Exploring the influence of edible flowers on the organoleptic and antioxidant properties of white wine vinegar

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Resumo

Na sociedade atual verifica-se globalmente uma maior consciência e zelo pela saúde e preocupação com os padrões alimentares, justificando assim uma atenção redobrada aos polifenóis. Uma maior pesquisa destes metabolitos secundários de origem vegetal que se destacam devido aos seus inúmeros benefícios para a saúde é então vivamente encorajada. As vantagens associadas à sua ingestão favorecem a escolha de alimentos ricos em polifenóis em relação a alimentos pobres em polifenóis, com as pessoas agora privilegiando opções alimentares mais saudáveis por existir um maior interesse no perfil nutritivo e impacto dos alimentos na saúde humana. As flores comestíveis possuem uma longa história de uso na culinária em muitos países, e além do seu propósito decorativo, de aumentar a beleza de uma variedade de pratos também constituem uma fonte significativa de polifenóis na dieta daí a sua inclusão poder levar a um maior consumo de compostos fenólicos pela população geral. Os polifenóis presentes nas flores comestíveis possuem a capacidade de moldar várias propriedades organoléticas, nomeadamente a cor, aroma e adstringência. As cores vibrantes exibidas devido a presença de polifenóis específicos podem ser muito apelativas para os consumidores, aumentando, portanto, o valor comercial destes produtos alimentares.

Além deste maior apelo estético, polifenóis também modelam a adstringência que é uma sensação tátil complexa caracterizada pela secura, enrugamento e aspereza sentidos na cavidade oral, que impacta como é percecionado o sabor. Um nível equilibrado de adstringência é desejável em alguns produtos, como no vinho e na cerveja, em que a sua presença é expetável e apreciada por muitos, enquanto que em outros produtos a sua presença é desagradável e não intencional. Dos vários mecanismos utilizados para tentar explicar a indução de adstringência, o mais estudado refere-se à interação entre as proteínas salivares e os polifenóis, resultando na precipitação de complexos e, consequentemente, na perda de lubrificação, a interação entre esses compostos sem que haja precipitação também está relacionada com o aumento da perceção de adstringência. Outro mecanismo de evocação de adstringência está dependente da interação entre polifenóis e células epiteliais orais. A adstringência é postulada como uma sensação trigeminal, sendo que as interações de adstringentes com os mecanorreceptores, com os quimiorreceptores e com os recetores acoplados à proteína G possuem um importante papel na perceção de adstringência.

A pesquisa conduzida teve como intenção melhorar a compreensão do papel inovador da adição de flores comestíveis no vinagre de vinho branco, relativamente ao teor de polifenóis totais, a capacidade antioxidante e a modulação da adstringência. A

progressão ao longo do tempo da cor dos vinagres infundidos com flores comestíveis ricas em polifenóis nomeadamente *Viola tricolor, Centaurea cyanus e Cosmos bipinnatus* foi monitorizada. Foi demonstrado que a adição de flores comestíveis causa alterações de cor com um grau variável de intensidade dependendo do seu perfil fenólico. Foi efetuada a determinação do teor fenólico de cada variedade de vinagre com infusão de flores comestíveis e dos seus extratos concentrados ricos em polifenóis em diferentes concentrações, mostrando que a infusão conduz a uma melhoria significativa do teor de polifenóis, sugerindo que as flores comestíveis têm um grande potencial como fonte dietética de polifenóis. O efeito positivo observado varia consoante a concentração e a espécie de flor comestível. A tentativa de identificação dos perfis fenólicos de cada vinagre com infusão de flores comestíveis evidencia a variabilidade fenólica em termos de concentração e heterogeneidade, contendo polifenóis de complexidade variada, com padrões de substituição distintos, sendo que muitos sofrem glicosilação ou acilação. Foram conduzidos estudos antioxidantes para elucidar sobre a capacidade antioxidante dos extratos de vinagres infundidos com flores comestíveis e dos extracts de flores comestíveis que revelaram um aumento considerável da atividade antioxidante à luz da infusão com flores comestíveis. Um importante objetivo deste trabalho baseia-se no estudo das interações entre os polifenóis e as proteínas salivares, comumente associadas à indução da adstringência, apesar de ainda não se ter chegado a um consenso sobre os mecanismos responsáveis pela adstringência. As famílias de proteínas salivares estudadas são as proteínas básicas ricas em prolina (bPRPs), as proteínas ricas em prolina glicosiladas (gPRPs), as proteínas ricas em prolina ácidas (aPRPs), o péptido estaterina/P-B e as cistatinas. Este estudo foi efetuado através da comparação das áreas dos picos cromatográficos destas proteínas na ausência e presença de compostos fenólicos em três concentrações diferentes, demonstrando que a presença de flores comestíveis intensifica a precipitação, condicionada pela espécie de flor comestível e pela concentração. Uma maior compreensão da modulação da adstringência é crucial para o desenvolvimento da produção de alimentos, daí a importância desta investigação como uma forma de melhor apelar às preferências dos consumidores.

Palavras-chave: adstringência, antioxidante, antocianinas, flores comestíveis, polifenóis, proteínas salivares, vinagre.

Abstract

In contemporary society, there is a greater global awareness and zeal for health and focus on dietary patterns, thus justifying an increased attention in polyphenols. Further research into these plant-derived secondary metabolites that have drawn attention due to their countless health benefits, is heavily encouraged. The advantages associated with their intake favors the choice of polyphenol-rich foods over polyphenol-poor foods, with people now opting for healthier food options as there is greater interest in the nutritional profile and food´s impact on human health. Edible flowers have a long history of culinary use in many countries, in addition to their decorative purpose, of enhancing the beauty of a variety of dishes, they also constitute a significant source of polyphenols in the diet, which is why their inclusion could lead to a greater consumption of phenolic compounds by the general population. The polyphenols present in edible flowers have the ability to shape various organoleptic properties, namely color, aroma and astringency. The vibrant colors exhibited due to the presence of specific polyphenols can be very appealing to consumers, thus increasing the commercial value of these food products. In addition to this greater aesthetic appeal, polyphenols also shape astringency, which is a complex tactile sensation characterized by dryness, wrinkling and roughness felt in the oral cavity which impacts how taste is perceived. A balanced level of astringency is desirable in some products, such as wine and beer, in which its presence is to be expected and appreciated by many while in other products its presence is unpleasant and unintentional. Of the various mechanisms used to try to explain the induction of astringency, the most studied one refers to the interaction between salivary proteins and polyphenols, resulting in the precipitation of complexes and, consequently, the loss of lubrication; the interaction between these compounds without precipitation is also related to the increase of the perception of astringency. Another mechanism for evoking astringency depends on the interaction between polyphenols and oral epithelial cells. Astringency is postulated as a trigeminal sensation with the interactions of astringents with mechanoreceptors, chemoreceptors and G-protein coupled receptors possessing an important role in eliciting astringency.

The research conducted herein intended to improve the understanding of the innovative role of adding edible flowers to white wine vinegar, regarding the total polyphenol content, antioxidant capacity and modulation of astringency. The progression over time of the color of vinegars infused with polyphenol-rich edible flowers, namely *Viola tricolor*, *Centaurea cyanus* and *Cosmos bipinnatus*, was monitored. It was shown that the addition of edible flowers causes changes in color with varying degrees of

intensity, depending on its phenolic profile. The ascertaining of the phenolic content of each variety of edible flower infused vinegar and of their concentrated polyphenol-rich extracts in different concentrations was carried out, showing that the infusion leads to a significant improvement of the content of polyphenols, suggesting that edible flowers have great potential as a dietary polyphenol source. The positive effect observed varies depending on the concentration and edible flower in question. The tentative identification of the phenolic profiles of each edible flower infused vinegar showcases the phenolic variability in concentration and heterogeneity, with all of them containing polyphenols of differing complexity, with distinct substitution patterns, with many of compounds present suffering glycosylation or acylation. Antioxidant studies were performed to elucidate on the antioxidant capacity of the edible flower infused vinegars extracts and of the edible flower extracts that revealed a considerable increase in antioxidant activity in light of infusion of edible flowers.

An important aim of this work is based on the study of the interactions between polyphenols and salivary proteins, which is commonly linked to the induction of astringency, despite a consensus over the mechanisms responsible for astringency not having yet been achieved. The salivary protein families studied are basic proline-rich proteins (bPRPs), glycosylated proline-rich proteins (gPRPs), acidic proline-rich proteins (aPRPs), statherin/P-B peptide and cystatins. This study was carried out by comparison of the areas of the chromatographic peaks of these proteins in the absence and presence of phenolic compounds in three different concentrations, demonstrating that the presence of edible flowers intensifies precipitation, conditional on the species of edible flower and concentration. A greater understanding of the modulation of astringency is crucial to the development of food production, hence the importance of this research as a way to better appeal to the preferences of consumers.

Keywords: anthocyanins, antioxidant, astringency, edible flowers, polyphenols, salivary proteins, vinegar.

