Neves et al., 2021). Natural sources of blue pigments include many flowers such as *Gardenia jasminoides*, *Indigofera*, and *Allium.* Blue flowers generally contain high amounts of anthocyanins and a pH of around 6-7 that allows the anthocyanins to get this color. Despite this, the use of anthocyanins, possibly extracted from some edible flowers, as a blue dye is a challenge that science is trying to solve. Blue anthocyanins are extremely unstable and need specific conditions in order not to lose their color. Sigurdson et al. (2017), studied obtaining blue colorant from anthocyanins, and they found that chelating metals and a pH between 5 and 7 are necessary to maintain the color. However, they found that after 60 days at 23°C, the blue color completely disappeared, showing extreme physical instability. Given its high instability, it will be a great challenge for the food industry to find suitable chemical and physical conditions for maintaining the blue color. Alternatively, its encapsulation in biopolymer matrices can minimize its exposure to pH changes, light, oxygen, high temperatures, and interactions with undesirable co-pigments during processing and storage (Bryła, 2015; Gabriela et al., 2016; Moura et al., 2018; Reis et al., 2018).

6. EXTRACTION OF ANTHOCYANINS FROM EDIBLE FLOWERS

Extraction is the first fundamental step in the purification and recovery of interesting substances from plant materials such as bioactive compounds or pigments. Anthocyanins fall into both categories, so evaluating the best methods for extracting anthocyanins from edible flowers is very important for subsequent application. Extraction methodologies could be several, and the different extraction conditions affect on extraction yields of anthocyanins from flowers and plant tissue in general. Many techniques for extracting anthocyanins from edible flowers have been developed. Anthocyanins are polar molecules, which allows them to be extracted using various polar solvents commonly used for polyphenol extraction such as methanol, ethanol, water, and acetone. On the other hand, considering food, pharmaceutical and environmental needs, more environmentally friendly solvents such as water, ethanol, or a mixture of the two are increasingly being used (Barani et al., 2022). In recent years, much attention has been paid to obtaining more environmentally friendly products, and this chapter will also look at these important aspects.

6.1. Solvent extraction as a traditional approach

Solid-liquid extraction of anthocyanins is traditionally the most widely used method for the extraction of anthocyanins, as it is easy to perform and does not require any special equipment. As previously described, the polarity of anthocyanins allows them to be easily extracted through the use of organic solvents such as ethanol, methanol, and acetone with or without the addition of weak organic acids such as citric acid, formic acid, and acetic acid, or low concentrations of strong acids (0.5−3.0% for trifluoroacetic acid and <1.0% for hydrochloric acid), which facilitates the solubilization of anthocyanins (S. Hosseini et al., 2016). Also, heat aids the extraction of anthocyanins; in fact, temperatures between 20°C and 60°C are often used to facilitate extraction. Much higher temperatures are hardly ever used as studies are showing that many anthocyanins begin to degrade significantly when the temperature exceeds 70°C (Patras et al., 2010). High temperatures could be used with short extraction times to minimize anthocyanin degradation. Methanol and acetone (mixed or not with water) are among the most commonly used solvents for the extraction of anthocyanins, but low-impact solvents are often used today that have less impact on the environment. There is a tendency to use solvents that are more environmentally sustainable but still interesting in terms of extraction yields; and water, ethanol, or their mixtures are still effective (Barani et al., 2022). Tri Nuth Pham et. al (2019) applied conventional solvent extraction methods using ethanol to extract anthocyanins from butterfly pea flowers (*Clitoria ternatea L. Flowers*). The above study used dried petals of the butterfly pea, which were ground and extracted using an ethanol 50° solution. They subsequently proceeded with centrifugation, collection of the supernatant, and filtered extract. Finally, monomeric anthocyanin was calculated as cyanidin-3-glycoside using a differential pH method. The paper showed that the highest anthocyanin level was obtained using 50% of ethanol (143.49 mg/L), while the lowest was obtained with water (90.9 mg/L). The temperature was found to be decisive in increasing the extraction yield and the best condition was seen at a temperature of 60°C. The optimum extraction yield, in this case, was found when the liquid/solid ratio until equilibrium arrived at the optimal ratio of 25:1 (mL/g).

However, recent studies show that anthocyanins can also be successfully extracted using only water as a solvent by varying the extraction conditions. As described above, solvent extraction is known to be environmentally unfriendly, but also expensive and sometimes linked to toxicity. Jeyaraj et al. (2021) found that in the extraction of anthocyanins from *Clitoria ternatea* flowers, heat-assisted water extraction was efficient and had similar extraction efficiency as the best solvent extract (50% ethanol), suggesting that this approach could be equally effective, more environmentally friendly, economical, and suitable for food applications than organic solvent extraction. Aqueous solvent extraction involves the use of high temperatures, even close to 100°C, but this does not cause anthocyanins degradation, they often remain stable despite the high temperature, and this is probably due to the high stability in the triacylated form. This may be an advantage over many other anthocyanin sources that degrade instead, allowing for potential application as a functional ingredient or as a natural dye, given the resistance to high temperatures during food processing.

6.2. Alternative techniques for anthocyanins extraction

Typically, solvent extraction processes require heat and a high extraction time. In a large-scale, industrial application, this leads to high energy consumption, increased costs, and a high environmental impact. Therefore, the development of new methods for extracting anthocyanins is a key focus in the industrial applications of these compounds. In recent decades, the trend has been to develop extraction techniques in line with 'green technology, allowing lower solvent consumption, faster extraction times, and better extraction efficiency. These techniques also meet the demand of consumers in terms of greener products, sustainable energy, and environmental protection. The use of non-thermal technologies for the extraction process has been widely studied, such as the application of ultrasonic-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and other extraction technologies.

6.2.1. *Ultrasonic-assisted extraction (UAE)*

Ultrasonic-assisted extraction (UAE) was considered an efficient and green technique for extracting anthocyanins from edible flowers. Ultrasound with frequencies ranging from 20 kHz to 50 kHz can produce cavitation, vibration, shattering, mixing, and other effects that can break down cell walls and enable the successful extraction of compounds such as anthocyanins (Wen et al., 2018). Mushollaeni & Tantalu (2021) successfully extracted anthocyanins from the flowers of *Hibiscus sabdariffa l.* using the UAE method, and the best-operating conditions for the extraction process were observed at a frequency of 24 kHz, a temperature of 40°C, and an extraction time of 5 minutes. It would be possible to increase yields by increasing extraction times and temperatures, but this could damage the pigments to be extracted. Ultrasonic-assisted extraction is widely used for the extraction of bioactive compounds from plants and flowers, and the results of various studies demonstrated that this system is rapid, convenient, and effective.

6.2.2. Microwave extraction (MWE)

This extraction technology involves the use of microwaves which cause continuous molecular movement and stirring of liquids leading to an increased temperature. It is a fast and efficient alternative extraction method for the extraction of analytes from solid matrices, especially secondary metabolites from plant materials (Barani et al., 2022). This is an efficient and modern tool with multiple benefits as compared to the traditional methods of extraction, such as reduction in cost, time of extraction, amount of solvent used, energy consumption, and low CO_2 emission (Akhtar et al., 2012). Microwave extraction technology is widely used in the extraction of bioactive substances such as anthocyanins from edible flowers. Pimentel-moral et al. (2018) used MWE for the extraction of bioactive compounds from *Hibiscus sabdariffa*, and Marsin et al. (2020) used this technique to extract anthocyanins from blue pea flowers (*Clitoria ternatea*), suggesting better retention of the blue pigment in the final product.

6.2.3. Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is based on the use of supercritical solvents with different physicochemical properties, such as density, diffusivity, viscosity, and dielectric constant. A substance goes into a supercritical state when it is forced to pressure and temperature above the critical point. Due to the low viscosity and relatively high diffusivity of the supercritical fluid, it has a stronger transport performance than the liquid and is more easily diffused in the solid material, which ultimately increases the extraction speed (L. Zhao et al., 2019a). The consumption of toxic solvents is drastically reduced compared to traditional extraction techniques and also has better selectivity (Justyna et al., 2017). The most commonly used supercritical fluid is CO2 for several advantages such as cost-effectiveness, non-toxicity, and convenience in operational terms. Despite being an innovative and promising technique, the extraction of bioactive substances from edible flowers with supercritical fluids has not yet been extensively developed, and more studies are needed to improve this technique for the extraction of bioactive substances or pigments such as anthocyanins.

6.2.4. *Other extraction methods and considerations*

There are other novel extraction methods (figure 6) such as pressurized liquid extraction, pulsed electric field (PEF), thermo-mechanical transient controlled voltage drop (DIC), ohmic accelerated steam distillation (OASD), and ohmic-assisted hydro-distillation (OAHD). Depending on the type of these techniques, they could be used either as the main extraction method or as a pretreatment before other extraction techniques (Ameer et al., 2017; Kumari et al., 2017; Rajbhar et al., 2015). For example, (Tzima et al., 2021) used the PEF technique as a pretreatment step before UAE of polyphenols from fresh rosemary and thyme byproducts, with excellent results. In fact, because of the ability of the pulsed electric field to electroporate cell envelopes, it could be used as a pretreatment to facilitate the recovery of polyphenols using a subsequent conventional or novel extraction method (Tzima et al., 2021). Furthermore, the usage of organic solvents or water mixtures in combination with some nonthermal extraction methods could provide promising results (H. Hosseini et al., 2018; Tzima et al., 2021). Analyzing the available studies about extraction methods of anthocyanins from edible flowers emphasizes the need to have a direct comparison between different techniques under the same conditions and with the same sample, so there is no ideal extraction system and conditions for anthocyanin extraction (Barani et al., 2022). In general, as seen above, traditional extraction with organic solvents has some disadvantages, such as longer times, high environmental impact, and the use of hazardous chemicals. Therefore, it is critical to study the efficiency of mild technologies in the extraction of anthocyanins from edible flowers. Another aspect that must be taken into consideration is the control of polyphenol-oxidase during the extraction process, a crucial point in maintaining the color and nutritional characteristics of the extracted anthocyanins. The most appropriate method should be selected by taking into consideration several important factors, including the production target, costs, effect on PPO, yields of extraction, environmental impact, and the characteristics of the sample.

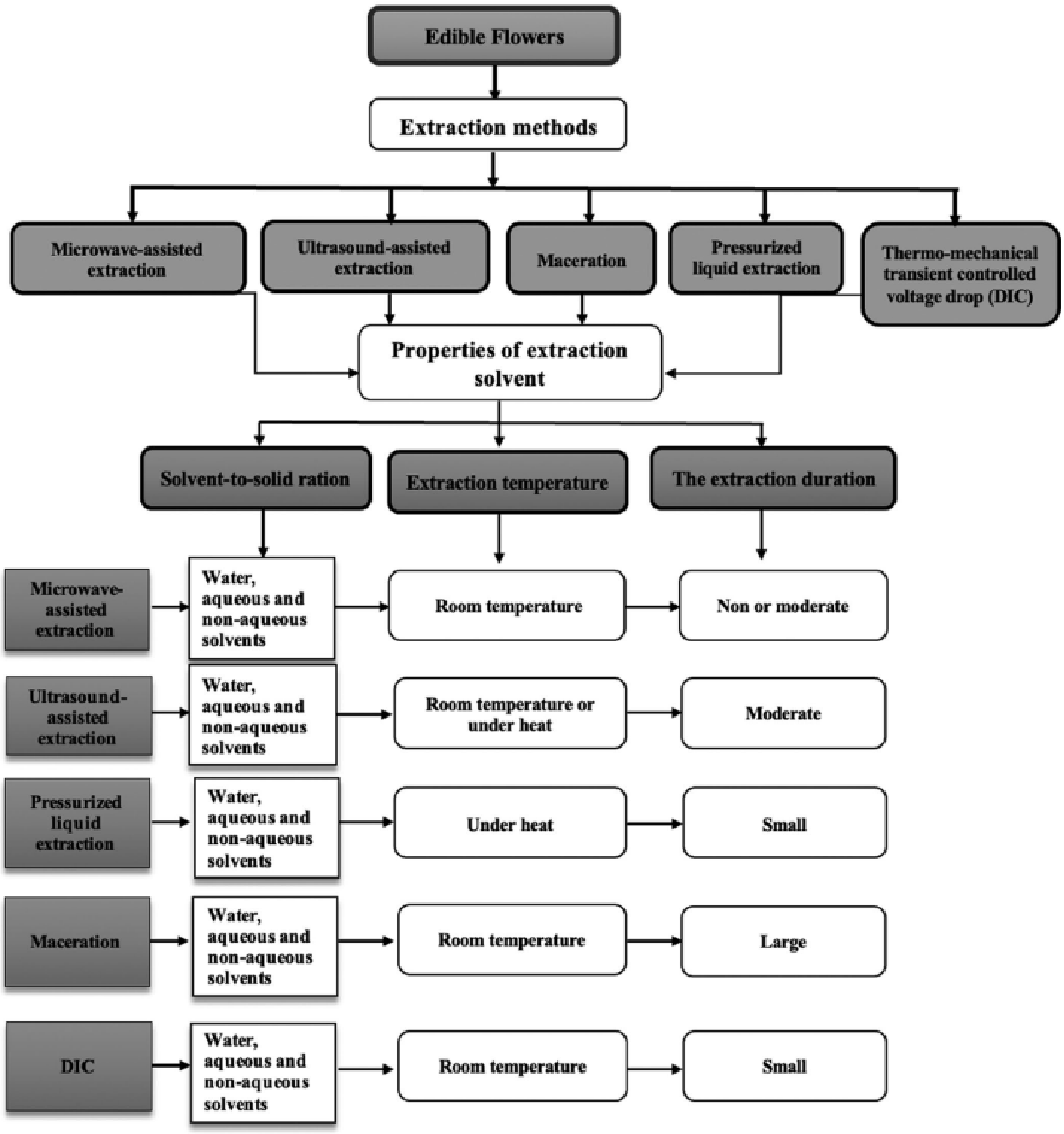

Figure 6. *Mechanism and properties of various extraction methods (Barani et al., 2022)*

7. POLYPHENOL-OXIDASE AND COLOR VARIATION IN EDIBLE FLOWERS

7.1. Properties and physiological functions of polyphenol-oxidase

Polyphenol-oxidase (PPO), also known as tyrosinase, cresolase, polyphenolase, phenolase, catechol-oxidase, or catecholase, is a generic name for a group of enzymes typically present in most flowers, plants, and fruits (Yoruk & Marshall, 2003). It is copper-containing oxidoreductase, belonging to group 1 of the European Classification. PPOs are essential oxidases in biological systems, where they are involved in defense mechanisms, biosynthetic processes, polymerization, and detoxification of plant phenolic compounds (Polak et al., 2020). PPOs are divided into three different groups:

- Tyrosinases (E.C. 1.14.18.1; monophenol mono-oxygenase)
- Catechol oxidases (EC 1.10.3.1; oxidoreductase also known as o-diphenol oxidase)
- Laccases (EC 1.10.3.2)

Although they belong to different groups, all polyphenol-oxidases have an overlapping spectrum of substrates (Faccio et al., 2012), oxidizing phenolic or polyphenolic compounds, particularly flavonoids (e.g. anthocyanins), which regulate all aspects of plant life (Pourcel et al., 2006). These enzymes are primarily responsible for browning reactions, which assume a major role in plant defense against biotic and abiotic stresses. In the case of tissue injury, a melanin layer is formed that acts as protection against microbial pathogens (Mayer et al., 2006). Melanin formation increases cell wall resistance to insect and pathogen attacks (*War et al., 2012*). In addition, quinones produced by the enzymatic reaction play a role in the digestibility and palatability of plant tissues as they covalently bind to leaf proteins and hinder protein digestion by invading insects (Constabel & Barbehenn, 2008).

7.2. Characteristics and mechanism of action of polyphenol-oxidase

Beyond physiological functions in plants, enzymatic browning reactions cause problems mainly in terms of color alteration and nutritional losses, so it is receiving a lot of attention from food science. The substrate of PPOs is phenolic compounds, typically contained in the vacuole of plant cells, and anthocyanins fall into this category. As phenolic compounds are the substrate of this class of enzymes, antioxidant activity can also be reduced by PPO activity. The enzymatic activity of PPO proceeds in two steps (Figure 7):

1) Cresolasic activity, where the PPO catalyzed the hydroxylation from monophenol to ortho-diphenol (with the formation of H_2O).

2) Catecholasic activity, where the previously formed ortho-diphenol is oxidized to ortho-benzoquinone.

Polyphenol-oxidases have oxygen as a cofactor, without which they cannot conduct the catalysis reaction, so both of the above steps must be conducted in the presence of O_2. The previously formed benzoquinone is unstable, tending to bind to other compounds and generally polymerizing or reacting (non-enzymatically mediated reaction) leading to the formation of brown compounds called melanins. The enzymatic browning leads not only to color alteration and antioxidant degradation but also organoleptic and nutritional losses because of the quinones' condensation with other compounds such as amino acids, proteins, phenols, and sugar (Queiroz et al., 2008). While phenolic substrates are found in the vacuole of plant cells, PPOs are found dispersed mainly in cytoplasmic organelles. This means that under normal conditions enzyme and substrate do not come into contact, so the browning reaction cannot take place. When plant cells, in the presence of O_2, are damaged during anthocyanins extraction, or by drying, peeling, trituration, or rupture, enzyme-substrate contact is made possible, and the catalysis reaction begins. The browning degrees vary with phenolic compounds, reducing substances, oxygen concentration, metal ions, temperature, pH, and kind of PPO (H. Sun et al., 2012). As a result, enzymatic browning reactions conducted by polyphenol-oxidases reduce the anthocyanin content of plant products, such as edible flowers, significantly altering the color while reducing shelf life and commercial value.