Table of Contents

Table of Contents vi

List of Tables ix

List of Figures x

List of Abbreviations xiii

General Introduction 14

1. Phenolic compounds 2

    1.1. Food sources of Polyphenols 3

2. Edible flowers 5

    2.1. Historical contextualization of edible flowers 6

    2.2. The present-day popularity of Edible flowers 7

    2.3. Edible flowers´ culinary use 8

3.The case of vinegar 9

Objectives 10

Chapter 1 - Phenolic quantification and characterization of the edible flower infused vinegars based on their physicochemical and antioxidant properties **..... 12**

1. Introduction 13

    1.1. Structures of Polyphenols 13

        1.1.1. Non-flavonoids 13

        1.1.2. Flavonoids 15

            1.1.2.1. Anthocyanins 18

    1.2. Phenolic compounds' effect on the physicochemical properties of flowers 20

        1.2.1. Anthocyanins-rich edible flowers 24

    1.3. Polyphenols´ health benefits 25

2. Materials and methods 30

    2.1 Production of polyphenol rich edible flower infused vinegars 'extracts 30

    2.2 Physiochemical characterization of the edible flower infused vinegars 31

        2.3.1 Quantification of the total phenolic content (TPC) of edible flower infused vinegars 32

2.3.2 Quantification of the TPC of polyphenol-rich vinegar extracts made from vinegars infused with flowers 32
2.4 Statistical analysis 33
2.5 LC-DAD/ESI-MS analysis 33
2.6 Antioxidant Power of the Extracts 34
2.6.1 FRAP (Ferric Reducing Antioxidant Power) Assay 34
2.6.2 DPPH (Diphenyl-1-Picrylhydrazyl) Assay 35
3. Results and discussion 36
3.1 Colorimetric analysis 36
3.2 pH analysis 39
3.3.1 Quantification of the total polyphenols content (TPC) of the edible flower infused vinegars through a F–C assay 40
3.3.2 Quantification of the total polyphenols content (TPC) of the polyphenol rich freeze-dried through a F–C assay 42
3.4 Phenolic characterization of the edible flower infused vinegars 45
3.5 Antioxidant studies 64
Chapter 2 - Astringency experiments **..... 67**
1. Introduction 68
1.1 Astringency 68
1.2 Influence of polyphenols on astringency 69
1.3 The mechanisms behind astringency 70
1.4 Saliva 75
1.4.1 Proline-rich proteins 76
1.4.1.1 Acidic proline-rich proteins 76
1.4.1.2 Basic proline-rich proteins 77
1.4.1.3 Glycosylated proline-rich proteins 78
1.4.2 Statherin/ P-B peptide 78
1.4.3 Cystatins 79
2. Materials and methods 80
2.1. Collection and treatment of saliva 79

2.2. Bicinchoninic acid (BCA) assay 81
2.3. Analysis of the salivary proteins upon interaction with polyphenols through HPLC-DAD analysis 81
3. Results and discussion 84
3.1. Bicinchoninic acid (BCA) assay 84
3.2. Quantification of the precipitation of salivary proteins upon interaction with polyphenois 84
4. Conclusions and future perspectives 96
References 99

List of Tables

Table 1. Values of delta E from day 0 to day 6, day 6 to day 11, day 11 to day 14, day 14 to day 17, day 17 to day 21 and day 21 to day 30. 37

Table 2. UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Control vinegar at 280 nm. retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented. 47

Table 3. UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main phenolic compounds identified in control vinegar at 280 nm and 520 nm. Retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented. 48

Table 4. UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Viola vinegar at 280 nm. retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 50

Table 5. UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main phenolic compounds identified in Viola vinegar at 280 nm and 520 nm. Retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 52

Table 6. UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Centaurea vinegar at 280 nm. retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 54

Table 7. UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main phenolic compounds identified in Centaurea vinegar at 280 nm and 520 nm. Retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 56

Table 8. UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Cosmos vinegar at 280 nm. retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 58

Table 9. UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main phenolic compounds identified in Cosmos vinegar at 280 nm and 520 nm. Retention time (rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented 60

List of Figures

Figure 1: General basic structures of non-flavonoids: a) Hydroxybenzoic acid, b) Hydrocinnamic acid, c) Stilbene and d) Lignan 14

Figure 2: General structures of classes of flavonoids. a) Flavonoid b) Flavone c) Flavonol d) Flavan-3-ol e) Flavanone f) Isoflavone g) Chalcone. 17

Figure 3: Basic structure of an anthocyanin 20

Figure 4. Visual representations of the edible flowers: *Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus* respectively 25

Figure 5. Graph representing the values of Δe measured over time, throughout 30 days, of vinegar in the absence of flowers and of each edible flower infused vinegar 37

Figure 6: Visual representation of the colors of edible flower infused vinegars over time (30 days) 38

Figure 7. Evolution of the values of pH of the vinegars over time (30 days). 39

Figure 8. Progression expressed as (μg/mL) Gallic acid equivalents of the phenolic content (μg/mL) of vinegars throughout the month (day 0, day 7, day 15, day 21 and day 30. The data presented were expressed as mean ± standard deviation (SD) * means that the flower infused vinegar and control vinegar are statistically different $p<0.05$; ** means that the flower infused vinegar and control vinegar are statistically different $p < 0.01$; *** means that the flower infused vinegar and control vinegar are statistically different $p< 0.001$. 40

Figure 9. Content in phenolic compounds in edible flower infused vinegars on the 30th day of infusion. The data presented were expressed as mean ± standard deviation (SD). * means $p<0.05$; *** means $p< 0.001$ 41

Figure 10. Total phenolic content determined by F-C assay expressed as (mg/mL) gallic acid equivalents A) concentration of polyphenols, mg in mL, in a polyphenol rich vinegar extract with a concentration of 2 mg/mL made after the 30 days. B) purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 2 mg/mL. C) concentration of polyphenols, mg in mL, in a polyphenol rich extract with a concentration of 0.5 mg/mL. D) purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 0.5 mg/mL E) concentration of polyphenols, mg in ml, in a polyphenol rich extract with a concentration of 0.25 mg/mL. F) purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 0.25 mg/mL. the results presented were expressed as mean ± standard deviation (SD). * signifies a statistical difference of $p< 0.05$; ** signifies a statistical difference of $p< 0.01$; *** signifies a statistical difference of $p< 0.001$; ****

signifies a statistical difference of p< 0.0001; # signifies that *viola tricolor* is statistically different from the other vinegars p<0.0001 43

Figure 11. Chromatogram of Control vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS. 46

Figure 12. Chromatogram of Control vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS 48

Figure 13. Chromatogram of Viola vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS. 50
Figure 14. Chromatogram of Viola vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS. 51

Figure 15: Chromatogram of Centaurea vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS. 53

Figure 16: Chromatogram of Centaurea vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS. 56

Figure 17: Chromatogram of Cosmos vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS. 58

Figure 18: Chromatogram of Cosmos vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS. 60

Figure 19: Box plot representation of the antioxidant power; FRAP and DPPH; respectively of polyphenol rich edible flower infused vinegars with a concentration of 0.6 mg/mL. Comparison of the antioxidant activity measured for Control vinegar, Viola vinegar, Centaurea vinegar and Cosmos vinegar. Data normalized to Trolox equivalents. White wine vinegar (control vinegar) is represented in blue; *Centaurea cyanus* is represented in green; *Cosmos bipinnatus* is represented in purple and *Viola tricolor* is represented in pink. * means statistical difference of p<0.05; ** means statistical difference of p< 0.01; *** means statistical difference of p< 0.001; # means statistical difference of p< 0.0001 64

Figure 20: Box plot representation of the antioxidant power; FRAP and DPPH; respectively of edible flower extracts with a concentration of 0.6 mg/mL. Comparison of the antioxidant activity measured between *Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus.* Data normalized to Trolox equivalents. *Centaurea cyanus* is represented in green; *Cosmos bipinnatus* is represented in purple and *Viola tricolor* is represented in pink. * means statistical difference of p<0.05; ** means statistical difference of p< 0.01; *** means statistical difference of p< 0.001; # means statistical difference of p< 0.0001 66

Figure 21: Proposed mechanisms linked to the triggering of astringency. 1- Interactions between polyphenols and SPs lead to the formation of soluble complexes, and thereafter, insoluble aggregates. 2- The interactions between polyphenols and SPs provoke the disarranging of the salivary film. 3- Polyphenols binding directly to oral epithelial cells and interactions between polyphenols and proteins in the mucosal pellicle sparks modifications in the pellicle due to the aggregation and sedimentation observed. 4- The mechanoreceptors present in the trigeminal nerve are stimulated and therefore activated by the astringent polyphenols. All of the mechanisms listed are simultaneously involved in eliciting an astringent sensation. Source: (Guerreiro et al.) 73

Figure 22: Chromatogram of the protein profile of saliva detected at 214 nm, with identification of the principal families of SPs. The chromatogram is divided into six zones, with each zone corresponding to a protein family. Adapted from (Soares et al., 2011). 82

Figure 23: Influence of the interaction with different vinegars on the percentage of precipitated proteins in saliva (%) at the concentrations 0.30; 0.45; 0.60 mg/mL. *Viola tricolor* is represented in pink, *Centaurea cyanus* represented in green and *Cosmos bipinatus* in purple and when it comes into contact with Control Vinegar which does not possess edible flowers. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard deviation (SD)..... 85

Figure 24: Influence of different concentrations (0.30; 0.45: 0.60 mg/mL) on the percentage of precipitated proteins in saliva (%). 0.30 mg/mL is represented in orange, 0.45 mg/mL is represented in yellow and 0.60 mg/mL represented in green. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard deviation (SD). 88

Figure 25: Influence of the interaction with edible flowers extracts on the percentage of precipitated proteins in saliva (%) at the concentrations 0.30; 0.45; 0.60 mg/mL. *Viola tricolor* is represented in pink, *Centaurea cyanus* in green and *Cosmos bipinatus* in purple. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. the results presented were expressed as mean ± standard deviation (SD)..... 91

Figure 26: Influence of different concentrations (0.30 mg/mL; 0.45 mg/mL: 0.60 mg/mL) on the percentage of precipitated proteins in saliva (%). 0.30 mg/mL is represented in orange, 0.45 mg/mL is represented in yellow and 0.60 mg/mL represented in green. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. the results presented were expressed as mean ± standard deviation (SD) 93

List of Abbreviations

AAB	Acetic acid bacteria
ANOVA	Analysis of variance
aPRPs	Acid Proline-rich Proteins
BCA	Bicinchoninic acid assay
bPRPs	Basic Proline-rich Proteins
BSA	Bovine Serum Albumin
DPPH	2,2-Diphenyl-1-picrylhydrazyl
F-C	Folin-Ciocalteu
FCR	Folin-Ciocalteu reagent
FRAP	Ferric Reducing Antioxidant Power
gPRPs	Glycosylated Proline-Rich Proteins
HPLC	High-performance Liquid Chromatography
LC-MS	Liquid Chromatography–Mass Spectrometry
LDL	Low Density Lipoproteins
PBS	Phosphate Buffered Saline
PI	Isoelectric Point
PRPS	Proline-Rich Proteins
PUFAS	Polyunsaturated fatty acids
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RSS	Reactive sulfur species
SD	Standard Deviation
SP	Salivary Protein
STATH/P-B PEPTIDE	Statherin/P-B peptide
TAC	Total antioxidant capacity
TFA	Trifluoroacetic Acid
TPC	Total Polyphenol Content

General Introduction

1. Phenolic compounds

Polyphenols consist of a group of naturally occurring compounds widespread in many plant-based foodstuff with a myriad of health benefits that have been amply described (Prabhu et al., 2021). In plants, these compounds tend to exhibit functions related to the defense of the organism against abiotic stresses like drought, UV radiation, heat and heavy metals and biotic stresses like pathogens that might attack said plants (Šamec et al., 2021; Tuladhar et al., 2021) .They are celebrated as agents with antioxidant, anti-allergic, anti-inflammatory, antimicrobial, anticancer and antihypertensive properties, additionally they can act on a multitude of cell receptors and enzymes with the capacity of modifying their activity (Daglia, 2012; Middleton et al., 2000).