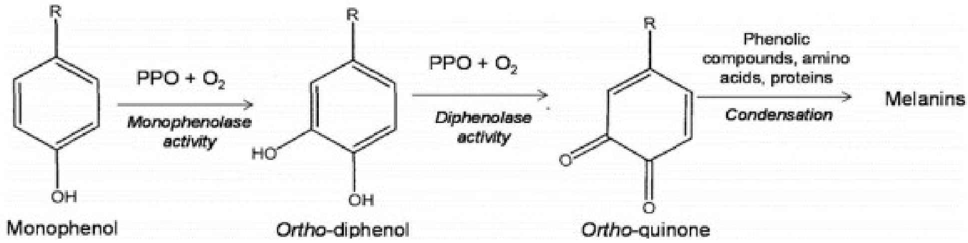

***Figure 7**. Browning reaction of polyphenol-oxidase*

7.3. Relationship between polyphenol-oxidase and anthocyanins

In contrast to the detailed molecular knowledge available on anthocyanin synthesis, little is known about its catabolism in plants. The mechanisms of enzymatic degradation of anthocyanins in plants are not yet fully clear, but three enzyme families have involved in this process: polyphenol-oxidase, class III peroxidase, and β-glycosidase (Luo et al., 2017; Luo et al., 2019). The collective name for this group of enzymes is anthocyanases and it is worth noting that glycosidases directly affect anthocyanins, while peroxidases and polyphenol-oxidases have indirect effects on their stability (Marszałek et al., 2017). Specifically, glycosidases break the covalent bond between the glycosyl residue and the aglycone of an anthocyanin, leading to the formation of unstable anthocyanidins. At this point, polyphenol-oxidases and peroxidases, which are plant-specific enzymes, once removed from cellular compartments (e.g., anthocyanins extraction or cells rupture), accelerate the degradation of these compounds. As described in the previous section, polyphenol-oxidase is the enzyme that will catalyze two types of reactions: o-hydroxylation of monophenols in o-diphenols and oxidation of o-diphenols in o-quinones. Quinones are highly reactive electrophilic compounds that can covalently alter nucleophilic molecules like anthocyanins to produce brown pigments known as melanin. Polyphenol-oxidases and peroxidases are mainly located in the plastids of photosynthetic and non-photosynthetic tissues, or the cytoplasm, so it is unlikely to degrade anthocyanin in living tissues, in which no cell decompartmentation occurs (Y. W. Zhao et al., 2021). In summary, it is assumed that the first enzyme that affects the stability of anthocyanins is β-glucosidase, which forms anthocyanidins that can be further oxidized by polyphenol-oxidase and/or peroxidase (Marszałek et al., 2017). Following the action of these enzymes, the solubility of anthocyanins decreases, and their transformation into colorless compounds takes place, thus losing the typical color intensity of these pigments (Enaru et al., 2021).

7.4. Advances in the research on the effect of PPOs on anthocyanin's degradation

Due to the problems mentioned above, the activity of PPOs on plant products has been extensively documented in recent decades. Examples of fruits, flowers, and other fresh or processed plant products with high anthocyanin content are numerous, and each of these can be affected more or less by the action of polyphenol-oxidases. Blueberry juice is an example of a product that undergoes heavy browning after processing and cold storage. Ning et al. (2019) studied the anthocyanin content and color change in high hydrostatic pressure (HHP) blueberry juice due to endogenous polyphenol oxidases, then looked at the effect that different antioxidants could have. The color was expressed in L*, a*, and b* values, and the total color difference (ΔE) in the various samples during the storage period was calculated. After storage of 4 days, the control juice had a ΔE of 2.30, indicating a noticeable change. At the same time as measuring color, a measurement of anthocyanin content was conducted in the control juice, which dropped by 36% in 4 days. However, after adding different antioxidants in the juice (ascorbic acid, gallic acid, ferulic acid, tea polyphenol, and α-tocopherol) at 1 g/L, the ΔE values were within the range of 0.5 ∼ 1.5, indicating a protective effect against color change. The degradation of the anthocyanins of HHP juice during storage might be due to the incomplete inactivation of polyphenol-oxidase by the HHP, leaving them to react with unstable anthocyanins in the juice and form quinones, leading to browning and a decrease in the anthocyanins content (Aaby et al., 2018).

A large part of the research on the degradation of anthocyanin by polyphenol-oxidase has been mainly conducted in litchi (Litchi chinensis Sonn.). Litchi is a typical fruit from China, but now worldwide cultivated, with high anthocyanin content in the pericarp. After harvesting, the pericarp tends to change its color from a pink/red typical of the species to brown, accompanied by a certain degree of anthocyanin degradation. To elucidate enzymatic anthocyanin degradation in litchi, anthocyanin degradation enzyme (ADE) was obtained by purification and identified as laccase (LAC). Epicatechin was found to be the favorable substrate for the ADE/LAC. It was noted that there is a relationship between the degradation of epicatechins and anthocyanins content that suggests an ADE/LAC epicatechin-coupled oxidation model. This model was supported by a dramatic decrease in epicatechin content in the pericarp parallel to anthocyanin degradation. It was therefore concluded that the ADE/LAC is responsible for epicatechin-mediated anthocyanin degradation in litchi fruit pericarp (Fang et al., 2015; X. Zhang et al., 2018).

Truong et al. (2019) have studied the content of phenolic compounds in the processing of anthocyanin-rich juice and co-product recovery from purple-fleshed sweet potatoes. As expected, the presence of polyphenol-oxidases correlates with browning and reduced anthocyanins content. This drops the commercial value and consumer suitability of the product, due to the loss of color and important antioxidant properties related to the presence of anthocyanins. The study showed that acidification with citric acid proved to be a successful technique in controlling polyphenol-oxidase in purple-fleshed sweet potatoes resulting in the attractive reddish color of anthocyanin-rich extracts. Furthermore, with high polyphenolic content, sweet potatoes juice can be used as a functional ingredient in beverages and nutraceutical products.

There are many other articles describing the effect of PPOs on anthocyanins, with results often in line with each other, highlighting, in most cases, that the action of these enzymes cannot be overlooked. Although the impact of PPOs in plant-derived products is often problematic, there are still cases where they appear to have little effect in terms of color retention and anthocyanin loss during the process or shelf life. Teribia et al. (2021) revisited the role of polyphenol-oxidases in color retention in strawberry puree under storage conditions. Total anthocyanin content was monitored after 42 days of storage and a loss of 95% was observed. However, before the storage, a complete polyphenol-oxidases inactivation (activity below the limit of detection) was observed for all the samples after pasteurization, and this suggests that the impact of these enzymes on anthocyanin and color change during the shelf life of the puree may be limited, and non-enzymatic reactions should be considered as the main pathway for anthocyanin degradation. Therefore, in some cases, non-enzymatic reactions could play an important role in browning during the shelf-life of some products and should be further studied. Despite this, it is widely recognized that most plant products with high anthocyanin content suffer from the effect of PPOs during their storage, so there is a need to study and develop methods to solve this problem or, at least, keep it under control. Consequently, research is focusing primarily on treatments that can be carried out to effectively inactivate this class of enzymes. A better understanding of these issues, along with the development of the best techniques is crucial for eliminating or keeping under control color alteration in plant products such as edible flowers, as well as for avoiding other negative effects (e.g., loss of antioxidant activity and other benefits of bioactive compounds). Methods and technologies to inactivate polyphenol-oxidases and prevent anthocyanin decrease will be discussed in the following chapter.

8. PROGRESS OF PROCESSING TECHNOLOGIES TO INACTIVATE POLYPHENOL-OXIDASE IN EDIBLE FLOWERS

Food science is particularly interested in inhibiting the action of PPOs, and in developing different methods to ensure such inhibition to improve the stability of plant products. As analyzed above, inhibiting the action of these enzymes would ensure better color retention by improving the consumer acceptability of the product. Edible flowers are hardly commercialized as a fresh product, and drying is one of the effective ways to reduce the moisture content and prolong shelf life, but, despite this, the drying process can also cause color change and the fading of flowers (Ran et al., 2021). Many flowers contain significant amounts of anthocyanins, which are responsible for the color of the flowers and their antioxidant properties. Anthocyanins show low stability during processing or storage, and it was demonstrated that could be influenced by temperature, moisture content, pH, oxygen, enzymes, metal ions, and other factors. Edible flowers are very perishable, because of their high anthocyanin instability, and without processing cannot be preserved for a long time. Thus, pre-treatment was necessary before drying to inhibit browning and maintain the content of anthocyanins in fruits and vegetables (Doymaz, 2009). Thermal or non-thermal processing can be used to inactivate polyphenol oxidase. Many of the systems used to control browning and color losses include physical and chemical methods to inhibit PPO activity by focusing on the essential components for a reaction such as oxygen, copper ion, substrate, products, or even the enzyme itself (Queiroz et al., 2008). There are traditional and more innovative methods to process the flowers but it should be considered that today's trend and also customers tend to encourage the usage of methods that are natural and environmentally friendly (O.Y. Alabbasy et al., 2021). In addition, to apply anthocyanins as a natural pigment, it is necessary to extract them from their matrix. The polyphenol extraction process brings the polyphenols themselves into contact with PPOs, causing their oxidation. Therefore, it is essential to provide appropriate solutions to reduce the inconvenience of PPOs and prevent the oxidation of anthocyanins during the extraction process. All the methods used to inactivate PPOs are described and discussed in detail in the next sections.

8.1. Conventional heating pre-treatment

Thermal technologies are the most commonly used physical-based preservation systems for processing plant products and can also be used as a pre-treatment before drying in edible flowers. These treatments are a classic method for destroying bacteria and inactivating enzymes. Blanching is a classic example of heat treatment and it is usually applied before processing, such as freezing, drying, frying, and canning (Barani et al., 2022). It is a typical primary step in vegetable processing and can be briefly described as the process of heating vegetables to a high enough temperature to destroy enzymes in the tissue (Q. Chen et al., 2015). Thermal processes such as blanching cause the inactivation of enzymes such as PPOs but also stabilizes the color of flowers and reduce subsequent drying and dehydration time. In accordance with Pimpaporn et al. (2007), the main objectives of the blanching process in plants products are as follows:

- to inactivate enzymes and quality deterioration
- to enhance product quality and drying rate
- to minimize non-enzymatic browning reactions
- to remove pesticide and toxic materials

Certain flowers such as daylilies present toxicity problems when eaten fresh, and blanching has proven to be an excellent technique to make their consumption safe. Due to the improperly edible methods of daylily flower, many poisoning cases were reported every year in humans and animals, which were at risk for gastrointestinal distress and acute renal failure (Poppenga, 2010). In the case of daylily flowers, the problem is colchicine, an alkaloid that can be easily destroyed during the blanching process, rendering the flowers edible (Hong et. al, 2003).

8.1.1. *Hot water blanching*

Hot water blanching is defined as the most used and commercially adopted blanching method, because of its simplicity and ease of carrying out. In a typical hot water blanching, products are immersed in hot water (70 to 100 °C) for several minutes, and then, blanched samples are drained and cooled before being sent to the next processing operation (Xiao et al., 2017). In the flowers, the process is the same, with time-temperature combinations that may differ from case to case. Despite the ease of conducting the process, hot water blanching has several limitations such as the consumption of high amounts of water and energy. Besides this, to preserve the color of the product and inactivate microbial activity, sodium sulfite and sodium metabisulfite are often added to the blanching water (Xiao et al., 2017). Hot water blanching cause also the loss of water-soluble nutrients such as vitamins, minerals, and sugars, due to leaching or diffusion, from plant tissues to blanching water (Pimpaporn et al., 2007). Numerous studies showed also that blanching in boiling water causes the degradation of total phenolics compounds and loss of antioxidant activity (Arroqui, 2002; Cies et al., 2008; Gawlik-dziki, 2008; Ismail et al., 2004). Ran et al. (2021) demonstrated that blanching would lead to the degradation of pigment in rose petals due to the high temperature of hot water and the water solubility of rose anthocyanin. Nowadays, there is also a general tendency to use practices that are green and environmentally friendly. The discharged wastewater from hot water blanching contains high concentrations of biochemical, soluble solids, and chemical oxygen demand due to the leaching and dissolution of sugars, proteins, carbohydrates, and water-soluble minerals (Xiao et al., 2017). This treatment is now recognized as a water-intensive industry and wastewater can cause environmental pollution, such as eutrophication, if not well treated before discharge (Liu and Yang, 2012). The loss of phenolic compounds such as anthocyanins, together with other aspects mainly related to the environment and costs, suggests that the use of this treatment technique is not among the most suitable for inactivating PPOs in edible flowers. To alleviate these problems, it would be better to choose a more appropriate blanching method, more energy efficient and allows for better flower quality.

8.1.2. Steam water blanching

Steam water blanching alleviates in parts the problems of the traditional hot water blanching method. It is used superheated steam as a heating medium. The vapor condenses on the surface of the product to be treated, transferring a large amount of latent heat due to the temperature difference. The increasing temperature causes the denaturation of the enzymes present in the matrix. It is believed that steam blanching is relatively inexpensive and retains most minerals and water-soluble components when compared with water blanching due to the negligible leaching effects (Kumar et al., 2009). This method is often used in the inactivation of PPOs in edible flowers, it has some advantages over water blanching but on the other hand, there are also some disadvantages. There is often a need for longer heating times due to the lower heat transfer than with hot water blanching and this can cause undesirable quality changes (Xiao et al., 2017). Chen et al. (2015) studied the effect of two blanching methods (blanching in steam or hot water) in fresh daylily (Hemerocallis fulva L.) flowers. The results showed that phenolic compounds were affected significantly by blanching treatments, but steam blanching had higher antioxidant activity and compounds retained than those of hot water blanching. Steam blanching, at an industrial level, is carried out on moving belts and has often resulted in non-uniform blanching effects (Mukherjee & Chattopadhyay, 2007) as well as the fact that it needs long blanching time and therefore it affects the capacity and the economics of processing. Often steam blanching technology is combined with infrared or other novel blanching methods to make the treatment more effective, faster, and less costly

8.2. Novel thermal processing

Given the disadvantages of classical blanching processes, new blanching and heating technologies with higher energy efficiency, less nutrient loss, and lower environmental impact are being developed and applied in recent years. The most commonly used emerging heating technologies are infrared, microwave, and ohmic blanching.

8.2.1. Infrared blanching

The infrared dry blanching technology can inactivate enzymes such as PPO with high-energy efficiency and no leaching of solids and nutrients. Rastogi (2012) concluded that IR heating offers many advantages over conventional heating such as steam or hot water blanching under similar conditions, including uniform heating, reduced quality losses, and significant energy savings. Compared to the classical thermal methods described above, infrared blanching is more convenient as a pre-treatment to inactivate PPOs in edible flowers, or it may be convenient if the techniques are combined. Although the aforementioned advantages of infrared blanching, it does have some limitations such as surface deterioration due to overheating, non-uniform heating due to poor penetration, oxidation, charring due to the surface temperature of food products increasing rapidly and overheating with time, and low yields due to loss water (Xiao et al., 2017). Li et al. (2019) and Yuan et al. (2009) studied the effect of combined infrared and steam blanching on enzyme inactivation and quality of Chrysanthemum indicum L. flower. The results indicated that the color values of infrared steam-blanched, and steam-blanched chrysanthemum flowers showed no significant differences, whereas infrared-blanched and no pre-treated flower samples showed higher blue color than the fresh samples. For the enzyme inactivation, the results demonstrated that combined infrared steam blanching can be an alternative to conventional infrared blanching or steam blanching for the inactivation of polyphenol-oxidase and peroxidase in fresh plants with the added values of higher retention of bioactive compounds, better external appearance, lower evolution of Maillard reaction and shortening the processing time.

8.2.2. Microwave blanching

In microwave heating, heated materials absorb microwave energy and convert it into heat by the dielectric heating effect caused by molecular dipole rotation and agitation of charged ions within a high-frequency alternating electric field (Chandrasekaran et al., 2013). This causes the release of moisture due to the agitation of water molecules. Compared to conventional heating methods applied in the food industry, microwave heating has several advantages such as volumetric heating, high heating rates, and short processing times (Xiao et al., 2017). Moreover, compared to traditional blanching methods, the amount of nutrients lost by leaching is significantly reduced. Microwave heating can be successfully applied not only in blanching but also in drying, pasteurization, thawing, tempering, and baking, which makes it very versatile (Bai et al., 2013; Ramesh et al., 2001). To improve the quality and enhance the drying productivity of *Lonicera japonica* flowers, Li et al. (2017) study the application of microwave blanching to inactivate polyphenol oxidase before hot air drying. In *Lonicera japonica* flowers, hot air-drying causes the oxidation of phenolic acids by endogenous polyphenol oxidases that are not inactivated. Microwave blanching is a very fast method for blanching and generates a low amount of heat. In this study, microwaves were applied to rapidly inactivate polyphenol-oxidases, followed by drying at a higher temperature to reduce the drying time. The results showed that a 2-minutes microwave treatment was sufficient to completely deactivate polyphenol oxidases. Microwave-treated samples had a higher chlorogenic acid content, and a brighter appearance, and the processing will also make the product more stable during storage or extraction than samples treated only with hot air drying.

8.2.3. Ohmic blanching

In ohmic blanching, food products are placed between two electrodes behaving like an electrical resistor, in which heat is generated and the temperature of the product rises rapidly (*Sastry and Barach, 2000*). It is widely known that the frequency of the voltage determines the results of the ohmic heating. The heating rate decreases with increasing frequency, so low frequency is frequently used (Xiao et al., 2017). Compared to conventional hot water blanching, ohmic blanching requires a shorter time and it yields better product quality as it reduces solids and nutrients leaching and preserves color and texture (Himoni, 2005; Mizrahi, 1996). Moreno et al. (2013), studied the influence of combining ohmic heating and osmotic dehydration in apples and they observed a complete inactivation of polyphenol oxidase and an extended shelf life of 4 weeks when stored at 5 °C. Sarkis et al. (2013) studied the effects of ohmic and conventional heating on anthocyanin degradation during the processing of blueberry pulp. It was observed that the degradation of anthocyanins increased with the increase of voltage and solids content. In addition, when ohmic blanching under low voltage gradients, the percent of anthocyanin degradation was similar to that obtained with conventional hot water blanching or even lower. The application of ohmic heating has been studied in many other fruits and vegetables such as carrots, acerola fruit, strawberry, vegetable juices, etc. As the edible flower market is an emerging market, there is a lack of information on the application of this technology as pre-treatment in edible flowers and more studies will be required to better understand the effect on PPOs and anthocyanin's content.