The number of polyphenols described in literature continues to increase, as of now around 10 000 phenolic compounds have been identified in spite of the complications associated to this undertaking (Kawa-Rygielska et al., 2018). The setbacks to this research are related to their considerable diversity and complexity in terms of chemical structure and the difficulty in extracting these secondary metabolites from complex plant-based matrices (Guerreiro et al., 2020; Mir-Cerdà et al., 2023).

Polyphenols can differ greatly, and can be categorized into distinct classes according to specific characteristics namely their nature of action, their number of phenol rings and their structural elements albeit they all also share collective features. All of them possess at least two hydroxyl substituent groups (-OH), attached directly to at least one aromatic or benzene ring (Kasprzak-Drozd et al., 2022; Šamec et al., 2021). They tend to be found in foodstuff in their conjugated form, either with sugar residues by β-glycosidic bonds or due to bonds of the sugar to one of the carbon atoms present in the aromatic ring (Gutiérrez-Escobar et al., 2021; Pandey & Rizvi, 2009).The biosynthesis of polyphenols can be derived from distinct pathways: pentose phosphate, shikimate, and phenylpropanoid (de Paulo Farias et al., 2020) .

The major classes of polyphenols are flavonoids and nonflavonoids. Non flavonoids encompass phenolic acids, stilbenes, and lignans. However not all polyphenols fit into these classes, those are considered “other polyphenols”. Phenolic acids can be subdivided in hydroxybenzoic and hydroxycinnamic acids. The metabolites of benzoic acid can be monohydroxy, dihydroxy or trihydroxy derivatives (Ivanova et al., 2002). Flavonoids can be further categorized as flavones, flava-3-ols, flavonols, isoflavones, flavanones, chalcones, condensed tannins and anthocyanins. Other

polyphenols encompass lignins, xanthones, chromones and anthraquinones (Pandey & Rizvi, 2009; Tresserra-Rimbau & Lamuela-Raventós, 2017).

1.1. Food sources of Polyphenols

Dietary polyphenols encompass compounds of varying complexity: monomers, oligomers and polymers, with the dietary sources of these molecules including many products regularly ingested across the world such as tea, coffee, wine and fruits (Scalbert et al., 2005).

There´s an elevated variability pertaining both to the concentration and the type of polyphenols present in different vegetal species, with many of them containing the presence of diverse groups of phenolic compounds, some are present pervasively in many foodstuffs and other are exclusive to certain plant families (Cheynier, 2005). Within the individuals of each vegetal species the climate, the geographical region, the time of harvest and the biotic interactions will also factor into its polyphenol composition (Manach et al., 2004; Vong et al., 2022). Two thirds of the intake of polyphenols revolve around flavonoids while the other third of them is taken up by various phenolic acids, of the flavonoids available : flava-3-ols and their oxidation products dominate (Chun et al., 2007).

Hydroxycinnamic acids can be found in a large multitude of foodstuff for example: in apples, cereal grains, coffee, cinnamon, ginger, grapes, pineapple, potatoes, lettuce , spinach and olives (Martinez et al., 2017). Monohydroxybenzoic acid derivatives' only notable dietary source is olives whilst large amounts of both dihydroxybenzoic and trihydroxybenzoic acids derivatives are available in cloves and also in chestnuts at concentrations of 250-900 mg per 100 g and more modest amounts can be seen in berries, black tea and other spices (Vong et al., 2022). Red wine, grapes, berries, dark chocolate, peanuts and pistachios comprise the main sources of stilbenes (El Khawand et al., 2018; Fernández-Marín et al., 2012). Lignans are mainly isolated in: cereals and grain products namely wheat, oats, rye, and barley, with flaxseed and sesame seeds constituting sources of lignans of particular note (Durazzo et al., 2018; Tetens et al., 2013), the content of lignans in cereals and products derived from cereals oscillates from 23 - 401 µg per 100 g of its dry weight (Durazzo et al., 2013).

Flavonoids can be found in a number of different sources. Flavones constitute part of the consumer´s diet thanks to both spices and teas, its intake per person is estimated to be around 1−5 mg per day but is projected to increase to 5−20 mg if all dietary sources are accounted for (Vong et al., 2022). Whereas the principal sources of flava-3-ols are

chocolate and cocoa with values of flava-3-ols reaching around 920-1220 mg per 100 g, tea in which it´s possible to find up to 300 mg of flava-3-ols per infusion and apples with concentrations of 120 mg per 200 g. It´s been estimated that the average person´s daily intake of flava-3-ols fluctuates around 50 to 100 mg (Heiss et al., 2010). Flavonols are another subclass that exists more plentifully in plant-based food, predominantly in leafy vegetables, broccoli, onions, tea and apples (Wang et al., 2009). Dietary sources are contingent on the eating habits of the inhabitants of different countries, with studies pointing to green tea being the main source of flavonols in Japan and fruit and vegetables being the main one in European countries like Finland, with the principal flavonol quercetin being present abundantly in onions (Aherne & O'Brien, 2002).Flavanones are the most prevalent in citrus fruit for example oranges, grapefruits, tangelos and mandarins with an average daily intake around 20−60 mg per day, they´re also common in tomatoes and mint but in lower concentrations (Barreca et al., 2017; Escobar-Cévoli et al., 2017). Isoflavones are commonly available in the *Leguminosae* family, markedly in soy, and also in smaller amounts present in nuts and chickpeas. Depending on the consumption of soy and its derivatives, the daily intake of isoflavones can reach values of 15−60 mg per person per day (Gómez-Zorita et al., 2020; Kuryłowicz, 2020; Vong et al., 2022). Chalcones are frequently found in foodstuff from *Moraceae, Leguminosae* and *Asteraceae* families (Rozmer & Perjési, 2016). Dietary sources rich in anthocyanins encompass different types of berries, grapes, purple varieties of vegetables like purple corn and purple sweet potato, with many anthocyanins being found in edible flowers (Hellstrom et al., 2009; Teixeira et al., 2023).The phase of maturation also affects the concentration of anthocyanins with its content amplifying amidst fruit ripening (Yan et al., 2023). In a study about anthocyanins intake conducted in the USA around 2001-2002 by NHANES (National Health and Nutrition Examination Survey) the daily consumption of anthocyanins was estimated to be 12,5 mg per day per person (Wu et al., 2006).

2. Edible flowers

Edible flowers are not only valuable for their ornamental merit as garnishes and decoration due to the captivating beauty and aroma associated to them and for being low on calories and low on fat (Drava et al., 2020; Kumari & Bhargava, 2021) but also for their health promoting profile, attributable to them being rich in many micronutrients and proteins, particularly for being sources of bioactive phytochemicals like polyphenols (Marchioni et al., 2020; Prabawati et al., 2021). In edible flowers the most frequently found biologically active compounds are carotenoids, phenolic acids, anthocyanins, and flavonoids. Oftentimes edible flowers possess quantities of vitamin C, carotenoids and polyphenols (specifically anthocyanins) superior to the quantities of these compounds present in common fruits and vegetables (Kumari & Bhargava, 2021). A hallmark of polyphenol rich flowers are their medicinal proprieties, the strong antioxidant potential associated to them makes them valuable anti-inflammatory, antinociceptive and anti-mutagenic agents (Chen & Wei, 2017; Umme, 2024).

According to current nomenclature It´s possible to pinpoint worldwide around 97 different families, 100 genera and 180 species of edible flowers (Lu et al., 2016). The choice of flowers for human consumption is pivotal in order to guarantee the safety of consumers, flowers in order to be considered edible must be devoid of toxicity and be nutritious, it is also recommendable to give preference to flowers with a multitude of polyphenols (Nowicka & Wojdyło, 2019; Takahashi et al., 2020). The edibility of flowers also ranges from being totally edible to being only partly edible, with partly edible flowers having certain parts that are edible and others that are not, the content of edible flowers present is also vital since certain flowers can be eaten only within certain quantities (Kumar et al., 2022).

Nutritionally, edible flowers are mostly water, with water factoring in as 70-95% of their composition, they also contain carbohydrates that are the most amply present macronutrients, potassium, calcium , magnesium and phosphorus all stand out as the foremost minerals present which oftentimes are not provided in sufficient numbers in the diet (Fernandes et al., 2017; Pires et al., 2019). Edible flowers' content in fatty acids oscillates between 0.1 to 15% of its dry weight, they contain unsaturated fatty acids that are essential fatty acids such as linoleic acid (C18:2) and α-linolenic acid (C18:3) and the most frequently present saturated fatty acid that's also part of the essential fatty acids ,palmitic acid (C16:0), they need to be included in the diet of consumers because it´s impossible for them to be synthesized by the body (Jadhav et al., 2023; Rivas-García et al., 2022). Edible flowers' dietary content in fibers is highly variable being very

dependent on the species in question, in some species a considerable source of fiber can be witnessed and their content in proteins tends not to be particularly high, with cornflowers being a noteworthy exception (Jadhav et al., 2023).