8.3. Non-thermal technologies

As previously described, blanching processes, both conventional heating and novel thermal processing have proven effective in inactivating PPO in plant products and edible flowers. For a long time, thermal food preservation methods have dominated the food processing sector because they are effective and easy to carry out and are therefore widely used both in industry and experimentally. Besides that, as is the case with all thermal technologies, some studies report a negative impact of heat processing on the organoleptic and nutritional qualities of final products by significantly decreasing the content of bioactive compounds such as phenolic compounds and vitamins (Rawson, Patras, et al., 2011). A classic example is the fruit juice industry. Among the various sectors of processed plant-based products, fruit juices are among the most affected by PPOs, and therefore industry and researchers have developed non-thermal food preservation techniques in response to consumer demand for safe, colorful, and nutritious juices (Zawawi et al., 2022). In accord with Irondi et al. (2016), flavonoids such as anthocyanins are susceptible to heat and they are also usually and mostly present in external layers of flowers and are mostly heat labile. Non-thermal technologies represent an emerging alternative in ensuring food processing and preservation with many advantages over heat treatments. Using these processes allows for improving food safety and shelf-life thanks to the inactivation of enzymes and microorganisms (Barba et al., 2017; Ma et al., 2017), improves the retention of bioactive compounds (Khan et al., 2018; Oms-oliu et al., 2012; Rawson, Patras, et al., 2011), minimize sensory and nutritional losses (Barba et al., 2017) and reduce the environmental impacts of pre-treatment in terms of water waste, emissions, and energy footprint (Pereira & Vicente, 2010). New food processing techniques have therefore made it possible to reduce the environmental impact of food processing and in a world that demands environmental sustainability and food security, innovation is also the key to sustainable growth in the food industry. Non-thermal technologies include innovative processing to control polyphenol-oxidase in fruit and vegetable products such as high hydrostatic pressure (HHP) processing, high-pressure carbon dioxide (HPCD) processing, ultrasound processing, pulsed electric fields (PEF) processing, UV–Vis radiation processing, pulsed light (PL) processing, cold plasma processing, Ozone (O_3) processing (Federica Tinello & Lante, 2018a). Although there are many types of non-thermal treatments, the technology most widely used and of which we have the most evidence to date in the pre-treatment of edible flowers is ultrasound processing. This suggests that in the coming years there will be a need to focus research on implementing the use of these novel treatments in edible flowers.

8.3.1. Ultrasound pre-treatment (US)

Publications on the use of ultrasound as a treatment in the food industry are several (Ebrahimi & Lante, 2022; Y. Sun et al., 2019; Wen et al., 2018) and show that this technique is effective in reducing PPO activity and producing high-quality products. Among nonthermal techniques, ultrasound (US) processing produces a cavitation process that increases the polyphenols of treated samples (Manzoor et al., 2021). Ultrasonic or ultrasound technology allows plant products to be processed without the use of heat, improving their quality and safety by converting electrical energy into mechanical energy through piezoelectric materials (Abdullah & Chin, 2014). When sound waves pass through the fluid of the matrix, continuous compression and relaxation will occur, and then the negative pressure overcomes the tensile force, the transmission of sound waves to the relaxed position causes the formation of micron-sized bubbles and spaces (Ba et al., 2016). Strong physical effects lead to continuous compression and expansion of the liquid, high local temperature, and pressure, accompanied by the production of hydrogen and hydroxyl radicals from water decomposition will chemically damage proteins, causing the loss of enzyme activity (Xu et al., 2022). The use of this technique in the food industry may involve the use of low or high-energy ultrasound. Low-energy ultrasound has an intensity of less than 1 $W \cdot cm^{-2}$, and frequencies above 100 kHz; while high-energy ultrasound has an intensity higher than 1 W·cm−2, and the frequency range is from 20 to 100 kHz (Abdullah & Chin, 2014). Ultrasound can be applied to a product using two methods: submergence in an ultrasonic bath (Wu et al., 2008), or direct treatment of the product using a probe sonicator (Marina et al., 2014; Rawson et al., 2011). This technology has a direct effect on the structure of the enzyme itself. Xu et al. (2022)

examined the influence of this treatment on the activity, structure, physicochemical properties, and molecular microstructure of PPO. The results showed that ultrasound treatments reduced the activity of PPO, and the dual-frequency mode treatment of 22/40 kHz had the most noticeable effect in this study. The inactivation followed first-order kinetics and was successfully fitted to the Weibull distribution model. Structurally, the α-helix and β-turn folding of PPO is decreased, resulting in the unfolding of the secondary structure. After analysis by fluorescence spectroscopy, the destruction of the tertiary structure of the enzyme was noted, which destroyed surface roughness resulting in aggregation of the protein. This caused the active center of the enzyme covered, which ultimately led to the loss of the catalytic function of the enzyme and the reduction of its activity.

Many studies in the literature have investigated the positive effect of ultrasound on enzyme inactivation, and ultrasonic treatment can also be combined with heat, taking the name of thermosonication (TS), with other nonthermal processes such as pulsed electric field, or with lactic acid or ultraviolet, thus creating a useful combination of factors. Zawawi et al. (2022) have studied polyphenol-oxidase inactivation by ultrasonic treatment in various fruit cultivars. It was found that the higher the intensity of the ultrasound, the greater the effect on the enzyme, as greater intensity results in greater energy released. Furthermore, PPO inactivation could be affected by different food matrices and enzyme sources. Temperature-sensitive parameters, such as z-values and Ea, were evaluated through slopes by plotting the linearized form of the Arrhenius equation. The differences in PPO inactivation could be due to a difference in fruits' cultivar or the fruits' form, such as puree and juice. Different cultivars or forms have different D, z, and Ea-values, showing different reaction rates (e.g., processes with high activation energy and low z-values are extremely temperature sensitive). Figure 8 shows how frequencies between 20 and 24 kHz cause polyphenol-oxidase deactivation of more than 80 percent at temperatures between 60 and 72 °C, and treatment times between 3 and 20 min (Abid et al., 2014; Bot et al., 2018; Cao et al., 2018; Illera et al., 2018; Sulaiman et al., 2015; Zawawi et al., 2022).

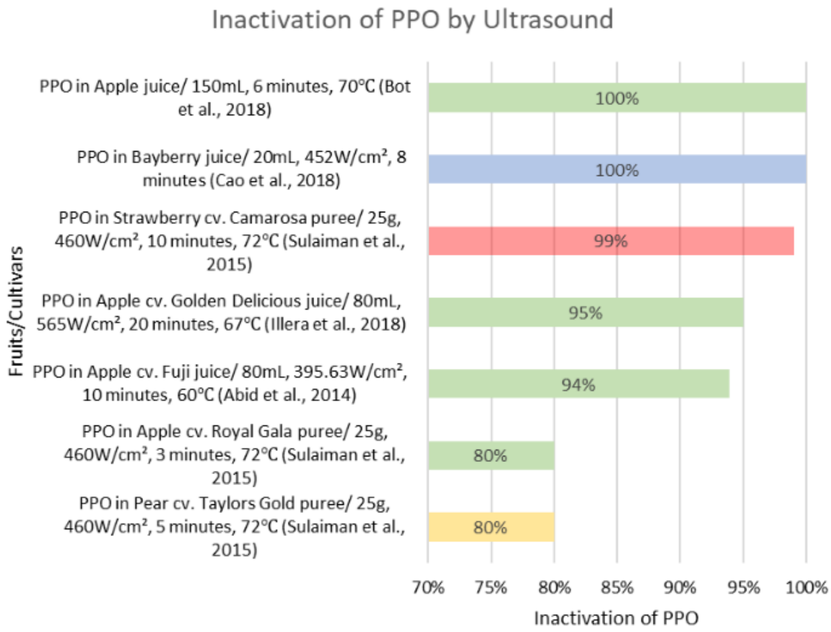

***Figure 8.** Inactivation of polyphenol-oxidase by ultrasound treatment in different cultivars (Abid et al., 2014; Bot et al., 2018; Cao et al., 2018; Illera et al., 2018; Sulaiman et al., 2015; Zawawi et al., 2022)*

Some studies in the literature have been conducted on various edible flowers, showing that the activity of PPOs was significantly reduced after ultrasonic treatment. Mounir et al. (2017) observed that ultrasound combined with low-intensity pre-treatment was effective in inhibiting the enzymatic activity of PPO and POD in daylily flowers compared with thermal pre-treatment. The study showed that the browning index of heat-treated flowers increased significantly compared with those treated with ultrasound, emphasizing that this new technology can be not only effective but better than classic heat treatment. In addition, it cannot be overlooked that low-frequency ultrasound has been shown in the literature to have a positive effect on total phenolic content and total flavonoid content (Barani et al., 2022).