2.1. Historical contextualization of edible flowers

Around the world the consumption of edible flowers, known as flowerphagia, is already part of the traditional gastronomies of several countries, their ingestion dating back to Ancient Greece, Rome, Egypt and China, they´re thought of as a delicacy by many and frequently associated to traditional festive celebrations (Takahashi et al., 2020; Umme, 2024).The first documented use of edible flowers can be traced all the way back to 3000 B.C, there´s reports of recipes in ancient China incorporating edible flowers in food preparations such as recipes utilizing petals of lotus flower (*Nelumbo nucifera*), in Ancient Rome people used lavender in sauces and flowers such as violets and roses were used in several recipes to sweeten purees and omelettes. Edible flowers were even referenced in Holy texts like the Bible which touches upon the consumption of dandelions (*Taraxacum officinale L.)* (Janarny et al., 2021; Takahashi et al., 2020). According to literature, they were used in distinct manners in various countries namely in the vein of the Victorian English tradition of candying violets for decoration of desserts and the French tradition of carnations being utilized in the making of the classic liquor Chartreuse (Fernandes, Casal, Pereira, Saraiva, et al., 2020) . Flowers like squash blossoms were consumed in Zuni tribes in the north of the American continent and by the Aztecs in the south of the American continent (Fernandes et al., 2017; Newman & Kirker, 2016).

Traditionally, edible flowers have been used as folk remedies in the treatment of a series of maladies, such as chamomile (*Matricaria chamomilla* L.*)* , usually in the form of an infusion, which can be used for gastrointestinal issues because of its association with the diminishment of both sugar absorption and carbohydrate digestion (Villa-Rodriguez et al., 2018), and roses which have been ingested as a way to improve blood circulation and to ease menstrual pain (Yang & Shin, 2017). In Nepal Mountain Ebonies (*Bauhinia Variegata L*.) were utilized in the treatment of diarrhoea and wild orchids were used to help with conditions ranging from broken bones, fevers, wounds and burns (Subedi et al., 2013).

Several traditional dishes that are still being made today use edible flowers including: Vazhaipoo Poriyal a traditional Indian dish made from cooked banana flower seasoned with green chili, mustard, and onion (Sarkar et al., 2015) ; rosewater which is very popular in several middle-eastern countries; cynara flowers (*Cynara cardunculus*

var. scolymus L.) that are traditionally used in the Iberian Peninsula for their enzymes that promote coagulation in the manufacturing of sheep cheese; the Italian dish Fritelle di Fiori d'Acacia that contains flowers powdered with sugar and frittered petals; buds of daylilies are in Hong Kong commonly known as the golden needle vegetable and the bulbs are utilized in several dishes such as soups and stir-fries in Shanghai (Cristóvão et al., 2022; Fernandes, Casal, Pereira, Saraiva, et al., 2020). These dishes are frequently associated to old traditional festive celebrations and prior to their resurgence in popularity that has been taking place these past few decades, the consumption of edible flowers was in fact actively decreasing due to globalization (dos Santos & Reis, 2021). According to a survey, nowadays many young people who are not well versed in the culinary history of the traditional edible flowers famous in their country of origin are more likely to learn about them through online platforms, requiring for the growth of this niche market to happen, a greater dissemination of data about them (Kumar et al., 2022).

2.2. The present-day popularity of Edible flowers

Globally, consumers are more prone to prioritizing well balanced diets and high-quality food which increases their concern with the nutritional profile of the food available to them, motivating the consumption of foodstuff with a positive impact on human health and on their wellbeing (Guerreiro et al., 2020; Zitka et al., 2011). This heightened interest in healthy eating and a larger demand for nutritious alternatives is a growing market in the current landscape. Stimulating new sources of income, inspiring a superior culinary progress by inciting a greater exploration of innovative food products (Benvenuti & Mazzoncini, 2021; Kinupp et al., 2021; Yasar et al., 2022). It´s an expanding market, having been projected from 2020 to 2026 a 90.5 million dollars market size growth of packaged edible flowers with a steady annual growth of 4.24% in 2021 and of 4.40% in 2022 (Pires Jr et al., 2023).

Conversely, it´s also imperative to consider that as reported by the World Health Organization (WHO) the fast paced lifestyle that many people lead nowadays is linked to having less time to adequately prepare meals, which contributes to the consumption of ready-to-eat very processed foods that are tied to conditions such as obesity and cardiovascular diseases, with edible flowers appearing as a plant-based nutrient-rich alternative to this issue so frequent in our hectic society (Jadhav et al., 2023; Umme, 2024).

From an environmental standpoint, the production and processing of edible flowers garners attention for having lower repercussions on the environment (Pires et al., 2019).

The production of these species is concordant with the need for urban cropping in cities, hence boosting urban diversity, the small-scale decentralized production of edible flowers facilitates the obtention of ready-to-eat locally produced food products fostering a favourable impact at the level of local commerce (Eigenbrod & Gruda, 2015; Pires Jr et al., 2023).

2.3. Edible flower´s culinary use

Edible flowers are commonly consumed fresh however it´s also possible to consume them in other forms such as cooked, dehydrated, lyophilised, or candied, they can be incorporated in numerous dishes both savoury and sweet such as sauces, salads, soups, jelly, and desserts. Not only adding to these dishes, a distinguishing flavor dependent on the species of edible flower chosen, along with providing pleasing colors intended to lure in consumers but also meeting the food restrictions associated with a vegetarian or vegan lifestyle (Mlcek et al., 2021; Takahashi et al., 2020). Sensorial studies are necessary to evaluate the public's reaction to food preparations containing edible flowers which will be contingent on their image, smell, texture and flavor, being paramount that these flowers come across as appetising to the general population which will boost their commercial potential (Takahashi et al., 2020; Teixeira et al., 2023). A study conducted in Portugal in 2019 about the knowledge surrounding the consumption habits of edible flowers shows that in a sample group of 247 participants, 94.3% of them had already heard of edible flowers and 69.0% of the participants had already consumed edible flowers at some point, thus showing an openness from the public to the consumption of these products (Guiné et al., 2019).

A possible hurdle to consider that weakens the appeal from a food industry perspective of working with edible flowers and limits their commerciality is their considerably reduced shelf life, since edible flowers are easily perishable (Benvenuti & Mazzoncini, 2021). A viable option to help enlarge their shelf life while simultaneously creating an innovating and exciting food product is to preserve them in vinegar such as white wine vinegar, which consists of a polyphenol-rich food matrix, by total immersion of the petals of flowers in vinegar, the conservation of their appealing colors and pleasant aromas is thus achieved. Thereby, permitting the fabrication of new varieties of white wine vinegars whose organoleptic properties(properties experienced by the senses, namely color, aroma, mouthfeel and flavor) are going to be influenced by the flowers inserted in them and afterwards could be used in different dishes, ergo the inclusion of

edible flowers plays a compelling role in the development of new products with added value (Kou et al., 2012; Nicolau & Gostin, 2016; Purohit et al., 2021; Szczesniak, 2002; Xia et al., 2022).

3. The case of vinegar

Vinegar is a well beloved acidic condiment greatly valued for the flavor that it adds to a variety of dishes, as well as for its function as a natural preservative (Perumpuli & Dilrukshi, 2022).

It was in ancient civilizations that vinegar was discovered accidently by leaving wine in the open air which subsequently became vinegar, with the first written testimony of it being around 5000 years old, its use was initially linked to the preservation of food (Román-Camacho et al., 2023). It has also been used medicinally throughout History as a cure for a number of maladies such as burns, rashes and stomach aches (Perumpuli & Dilrukshi, 2022).

Vinegar is generated through different raw materials via a fermentation process that incorporates two fermentations: the first is the alcoholic fermentation in which yeast converts sugars into ethanol and the second one is by the group of acetic acid bacteria (AAB) that oxidizes ethanol, yielding its crucial element: acetic acid (Chen et al., 2020; Gomes et al., 2018). For table vinegar, the concentration present of this acid will vary from 4% to 8% (Hailu et al., 2012). Wine vinegar, in specific, is the most widely consumed variety of vinegar in countries in the Mediterranean area, thus motivating a greater interest in including them in food product research (Paneque et al., 2017).

Vinegar is currently utilized in both pharmaceutical and nutritional areas because it has come to be associated with antioxidant, antibacterial and digestion promoting activities which furthermore increases the scientific curiosity around this condiment and the bioactive compounds contained in it, namely their content in polyphenols. These phenolic compounds can result from the raw material from which vinegar is originated from or they can form in the course of fermentation, with the amount and existence of polyphenols in vinegar being inconstant and as a result characterization and quantification of them sparking great interest (Bai et al., 2024; Kara et al., 2022; Melkis & Jakubczyk, 2024). It´s relevant to bear in mind that the huge instability of dietary polyphenols complicates the characterization of the constitution of polyphenols in any given food matrix because in the course of food processing the phenolic compounds continue to quickly transform into other compounds (Cheynier, 2005).

Vinegar can be perceived as astringent, with the phenolic compounds present in it playing a role associated to the modulation of this organoleptic property which can be described as a puckering mouthfeel, constituting the tactile component of flavor. It´s worth noting that the presence of this organoleptic property can influence positively or negatively a consumer´s preference or distaste for a particular food product. The underlying molecular mechanisms responsible for causing said astringency still remain somewhat unclear and subject to further research (Green, 1993; Huang & Xu, 2021; Laguna & Sarkar, 2017; Ousaaid et al., 2021).