Another interesting study on the application of ultrasound as a pre-treatment technique in edible flowers was conducted by Barani et al. (2021). In this paper, the researchers studied the effect of thermal and ultrasonic pre-treatment on enzyme inactivation, color, phenolics, and flavonoid contents of infrared freeze-dried rose flowers. The goal was to understand the effectiveness of the two pre-treatment techniques by comparing them with each other. The above study found that ultrasonic treatment conducted at 45 kHz was the only one to be effective in inactivating rose flower PPOs. Heat treatments conducted at 60, 70, and 80°C, as well as ultrasonic treatments conducted at 80 and 100 kHz, did not show relevant inhibitory activities toward polyphenol-oxidase (figure 9).

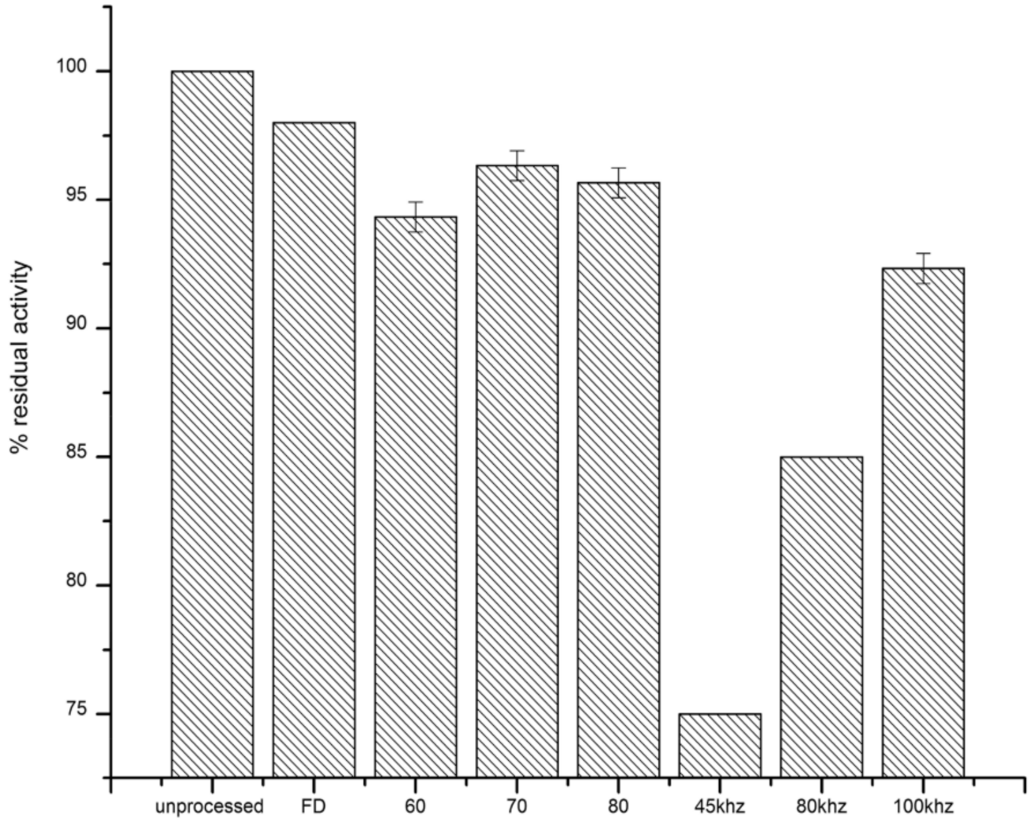

Figure 9. *Effect of infrared freeze-drying on PPO activity of pre-treated rose flowers after thermal and ultrasonic pre-treatments (Barani et al., 2021)*

Interestingly, although drying usually results in anthocyanin loss, ultrasound pre-treatment can help maintain a fairly close level to that of fresh samples. Compared with other pre-treatments, the anthocyanin content in the samples pre-treated by low-frequency ultrasound (45 kHz) was the highest, and the degradation index loss was the smallest. The main reason for the low degradation index of anthocyanins is due to increased inactivation of endogenous PPOs and PODs by ultrasound. This is also found by color analysis. Following the drying process, the ultrasonically treated samples showed a more vivid color and a lower browning index than the heat-treated samples. It is therefore noteworthy that ultrasonic pre-treatment could provide a unique opportunity for the production of high-quality products rich in anthocyanins and other bioactive phenolic substances.

8.3.2. High hydrostatic pressure pre-treatment (HHP)

High hydrostatic pressure (HHP) technology is another nonthermal technology that could be useful to inactivate polyphenol-oxidase in edible flowers. In accord with Zhao et al. (2019), high hydrostatic pressure (HHP) technology is all set to hold a prominent space in the future food industry given its food preservation potential as well as the advantages associated with the process and the product obtained. This technique is an emerging nonthermal food processing method that subjects liquid or solid foods, with or without packaging, to pressures between 50 and 1000 MPa (Tao et al., 2014). HHP is now a commonly used technology to inactivate microorganisms and enzymes, with the aim of preserving functional and nutritional properties. In general terms, HHP treatments are uniform, independent of the product geometry and size, use minimum chemical additives, and are environmentally friendly. HHP can extend product shelf-life whilst retaining the natural freshness, stability, and functionality of compounds and nutrients (Shantamma, Vasikaran, et al., 2021). Despite the ability to inactivate enzymes, it must be emphasized that PPO is a pressure-resistant enzyme, and pressures up to 800 MPa are required to inactivate it (González-Cebrino et al., 2013). Fernandes et al. (2017) conducted a study on the effect of HHP on four edible flowers (*Viola x wittrockiana, Centaurea cyanus, Borago officinalis,* and *Camellia japonica*) and showed that different flowers exhibit different tolerability to pressures. The above study concluded that high-pressure HHP treatment causes destructive effects on the matrix of some flowers such as camellia and borage, so the use of milder pressures is recommended. Edible flowers have different cellular structures, which cause a different behavior when submitted to HHP (Fernandes, Saraiva, et al., 2018). This phenomenon might be associated with the structural alteration of the cells provoked by the HHPs, yielding a higher amount of extracted metabolites or a physiological response of the flower to stress conditions at higher pressurization levels (Fernandes et al., 2017). In view of this, studies have shown that PPO not only cannot be deactivated by low-pressure treatment but can be activated more, due to conformational changes, membrane-bound enzyme release, proteolysis, and cell wall disruption that increases cell permeability and contact between enzyme and substrate (Izumi et al., 2015). Queiroz et al. (2021) studied the effect of high hydrostatic pressure in fresh-cut cashew apples and showed that HHP treatment at 250 MPa led to the activation of PPO threefold higher than the control sample. In broccoli, a pressure of 210 MPa at −20°C was insufficient to inactivate peroxidase and polyphenol-oxidase (Préstamo et al., 2004). In summary, HHP could be a promising technology for treating edible flowers and maintaining their quality during shelf life. However, each flower shows different behavior to pressure, and considering that polyphenol-oxidases need high pressures to be deactivated, further studies are needed to better understand the effects of this technology on different types of flowers.

8.3.3. Pulsed electric field pre-treatment (PEF)

PEF is an emerging technology based on the usage of an external electric field that yields reversible or irreversible electroporation in cell membranes (Ebrahimi & Lante, 2022). The electroporation phenomenon induces cell transformation or rupture under the utilization of a few to several hundred short pulses (intensity between 50 and 100 kV/cm2) with a period ranging from microseconds to milliseconds (Federica Tinello & Lante, 2018b; Zderic & Zondervan, 2016). In other words, the electroporation process leads to the formation of transmembrane pores, and this process can be reversible or irreversible depending on the intensity of PEF. When the electric field strength and treatment duration increase, the treatment intensity also increases, leading to the transformation of reversible membrane permeabilization into irreversible membrane breakdown (Ebrahimi & Lante, 2022). This technology is applied to liquid or semi-liquid foods placed between electrodes for effectively inactivate microorganisms and enzymes (Huang et al., 2012). The available literature has investigated the deactivation of PPOs by PEF technology in many plant products such as raspberry and blueberry purees (Medina-Meza et al., 2016), apple juice (Bi et al., 2013; Noci et al., 2008; Riener et al., 2008; Schilling et al., 2008) and grape juice (Marsellés-Fontanet & Martín-Belloso, 2007). PEF was shown to be an effective technology in inhibiting PPO, and similar to other nonthermal technologies it showed a synergistic effect when used with heat. Increasing temperature contributes to further improving anti-browning performance since PPO is resistant to certain PEF conditions (Federica Tinello & Lante, 2018b). Riener et al. (2008), studied the PPO inactivation in clarified apple juice treated by combining preheating to three temperatures (23, 35, and 50 °C) and PEF processing at different values of electric field strength (20 and 40 kV/cm2) and time (25, 50, and 100 μs), obtained at 50 °C the highest level of PPO activity reduction ranging from 25 to 45% at 20 kV/cm2 and from 45 to 70% at 40 kV/cm2. Moreover, the degree of PPO inactivation was shown to be higher than that obtained by pasteurization at 72°C for 26 seconds, where polyphenol oxidase activity dropped by 46%. However, Medina-Meza et al. (2016) found PPO residual activities of 98% and 80% in respectively raspberry and blueberry purees after the same processing conditions of 25 kV/cm2 and 66 μs in the temperature range of 25–28 °C, showed that PEF technology had very low impact on the activity of this enzyme. It should be kept in mind that the effect of an electric field on enzymes is strictly related to the applied conditions, and comparison between data needs to consider not only frequency and electric field strength but also the characteristic of the chamber, the length, and frequency of pulses, as well as the time of treatment (Zhong et al., 2007). In addition, the effectiveness of PEF technology against browning is also attributed to properties of the foods themselves, such as electrical conductivity, ionic strength, pH, or from the source of the enzyme, which can influence the resistance of PPO (Meneses et al., 2011; Federica Tinello & Lante, 2018b). Currently, there would appear to be no work that has studied the effectiveness of PEF technology on the inactivation of PPOs in edible flowers. Considering the above, further studies would be needed to define the optimal treatment conditions, also taking into consideration that different flowers might give different results in terms of enzymatic deactivation. Nevertheless, the results on other matrices, conducted under certain conditions, are encouraging and provide an incentive to study an application of this technology also to inactivate PPOs in edible flowers.