Objectives

The leading aim of the present research was to investigate the influence of the addition of three species of polyphenol-rich edible flowers (*Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus*) in white wine vinegar and creation of a new food product with added value. We proceeded to both quantify and identify the phenolic compounds present in vinegar after being infused with flowers with the goal of boosting the ingestion of polyphenols. Polyphenols have the ability of shaping several physicochemical properties, so we evaluated the progression over time of the colors of vinegars infused with edible flowers and assessed any potential modifications of pH linked to the incorporation of these polyphenol-rich flowers. In this thesis, it´s crucial as well the assessment of the antioxidant properties of this new food product through DPPH and FRAP assays, thus proving that value was added to white wine vinegar from a health benefit perspective.

The second part of the thesis aimed to study astringency which is a complex sensation that exerts a considerable effect over the perception of taste by consumers, influencing their choice for plant-based products. We delved into the modulation of astringency by interaction between specific families of salivary proteins and dietary polyphenols, intending to better understand surrounding how the infusion of edible flowers influences the astringency experienced by people when consuming vinegar. Taking into account, that this modulation is not necessarily going to either increase or reduce the astringency felt. We proceeded to study the interactions of polyphenols in the edible flower infused vinegars with the following salivary protein families: basic proline-rich proteins, glycosylated proline-rich proteins, acidic proline-rich proteins, statherin/ P-B peptide and cystatins.

The work was structured into two chapters exploring different effects of the incorporation of edible flowers in vinegars:

Chapter 1 - **Phenolic quantification and characterization of the edible flower infused vinegars based on their physicochemical and antioxidant properties**

- Preparation of polyphenol-rich edible flower infused vinegars
- Colorimetric analysis and pH measurement
- Quantification of phenolic content through Folin-Ciocalteu assay
- Identification of polyphenols through LC-DAD/ESI-MS analysis
- Measurement of free radical scavengers through DPPH assays
- Measurement of free radical quenchers through FRAP assays

Chapter 2 - **Astringency experiments**

- Saliva Isolation and Analysis
- Indirect quantification of salivary proteins that suffered precipitation upon interacting with polyphenols through HPLC-DAD analysis

Chapter 1 - Phenolic quantification and characterization of the edible flower infused vinegars based on their physicochemical and antioxidant properties

1. Introduction

1.1. Structures of Polyphenols

1.1.1. Non-flavonoids

Phenolic acids, stilbenes and lignans all fall under the umbrella of non-flavonoids (Bié et al., 2023). Phenolic acids are a class of dietary polyphenols that operate as antioxidants through free radical scavenging and chelation that are synthesized through the shikimate pathway from L-phenylalanine or L-tyrosine (Brglez Mojzer et al., 2016; Heleno et al., 2015). They can be divided into two subclasses: hydroxycinnamic acids, which are obtained from cinnamic acid, with a C3–C6 backbone frequently found in foodstuff esterified with quinic acid or glucose and into hydroxybenzoic acids, which are produced from benzoic acid with a C1-C6 backbone bound with lignin or in soluble state conjugated with sugars and organic acids (Kumar & Goel, 2019; Tsao, 2010). Hydroxycinnamic acids constitute a more commonly found subclass of polyphenols than hydroxybenzoic acids which are solely located in few foodstuffs consumed by human beings which diminishes the interest and, therefore the research applied to them (Manach et al., 2004). Caffeic acid and coumaric acid are common representative hydroxycinnamic acid derivatives, other representatives include ferulic acid and chlorogenic acid (Alam et al., 2016).Caffeic acid, 3,4-dihydroxycinnamic acid, is composed of a 3,4-dihydroxylated benzene ring linked to a phenylpropanoid structure attached to a carboxylic acid (COOH) and it´s one of the most abundant dietary dihydroxycinnamic acids, with ferulic acid constituting its methylated metabolite (De Ferrars et al., 2014; de Sousa Ferreira et al., 2022; Heindl et al., 1985). p-Coumaric acid, 4-hydroxycinnamic acid, is present in plants in cells walls bound to different lignocellulosic materials, most of the predominant linkages are ether linkages between phenolic hydroxyl groups and lignin (Zhao et al., 2011). p-Coumaric acid can thereafter be converted into other phenolic acids such as caffeic acid and chlorogenic acid or they can be transformed in other compounds like flavonoids (Pei et al., 2016).

Gallic acid, 3,4,5-trihydroxybenzoic acid, is a common representative of a trihydroxybenzoic acid derivative and it's the precursor of a high number of plant tannins, gallic acid and its ester derivatives such as propyl gallate, octyl gallate, and lauryl gallate are known for their multiple health benefits (Liu et al., 2020; Sharma et al., 2017; Zhao & Hu, 2013). Other representatives of derivatives of hydroxybenzoic acid include protocatechuic acid and vanillic acid (Gojak-Salimović et al., 2023).

Stilbenes are polyphenols with a 14-carbon skeleton (C6−C2−C6) with two aromatic rings connected through an ethylene moiety, with the diversity coming from the presence of distinct substituents, that are bound together by an ethylene bridge. Depending on the ethylene bridge, stilbenes can be isomers *trans*-stilbenes or *cis*-stilbenes which lack the stability of the *trans* isomers due to the steric interactions among the phenyl moieties (Duta-Bratu et al., 2023; Pecyna et al., 2020). Upwards of 400 stilbenes have been identified generated from *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) , with resveratrol being one of the main representatives of this group (Chong et al., 2009). Many stilbenes are only produced in plants in the eventuality of injury or infection occurring, acting as anti-fungal phytoalexins. These compounds have relevant health benefits namely inhibiting cellular events necessary for carcinogenesis to take place (Lall et al., 2015). Stilbenes have anti-inflammatory, antimicrobial, antifungal and anticancer activity with applications for the treatment of obesity and diabetes (Pecyna et al., 2020). Lignans are comprised of two phenylpropanoid C6–C3 units, thus possessing a 2,3-dibenzylbutane scaffold (C6−C3-C3−C6), they constitute one of the major classes of phytoestrogens known as antioxidants relevant for cell wall formation of plants (Brglez Mojzer et al., 2016; Owen et al., 2000). The most common representative lignans are pinoresinol, secoisolariciresinol, matairesinol, medioresinol, lariciresinol and syringaresinol (Durazzo et al., 2018).

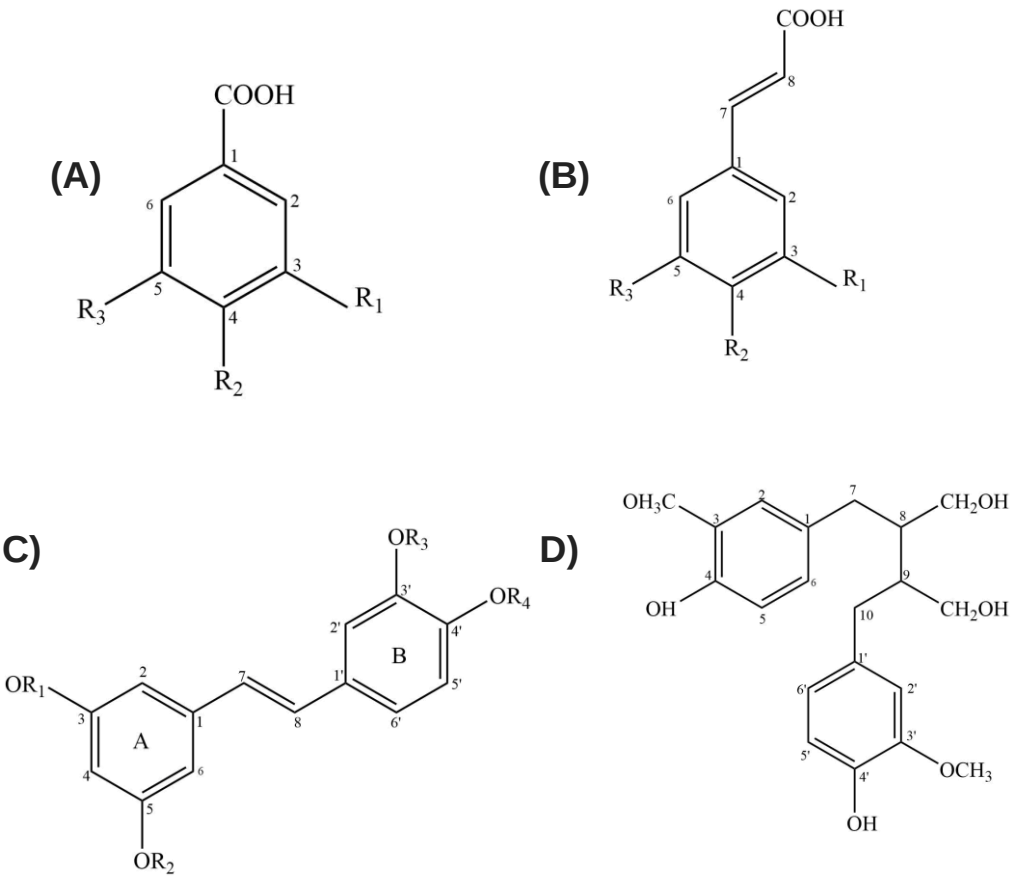

Figure 1: General basic structures of non-flavonoids: A) hydroxybenzoic acid, B) hydrocinnamic acid, C) stilbene and D) lignan.

1.1.2. Flavonoids

Flavonoids are C15 compounds with a C6-C3-C6 skeleton composed of two aromatic rings (Ring A and Ring B) with a three-carbon bridge between phenyl groups as illustrated in Figure 2 that are biosynthetically attained from acetate and shikimate. While all flavonoids display this basic structure, what distinguishes them from each other are the variations to the pyran ring created by cyclization with oxygen (Ring C) (Manach et al., 2004; Ramos, 2007). The alterations to the patterns of hydroxylation of Ring C, which is a process of insertion of hydroxyl groups (-OH) , is conditioned by the formation or absence of a carbonyl group (C=O) at the C4 position and the existence or lack thereof a double bond between C2 and C3 (Singla et al., 2019; Ullrich & Hofrichter, 2007). Through the scrutiny of the oxygenated heterocycle present, it´s possible to establish the distinct subclasses of flavonoids and within each subclass it's possible to distinguish the compounds of each one, based on the observation of distinct hydroxylation and methylation patterns of both rings A and B (de Araújo et al., 2021). Glycosylation has an important role affecting the properties of the subclasses of flavonoids in cells through the improvement of the solubility and stability of the compounds, O-linked glycosylation is the most abundant type in which the backbone via one of the -OH groups is attached to sugar moieties, direct C-linked glycosylation is also possible but it´s a less common form of glycosylation (Jiang et al., 2016). Flavonoids are highly important for plants because of their role of protection against stresses such as reactive oxygen species (ROS), tolerance to the cold, drought and heat, plus they´re floral pigments, antimicrobial agents and regulators of the growth of cells (Das et al., 2014; Dias et al., 2021). When consumed, they can perform as antioxidants mainly as free radical scavengers, with the antioxidant capacity of flavonoids being shaped by the phenolic hydroxyl groups present (Horvathova et al., 2003; Pannala et al., 2001), it´s important to consider that distinct classes of flavonoids that might vary considerably structurally will exhibit common health benefits (Brglez Mojzer et al., 2016).

Flavones (Figure 2B) are a subclass of flavonoids whose structural defining characteristic is the connection of the B ring to C2 position and double bond between carbon at position 2 and carbon at position 3, with their variability coming from processes of hydroxylation, *O*-/*C*-glycosylation, *O*-methylation, and acylation (Jiang et al., 2016). Apigenin and luteolin are representative flavones, with the structure of luteolin containing one more hydroxyl group than the structure of apigenin (Calderón-Oliver & Ponce-Alquicira, 2018).

The basic structure of isoflavones (Figure 2F) only differs from the structure of flavones in the position of the ring B, that is linked to the C-2 position of ring C in flavones and to C-3 position in isoflavones (Sohn et al., 2021). They´re highly polar flavonoid phytoestrogens whose structure closely resembles mammalian estrogens, with genistein, daidzein and glycitein counting among their most active compounds of this subclass (Setchell & Cassidy, 1999; Vitale et al., 2013).

Flavan-3-ols (Figure 2D) possess a C6-C3-C6 scaffold, the benzene rings are connected by a heterocycle that´s been oxygenated and hydroxylated at the C-3 position (Martín & Ramos, 2021), they can occur in monomeric, oligomeric and polymeric form , with the polymeric form being known as condensed tannins (Monagas et al., 2010) . It´s recurring the alteration of the hydroxyl group (-OH) of their flavan-3-ol core in tea specifically by addition of a gallate group (Heiss et al., 2010; Martín & Ramos, 2021). Examples of flavan-3-ols include (-)-epicatechin and (+)-catechin (Es-Safi et al., 1999).There´s two chiral centers: in the structure of the isomer with a *trans* configuration ,catechin, and the in the structure of the isomer with a cis configuration, epicatechin, attached to the C-2 position and C-3 position. Thus, allowing the existence of four diastereoisomers namely: (+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin (Colomer et al., 2017).

Flavonols (Figure 2C) are a group with a 3-hydroxyflavone scaffold, with quercetin, myricetin and kaempferol being the leading representatives (Chagas et al., 2022; Escobar-Cévoli et al., 2017). Quercetin, (3,3',4',5,7-pentahydroxyflavone), is an important antioxidant and plant pigment with a yellowish color utilized for the treatment of metabolic and inflammatory conditions as well as being recognized as a antitumor, antioxidant and antiviral agent (David et al., 2016). Myricetin has a similar structure to quercetin but with an additional -OH hydroxyl at the 5' of ring B, with a more elevated number of hydroxyl groups increasing its capacity as an antioxidant in comparison to quercetin. This compound is predominantly found in the families *Myricaceae, Pinaceae, Primulaceae, Polygonaceae* and *Anacardiaceae* (Agraharam et al., 2022). Rutin is devised from an aglycone quercetin substituted at the C-3 position of the C ring with a disaccharide rutinose linked by a glycosidic bond (Ademosun et al., 2016; Domitrović et al., 2012). Kaempferol stands out for its valuable role in cellular signalling pathways modulation, as well as a powerful promoter of apoptosis, it´s very promising in regard to cancer treatment due to its lower toxicity in contrast to the standard chemotherapy medication (Chen & Chen, 2013; Hikmawanti et al., 2023).

The structure of flavanones (Figure 2E) contains the ring A and ring B linked by a dihydropyrone ring characterized specifically by the absence of a lack of double bond between C2–C3 and a chiral carbon at the position of C-2, they're celebrated for their antihypertensive, antioxidant, anti-inflammatory properties with the ability to reduce the levels of lipids. Hesperidin, naringin, eriodictyol, isosakuranetin and taxifolin are important representative flavanones (Barreca et al., 2017; Chanet et al., 2012).

Chalcones (Figure 2G) are open-chain compounds with a 1,3-diaryl-2-propen-1-one core called chalconoid, the structure is composed by two phenyl rings with ring A being the one linked to the carbonyl group. It can adopt a more thermodynamically stable *trans* configuration or a more unstable cis one , with the instability being provoked by the steric effects between ring A and the carbonyl group (Salum et al., 2020; Zhuang et al., 2017). They´re recognized for their antioxidant, anti-inflammatory and antibacterial properties, with them being useful for the treatment of stomach ulcers (Hall et al., 2020).

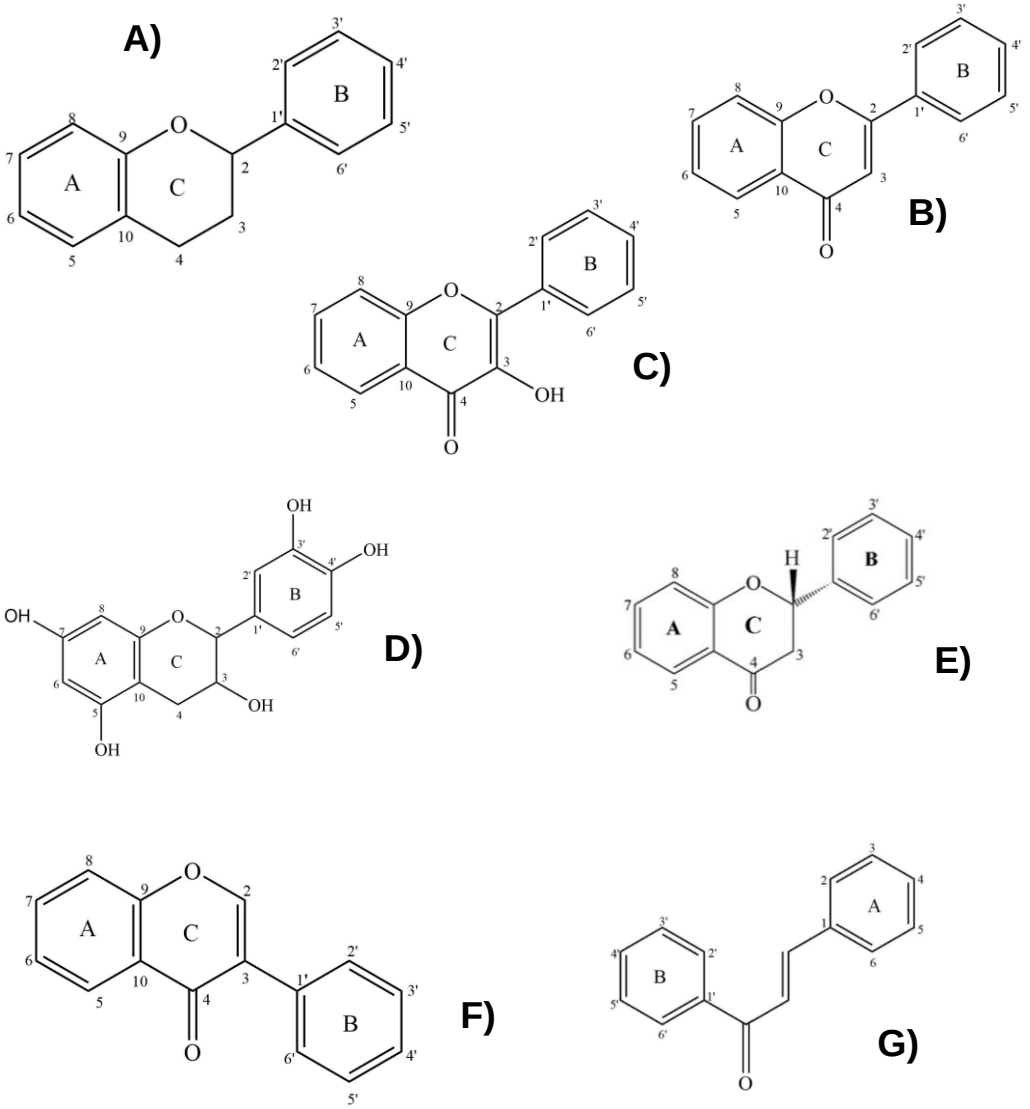

Figure 2: General structures of classes of flavonoids. A) flavonoid, B) flavone, C) flavonol, D) flavan-3-ol, E) flavanone, F) isoflavone and G) chalcone.