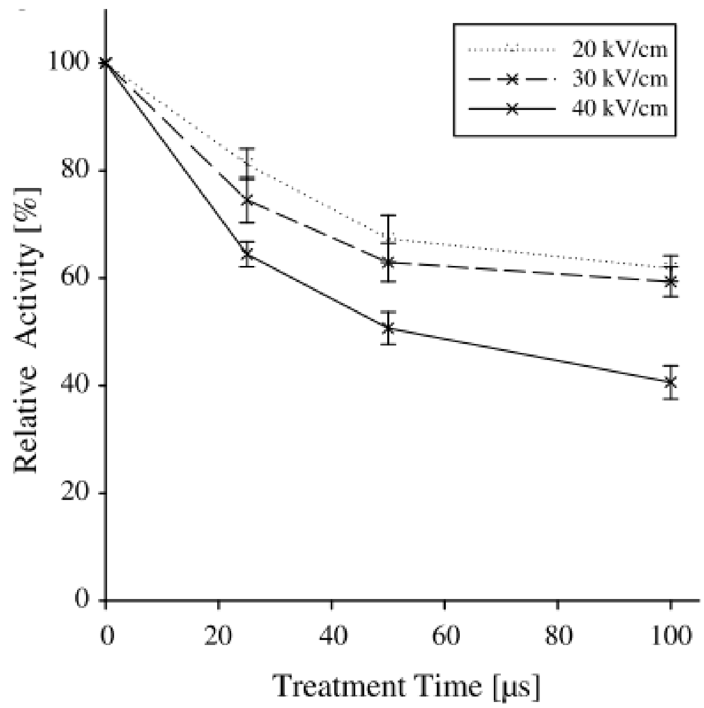

Figure 10. *Relationship between PPO residual activity and PEF treatment time on apple juice at 23°C (Riener et al., 2008)*

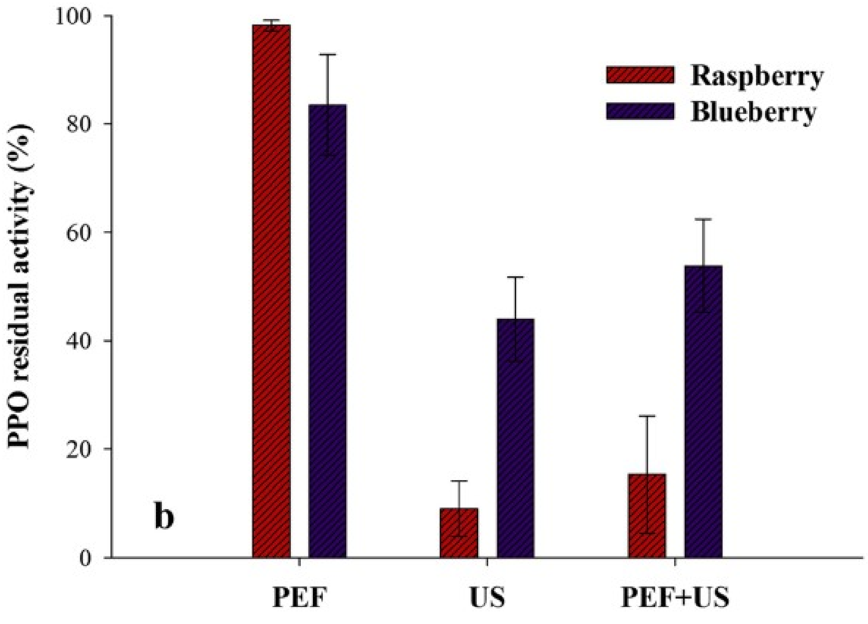

Figure 11. *Relationship between PPO residual activity and PEF, US, and PEF+US treatment on raspberry and blueberry purees at 25°C and 25 kV/cm2 for 66 μs (Medina-Meza et al., 2016)*

8.3.4. High-pressure carbon dioxide (HPCD) processing

High-pressure carbon dioxide (HPCD) processing is recognized as a valid alternative to heat treatments for liquid foods as it effectively destroys microorganisms and enzymes and retains sensory, nutritional, and physical properties (Reviews & Science, 2006). The inhibitory effect of HPCD processing on PPO activity depends mainly on the processing parameters such as pressure, CO2 level, and time (Federica Tinello & Lante, 2018b). In *Cucumis melo L.* juice treated with HPCD at 55°C for 60 minutes, PPO deactivation increased from 45% to 75% using a pressure of 8 to 35 MPa for 60 minutes. The treatment was effective in inactivating PPO in addition to inactivating microorganisms and preserving other important quality attributes such as color, browning, and aroma compounds (J. L. Chen et al., 2010). Fresh carrot slices treated with HPCD at different pressure values (1.5, 3, 5 MPa) and time (2, 5, 8, 12, 15 min) initially showed an increase and subsequently a 91% reduction in PPO activity with treatment at 5 MPa for 15 min (Bi et al., 2011). Similar to other non-thermal technologies, the combination of HPCD with mild heat treatment could further enhance PPO inactivation.

8.3.5. UV–Vis radiation processing

It has been shown in multiple food matrices that UV-Vis radiation is effective in inactivating polyphenol-oxidase. The inhibitory effect has been associated mainly with direct photo-oxidation arising from the absorption of light by amino acid residues (Trp, Tyr, His, Phe, Met, Cys), the resulting protein denaturation, and the formation of high molecular weight aggregates (Lante et al., 2013). UV–Vis light can effectively and safely control the enzymatic browning of fruit and vegetable products and simultaneously minimize organoleptic and nutritional losses (Federica Tinello & Lante, 2018b). Aguilar et al. (2018) conducted a study in peach juice, demonstrating that with a 460 W multi-wavelength emission lamp providing UV-Vis light in the range of 250-740 nm and an irradiation power of $4.49 \cdot 10^{-2}$ W/cm^2, PPO was effectively inactivated without affecting pH, acidity, vitamin C, sugars and brightness of the juice. In orange juice, after combined UV-C and thermal processing (65 °C for 10 min), Sampedro & Fan (2014) found a complete PPO inactivation.

8.3.6. *Cold plasma processing (CP)*

The use of plasma is an emerging technology that has also proven useful in enzyme inactivation. Plasma is defined as the "fourth state of matter" generated by applying energy in the form of heat, voltage, or electromagnetic field to a gas which is subsequently subjected to reactions such as ionization, excitation, and dissociation thus producing various active species *e.g.* electrons, free radicals, ions, UV radiation, *etc* (Federica Tinello & Lante, 2018b). Plasma causes the breakdown of specific bonds or chemical modification in the enzyme's side chains, causing the loss of secondary structure, resulting in the inactivation of the enzyme (Misra et al., 2016). Yi et al. (2022), investigated the effect of plasma treatment on fresh-cut mango and observed that this treatment was effective in inactivating PPO during storage (figure 12). Suppression of PPO and POD activities by CP treatment could contribute to reduced phenolic oxidation and amelioration of browning, as currently manifested by higher levels of total phenolics and flavonoids concomitant with an improved color appearance in CP-treated fruit (Yi et al., 2022). Illera et al. (2019) studied the effect of cold plasma on polyphenol-oxidase inactivation in cloudy apple juice and noted that the reduction in activity was significant. In the case of CP treatment for 4 or 5 min, the measured PPO activity was minimal, both after 24h and after 7 days. After 24h post-treatment, there was still residual activity in the juices that had been treated for 1, 2, and 3 min, but this was very low, e.g. residual activity changed from 63.5 ± 2.8 just after the treatment to 25.6 ± 0.6 after 24 h of storage (Illera et al., 2019).