1.1.2.1. Anthocyanins

Anthocyanins are a group of water-soluble flavonoids of particular high importance that can be useful as natural colorants in the food industry, these pigments provide color to many plant-based foodstuff, acting as red, blue or purple food coloring agents with functions in pollination and seed dispersal in plants. Anthocyanins' possible medicinal applications in diabetes and cardiovascular disease prevention are also a key aspect of these phenolic compounds (Burton-Freeman et al., 2019; Rodriguez-Amaya, 2019; Wu et al., 2006; Yang & Shin, 2017). Around 635 anthocyanins have been detected, which unlike their precursors anthocyanidins, of which there´s 23 known ones, retain a more robust stability thanks to their sugar moieties (Ali et al., 2016; He & Giusti, 2010; Kruger et al., 2014; Q. Qi et al., 2023). Anthocyanidins are aglycones and anthocyanins constitute their glycosides and acylglycosides, with the anthocyanins resulting of glycosylation of aglycones with sugar groups like glucose, galactose, and arabinose that can subsequently be acylated (Strack & Wray, 2017; Zhang et al., 2004). The core of anthocyanidins is the flavylium core so they are constituted by a C15 skeleton composed by three rings: the aromatic fused ring (ring A), the heterocyclic benzo-pyran ring (ring C) which results from the fusion of an aromatic ring and a pyran and one phenyl ring (ring B), they´re charged positively when they´re in the cationic form as in the flavylium ion form in which they possess two double bonds in the C ring with a positive charge at the oxygen atom (Das et al., 2019; Katritzky et al., 2008). It´s possible to obtain different anthocyanidins by hydroxylation and methylation in different positions of the flavylium cation skeleton (OH, H, or OCH_3), with cyanidin, malvidin, delphinidin, peonidin, petunidin, and pelargonidin being the most commonly encountered anthocyanidins (Mattioli et al., 2020).

Pertaining to the role of the degree of hydroxylation in anthocyanidins, it´s important to consider pelargonidin, cyanidin and delphinidin. Pelargonidin has one hydroxyl (-OH) group linked to C-4' position (R_2) of Ring B; Cyanidin which is the most vastly distributed anthocyanidin has two hydroxyl (-OH) groups attached to the C-3´and C-4´positions (R_1 and R_2) of Ring B; Delphinidin has three hydroxyl (-OH) groups linked to the C-3´, C-4´and C-5´ positions (R_1, R_2,R_3) (Table1) (Prasad et al., 2023; Praveena et al., 2022). The hydroxyl groups can suffer methylation mediated by *O*-methyltransferases (Gomez Roldan et al., 2014). Peonidin is derived from cyanidin and is characterized by a methoxy group at the C-3' position (R_1) and one hydroxyl group in the C-4'-position (R_2), malvidin is derived from delphinidin and possesses two methoxy groups linked to the positions C-3′ and C-5' (R_1 and R_3) and one hydroxyl group at the

C-4‘ position (R_2) (Du et al., 2015; Kähkönen & Heinonen, 2003; Seitz & Hinderer, 1988) and petunidin is derived from delphinidin by methylation and possesses a methoxy group attached at the position C-3′ and a hydroxy group at the C-5′ position (Jiang et al., 2021; Kamiloglu & Akgun, 2023).

The hydroxyl groups of anthocyanidins can be furthered altered by glycosylation, in which these groups suffer partial or total substitution by one, two or three sugars or even further acylation of the sugar moieties, in turn changing the reactivity, polarity, spatial distribution observed as well as the physical and chemical characteristics of the anthocyanins produced (Giusti & Wrolstad, 2003; Mattioli et al., 2020; Zhao et al., 2014). Differences of substituents bound to carbons of the basic skeleton allow these six anthocyanidins that vary only in regards to the substituents present in the ring B to form the majority of anthocyanins (Khoo et al., 2017; Teixeira et al., 2023).

The distribution of cyanidins in fruits and vegetables is around 50%, the distribution of delphinidin, pelargonidin, peonidin is 12% and of malvidin and petunidin is 7% (Khoo et al., 2017). All of these anthocyanidins are characterized in nature by distinct colors: cyanidin and peonidin showcase a magenta tone; delphinidin has a blue-reddish or purple hue; pelargonidin possesses an orange coloration; malvidin exhibits a purple tone while also being the predominant dark red pigment in red wine, with petunidin also manifesting a dark red or purple shade (Khoo et al., 2017; Mazza & Francis, 1995). In one regard, the diversity of anthocyanins relative to the structure coupled with them being very reactive compounds and amphoteric can constitute a hitch in the process of their identification and quantification, however, them being absorbed in a different range than the other polyphenols , meaning they absorb in the visible range around 500-550 nm, aids in their identification because they´re selectively detected from the other subclasses (Buljeta et al., 2024; Jackman et al., 1987).

Anthocyanins can operate as quality indicators regarding food ripening, when fruits like red apples showcase a sufficient quantity of anthocyanins in their skin it signifies that the fruit is in a ripe state with their high solubility facilitating their assimilation in aqueous food systems (Markakis, 2012).

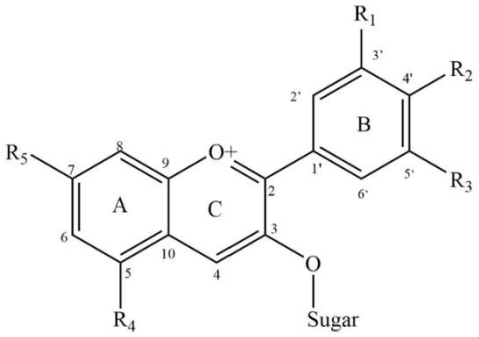

Figure 3: Basic structure of an anthocyanin. R_1, R_2, R_3, R_4 and R_5 in anthocyanins can be hydroxyl groups (-OH) or sugars.

List of the six major occurring in nature anthocyanidins. Adapted from (Wu et al., 2006)

	R1	R2	R3	R4	R5
Pelargonidin	H	OH	H	OH	OH
Cyanidin	OH	OH	H	OH	OH
Peonidin	OMe	OH	H	OH	OH
Delphinidin	OH	OH	OH	OH	OH
Petunidin	OMe	OH	OH	OH	OH
Malvidin	OMe	OH	OMe	OH	OH

1.2. Phenolic compounds' effect on the physicochemical properties of flowers

The visual appearance of edible flowers will be key in determining consumer´s choice considering that it is the first sensory quality of the flower noticed by the public, and sparking the curiosity of said consumer with the other sensory qualities like texture and flavor only comes into play later on, hence the color of a food product containing edible flowers must be thought of as a priority, making the presence of pigments particularly noteworthy (Takahashi et al., 2020).

Pigments have a chromophore that possesses the ability of absorbing light in specific wavelengths of the visible range of the electromagnetic spectrum, with the remaining ones being either reflected or refracted and thus being responsible for the flower colors that are by us perceived (Silva et al., 2020; Sousa, 2022).

The presence of polyphenols alters the color, aroma and flavor of the plants that contain them, therefore giving them unique traits. In the case of flowers, the color that they exhibit will be related to the existence of compounds like flavonoids, in particular, anthocyanins and also of certain non-phenolic pigments like carotenoids and betalains (Kumari & Bhargava, 2021). Both the quantity and quality of carotenoids, which is a subclass of over 750 naturally occurring terpenoids soluble in lipids, often provide yellow and orange hues that will affect the overall color of the flowers alongside the less common water soluble betalains that confer yellow, orange and bright red tones

(Benvenuti & Mazzoncini, 2021; Silva et al., 2023; Tanaka et al., 2008). Chlorophylls can also be present in certain flowers , they´re constituted by a chlorine ring bound to a metal ion, and bestow flowers a green hue by reflection of green light (Sousa, 2022).

Among flavonoids, anthocyanins whose name comes from the Greek “anthos” which means flower and “kyanos” which means blue, stands out for their ability to attribute flowers a wide spectrum of striking colors namely different hues of orange, red, blue and purple. The color provided by anthocyanins will depend on factors such as pH, the metal ion and the presence of co-pigments as well as their quantity and variety (Benvenuti & Mazzoncini, 2021; Prior & Wu, 2006; Tanaka et al., 2008). Cyanidin, malvidin, delphinidin, peonidin, petunidin, and pelargonidin are the most common anthocyanidins and they´re the ones that will comprise a sizeable range of anthocyanin-based flower colours (Gould et al., 2008).

The cultivars of highly pigmented anthocyanin rich flowers will also possess a more elevated antioxidant potential in comparison to less pigmented cultivars (Benvenuti & Mazzoncini, 2021). In anthocyanins, the chromophore is the 7-hydroxyflavilyum ion (Bueno et al., 2012).

A crucial factor influencing the color of flowers is the stability of anthocyanins that is dependent on the presence of hydroxyl or methoxyl groups in ring B, with an increased hydroxylation causing the formation of a blue coloration and a lower stability, conversely an increased methylation causes a red coloration and higher stability (Mattioli et al., 2020). Modifications of the coloration are elicited by functional groups since they influence the absorbance maxima, with the impact of an increased hydroxylation pattern provoking a considerable shift towards blue because it induces a shift towards longer wavelengths known as a bathochromic effect while the methylation pattern provokes a shift towards red because it causes a shift towards shorter wavelengths known as a hyperchromic effect (Enaru, Drețcanu, et al., 2021; Gould et al., 2008; Houghton et al., 2021; Vicente et al., 2022). The stability of anthocyanins and consequently the color observed is also reliant on the glycosylation, considering that glycosyl groups can be acylated with aromatic acids. Aromatic acylation is fundamental for the stability mainly due to its role in intramolecular copigmentation, allowing anthocyanins to showcase a higher color stability, particularly in mildly acidic pH values, it’s important factoring in that glycosylated anthocyanins present instability in the absence of acylation (Gould et al., 2008; Malien-Aubert et al., 2001).