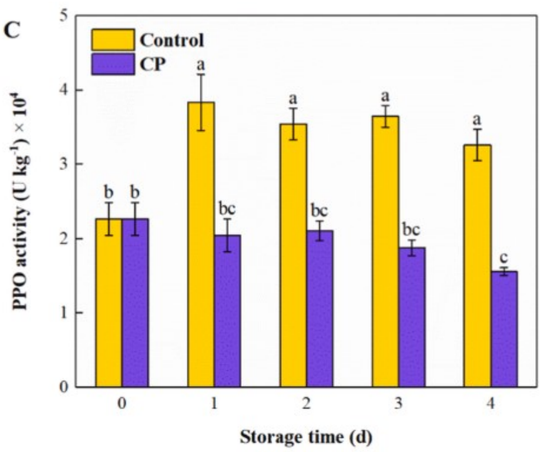

***Figure 12**. PPO activity of fresh-cut mango treated with CP (Yi et al., 2022)*

8.3.7. Other non-thermal pre-treatment and considerations

In addition to the non-thermal treatments discussed above, for the sake of completeness, it is only fair to mention others that have proven effective in inactivating PPO. Examples of these emerging new technologies include High pressure carbon dioxide (HPCD) processing, Pulsed light (PL) processing, or Ozone (O3) processing. All these techniques, despite in different matrices, have been shown to be effective in deactivating PPO, suggesting their possible application also as pre-treatment of edible flowers before the drying process. Deactivating PPOs using mild processing technologies that minimally affect color and organoleptic characteristics would be of great interest to the edible flower sector. Furthermore, it meets the consumer's demands of looking for minimally processed foods that are more similar to fresh products, without the use of chemical preservatives (Shantamma, Monica, et al., 2021). In the literature, studies related to the use of mild technologies in edible flowers are increasing, which shows a growing interest from the scientific community in this area. Today, analysing the available work, it is not clear which treatment is the most effective in terms of PPO deactivation; each of the treatments listed above is effective for this purpose, and it is therefore necessary to evaluate their application taking into consideration matrix to be treated, physiological characteristics of the flower, economic availability, and desired result in terms of deactivation. In addition, it is clear that using nonthermal technologies as a pre-treatment technique is more advantageous than traditional techniques such as blanching because they have proven to be effective and less environmentally impactful. Considering that the market for edible flowers is expanding, more research in this regard is expected in the coming years. Such research will be needed to develop the best technological, environmental, and economical solutions to inactivate polyphenol-oxidases, retard the degradation of anthocyanins in edible flowers, and ensure the consumer or industry a product of excellent quality.

8.3.8. Control of polyphenol-oxidase (PPO) during anthocyanins extraction

As described in previous chapters, the extraction of anthocyanins is a critically important process to employ these molecules in subsequent applications as natural dyes or as a source of bioactive compounds. During the process of anthocyanin extraction, the breakdown of the cell structure is caused, making it possible for PPOs to interact with the substrate, causing browning and loss of color of the anthocyanins themselves. Therefore, it is crucial to find an appropriate solution to reduce the drawbacks derived from PPO and prevent the oxidation of polyphenols during the extraction process (Ebrahimi & Lante, 2022). Nowadays, the research on sustainable food processing is concentrated on replacing conventional anti-PPO heating treatments with non-thermal techniques such as ultrasound treatments. Application of nonthermal technologies in the extraction of polyphenols may have different results for the enzymatic activity because they influence PPO by several factors, including the intensity, duration, mode of exposure, gas composition, electrical input, and degradation of enzymes or substrates (Paixão et al., 2019). In Chapter 8, it was extensively discussed how non-thermal treatments can inactivate polyphenol-oxidases thus causing increased anthocyanin retention. Reducing PPO activity has a direct impact on polyphenol retention and could increase the recovery yield of polyphenols during their extraction (Dayanne et al., 2021; Reza et al., 2017; F Tinello & Lante, 2015; Federica Tinello et al., 2018).

As an example, the pulsed electric field pre-treatment (PEF) is helpful in the extraction of intracellular compounds such as anthocyanins but can also alter the composition of different enzymes. Enzymes such as polyphenol-oxidases or peroxidases are partially inactivated by PEF treatment, which causes a decrease in their activity. Presently, the exact mechanisms associated with enzyme inactivation by PEF are not fully explained, but it is assumed that PEF causes variances in enzymes' conformational state which causes their inactivation (Manzoor et al., 2021). This has been noted, for example, in lysozyme, where there is an association between the loss of secondary and tertiary structures of lysozyme by PEF and its inactivation (Hang et al., 2007). In spinach juice, PEF can polarize the protein molecule and alter its conformation, appearing in the protein inactivation, which could demonstrate the inactivation of POD and PPO in the juice (Manzoor et al., 2021).

Cold plasma pre-treatment (CP) is another example of non-thermal treatment that is useful in inhibiting PPO before the extraction process. In the CP process, the higher the frequency, the further the anti-PPO activity. The loss of polyphenol-oxidase in the CP treatment may be due to the activity of free radicals produced in this process on protein bands (Ebrahimi & Lante, 2022). Loss of enzyme activity could also be caused by the modification of the secondary structure and side chain of amino acids of proteins, due to CP free radicals (Raquel et al., 2020). Dayanne et al. (2021) evaluated the effect of plasma pre-treatment in avocado pulp with the addition of lime extract. The results showed that this combination can significantly reduce the activity of PPOs and increase the phenolic content of the pulp. This could be explained by the fact that the addition of lime extract causes slight acidification that can reduce polyphenol oxidase activity. This effect is combined with the generation of free radicals typical of CP treatment, which interact with the released enzymes and cause structural changes in them. From the available literature, there would appear to be no research that has evaluated the impact of non-thermal technologies on polyphenol-oxidase during the process of anthocyanins' extraction from edible flowers. More research will be needed in the future, but despite this, the available literature on non-thermal treatments has shown that they can significantly reduce the action of PPOs (Table 2).

Technique	Treated Food/Compound	Treatment Condition	Result	Reference
Flat sweep frequency and pulsed ultrasound	Mushroom PPO	The ultrasound was applied with a frequency that moved up and down within a predetermined range.	Treatment with dual-frequency of 22/40 kHz mode decreased PPO activity significantly.	(Xu et al., 2022)
Ultrasound	Potato	Power: 540 W, time: 15 min, temperature: 20 ◦C.	The optimal condition had the highest PPO inhibitory effect.	(M. Wang et al., 2021)
Ohmic heating	Water chestnut juice	Voltage: 220 V; electric field strength: 22, 27.5, and 36.7 V/cm; with a titanium electrode.	PPO activity decreased rapidly with ohmic heating treatment at the critical deactivation temperature (35 ◦C).	(X. Li et al., 2019)
Ohmic heating	Coconut water	Electric field strength: 10 and 20 V/cm, time: 3–15 min.	At 90 ◦C, PPO activity decreased to about 10% of its initial activity at only 3 min.	(Kanjanapongkul & Baibua, 2021)
Ultrasound	Spinach juice	Power: 180 W, frequency: 40 kHz, time: 21 min, temperature: 30 ◦C.	PPO activity decreased by 36%.	(Manzoor et al., 2021)
Cold plasma	Cut apple and potato	Time: 10 min, frequency: 2.45 GHz, power: 1.2 kW, gas flow: 20 L/min.	PPO activity was reduced by about 62% and 77% in fresh-cut apple and potato tissue, respectively.	(Bußler et al., 2017)
Pulsed electric field	Spinach juice	Electric field strength:9 kV/cm, frequency: 1 kHz, treatment time: 335 µs.	PPO activity decreased by 44%.	(Manzoor et al., 2021)
Ultrasound and pulsed electric field	Spinach juice	Ultrasound was performed before the pulsed electric field.	PPO activity decreased by 56%.	(Manzoor et al., 2021)
Microwave	Peach puree	Different power densities (4.4, 7.7, and 11.0 W/g) were applied, and cooking value was observed.	The PPO significantly decreased from around 50% to around 5% with increasing the cook value level, regardless of the power density applied.	(Zhou et al., 2022)

Table 2. *Effect of different novel technologies on PPO (Ebrahimi & Lante, 2022)*

CONCLUSIONS

In recent years, edible flowers are attracting the interest of many consumers because of their color, aroma, and content of bioactive compounds. Although statistical data on the market for these products are not yet available, it is assumed that demand will grow rapidly in the coming years. Among the various bioactive compounds, anthocyanins are mainly responsible for the gorgeous color of many flowers, and thus confer essential visual and nutraceutical qualities to this class of products. Thus, the presence of polyphenol-oxidase is a limiting factor that worsens the appearance of the flowers and may severely limit their use by the consumer or the food industry. Analysis of the available literature showed that it was essential to control polyphenol-oxidase to reduce color loss, except for a few cases where discoloration was mainly due to non-enzymatic reactions. There are many methods studied to deactivate PPO, but the demand for the application of environmentally-friendly techniques is increasing, which invites a preference to follow these alternatives. Among the new treatments available, ultrasound appears to be among the most widely used to decrease polyphenol-oxidase activity in edible flowers. The use of ultrasound has been shown to be more effective than heat treatments, also ensuring greater retention of anthocyanins during the process. Despite this, alternatives such as HHP, PEF, HPCD treatment, and others have still proven effective in multiple plant matrices. Considering that the market for edible flowers is expected to grow, it should be emphasized that more study is expected in the use of these techniques to develop the best technological, environmental, and economic solutions to inactivate polyphenol-oxidase, retard the degradation of anthocyanins, preserve the color, and ensure the consumer or industry a product of excellent quality.

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