It´s key to ponder on the role of pH in color stabilization , knowing that anthocyanins tend to collect in plant vacuoles (pH 5), which possess a lower pH than the rest of the

cytosol (pH 7.2), therefore they are more stable in slightly more acidic environments, with the distinct levels of acidification of the vacuoles impacting the color observed (Mattioli et al., 2020; Vong et al., 2022). It´s imperative to also consider that anthocyanins are amphoteric compounds that can react in both acid and alkaline media. In an acidic environment, anthocyanins will preferentially present a red coloration because the formation of the fully protonated red flavylium ion (AH^+) responsible for that color is favoured (Houghton et al., 2021; Khoo et al., 2017; Susanti et al., 2018). As the pH rises, it leads to the deprotonation of the flavylium ion by donation of protons to H_2O, transforming these ions into quinonoid bases, privileging the presentation of a purple hue in a neutral pH environment. When it rises above the neutral pH, the quinonoid bases are additionally deprotonated, leading to the formation of unstable blue anionic quinonoid bases (Houghton et al., 2021). The absorption spectra of these pigments and consequently the flower coloration is very influenced by vacuolar pH (Stavenga et al., 2021). Due to anthocyanins being unstable by virtue of their high reactivity it leads to instability color wise, with the degradation of anthocyanins resulting in faded colors, with copigmentation minimizing this degradation (Susanti et al., 2018).

Copigmentation consists of the association between anthocyanins and a cofactor. The weak complexes formed due to this association make the colored form of these anthocyanins more stable whilst also contributing to brighter colors than the ones observed in the presence of anthocyanins by themselves, supplying flowers with a wider color diversity. Cofactors exist naturally in plant cells and are often colorless or have an extremely pale yellow hue, they can be amino acids, polysaccharides, alkaloids, phenolic acids and flavonoids. (Bimpilas et al., 2016; Eiro & Heinonen, 2002; Jackman et al., 1987).

Intermolecular copigmentation is defined by the non-covalent interaction of copigments which have electron-rich pi systems with the relatively electron-poor flavylium core of the anthocyanin that possesses a planar structure, thus preventing a water nucleophilic attack on the flavylium core which would turn it into its colorless pseudobase form (Escribano-Bailón et al., 2019; Parisa et al., 2007).

An essential form of copigmentation is the intramolecular copigmentation ,in which the cofactor is part of the anthocyanin itself, with a higher efficiency in stabilizing anthocyanins than the intermolecular one and not contingent on concentration (Zhao et al., 2022). In intramolecular copigmentation, anthocyanins are first acylated by addition of acyl groups and then the acyl groups that are bound to the skeleton covalently interact with each other through non-covalent interactions, occurring conformational folding of

these acyl groups over the chromophore, motivating a bathochromic shift, leading to brighter blues (Jackman et al., 1987; Malien-Aubert et al., 2001; Zhao et al., 2022). Anthocyanins also have the capacity to interact via self-association, establishing bonds with other anthocyanins, leading to a better stability as well but resulting in more weakly connected complexes than the ones observed in the occurrence of intermolecular copigmentation, this process isn't related to the occurrence of any covalent bonds, being exclusively reliant on weak intermolecular interactions. The presence of a higher content of anthocyanins before this mechanism can be spotted is a requirement (Houghton et al., 2021; Wang et al., 2023).

Another significant form of copigmentation is the metal complexation originated by covalent coordination bonds between anthocyanins and metallic ions namely : metallic sodium (Na^{+}), iron (Fe^{2+}/Fe^{3+}), aluminium (Al^{3+}), magnesium (Mg^{2+}), and zinc (Zn^{2+}). These bonds allow metallic ion complexes to form , with this interaction being particularly relevant for the observation of the color blue (Wang et al., 2023). The aglycones with at least two free hydroxyl groups in ring B namely cyanidin, delphinidin, and petunidin chelate with metal cations, the hydrogen atoms of metal cations compete with the hydrogen atoms of hydroxyl groups shifting the equilibrium away from the red flavylium ion and towards the transformation into blue quinonoid base anions, that can be further stabilized through other forms of copigmentation (Houghton et al., 2021; Sigurdson et al., 2016).

Many of the blue shades of flowers found in nature are an object of study because of their need to be stabilized in order to maintain their color through a combination of the mentioned mechanisms: intramolecular co-pigmentation, intermolecular co-pigmentation, and metal chelation. This stabilization of blue quinonoid bases is required since without it, in environments that leaned acidic, these bases would immediately transform in their colorless form and then ultimately prompt the browning of these flowers (Houghton et al., 2021).

Anthocyanins can be transformed into the corresponding chalcones (Mattioli et al., 2020). Chalcones constitute a group of flavonoids that will impart color to the flowers, the very word chalcone derives from the Greek "chalkos" which means bronze, associated to the look of the color associated to most chalcones found in nature. Chalcones have shades ranging from yellow to orange, with the color and conformation of chalcones being dependent on the electronic interaction between the aromatic rings (Hall et al., 2020; Suharoschi et al., 2023; Zhuang et al., 2017). The presence of chalcones and yellow pigments like aurones exerts influence over the coloration of

flowers exhibiting shades of red both in the presence and absence of anthocyanins. Flowers with a yellow hue contain as pigments: chalcones, aurones and flavonols whereas white flowers which aren´t usually purely white but possess a hue leaning towards a very pale shade of yellow, often contain flavonols and flavones namely kaempferol, apigenin, luteolin and quercetin (Iwashina, 2015).

1.2.1. Anthocyanins-rich edible flowers

Edible flowers with high contents of anthocyanins are highly sought after by virtue of the vibrant shades they exhibit, comprising some of the most widely cultivated, processed and consumed edible flowers (Teixeira et al., 2023).The flowers that were the primary target of our investigation all contain anthocyanins: *Viola tricolor*, *Centaurea cyanus* and *Cosmos bipinnatus* (Koike et al., 2015; Kulichenko et al., 2022; Mishio et al., 2015).

Viola tricolor commonly known as heartsease, johnny jump up or wild pansy is part of the *Violaceae* plant family, commonly found in both European countries and in western Asia (Mittu et al., 2024) and employed for the sake of their health benefits in the treatments of numerous skin conditions such as dermatoses, eczema and urticaria. They're also used in the treatment of respiratory issues like asthma and in the treatment of rheumatism (Hassanvand et al., 2021; MARIAN et al., 2022). They´re widely renowned ornamental flowers that enhance the appearance of any dish that contains them, it´s particularly recommendable their addition to drinks, soups and salads or the inclusion of their petals to butter in order to achieve a more original aromatic flavor (Mittu et al., 2024). These tiny and delicate flowers are broadly cultivated for their charm as ornamental flowers, existing diverse varieties of wild pansies with distinct colors such as yellow, purple, blue, pink and white, being feasible that flowers of the same variety of *Viola tricolor* may contain simultaneous a mix of several of the colors listed, they possess a flavor that has been described as grassy but still leaning a bit sweet (Fernandes, Casal, Pereira, Pereira, et al., 2020; Koike et al., 2015; Vukics et al., 2008).

Centaurea cyanus, commonly known as cornflower or bachelors' button, is a decorative flower part of the genus *Centaurea* L., a group of more than 700 species of herbaceous and perennial plants, the family *Asteraceae* to which it belongs can be found across Asia and Europe (Pirvu et al., 2015; Sharonova et al., 2021). Several health benefits have been attributed to them such as supressing the act of coughing, relieving itchiness, reducing inflammation, as well as acting as diuretics and laxatives (Pirvu et al.,

2015). They´re particularly eye catching exhibiting a very intense shade of blue imparted by the phenolic pigment protocyanin, which was initially identified in these flowers by Bayer in 1958. The calyx of this flower is bitter but the petals are edible and can be characterized as having a mildly sweet slightly spicy flavor reminiscent of a clove (Gupta et al., 2018; Lockowandt et al., 2019; Newman et al., 2009; Takeda et al., 2005).

Cosmos bipinnatus is a member of the genus *Cosmos* that are native to both the South of the USA and Mexico but can be found all throughout Asia, Europe, Australia and Africa, this species is also part of the family *Asteraceae* like *Centaurea cyanus*. *Cosmos bipinnatus* has been applied in the treatment of jaundice, intermittent fever, and splenomegaly, it has also traditionally been used against headaches and as an insecticide for the elimination of bed bugs and lice (Jang et al., 2008; Olajuyigbe & Ashafa, 2014). They´re ornamental herbaceous annual short day flowers of medium size (Bhuiyan & Nahid, 2023), that can be pink, violet, lavender or white with a flavor that's been characterized as leaning a tad more bitter (Jang et al., 2008; Teixeira et al., 2023).

Figure 4: Visual representations of the edible flowers: *Viola Tricolor, Centaurea cyanus* and *Cosmos bipinnatus* respectively.

1.3. Polyphenols' health benefits

Over time, an increasing number of epidemiological studies and associated meta-analyses have indicated the numerous advantages for the human body of a long term polyphenol-rich diet (Pandey & Rizvi, 2009) because polyphenols' high bioactivity grants them a wide variety of favorable medicinal properties (Rathod et al., 2023).

Taking into account both their copious health promoting activities and their contribution to the decrease of the likelihood of the development of chronic illnesses, more and more interest has been placed on studying them, researching ways to increase their bioavailability and bioaccessibility is at the center of marked interest (Gutiérrez-Escobar et al., 2021; Li et al., 2023).

There´s a very famous phenomenon dubbed the “French paradox” in which the people who live in the Mediterranean area have a comparatively lower cardiovascular disease induced mortality due to the quite substantial quota of fruit, wine and vegetables in their diet even though their diet also contains a pretty sizeable amount of fat (Cieślik et al., 2006), this health appeal of the Mediterranean diet comes from regular and frequent consumption of plant-based foods with a high polyphenol content (Amiot et al., 2021). It has been shown by multiples studies polyphenols’ utility to the cardiovascular system by promoting endothelial cells’ formation of vasoprotective factors, by stopping the oxidation of LDL cholesterol, by slowing down blood vessels’ aging and assisting the blood vessels’ walls pertaining its resistance and elasticity, therefore resulting in less diseases related to the cardiovascular system (Cieślik et al., 2006; Oak et al., 2018).

1.3.1. Polyphenols’ Antioxidant properties

Polyphenols are bioactive metabolites known for their antioxidant capacity which attributes them therapeutic properties against metabolic, neurodegenerative and cardiovascular diseases whose incidence is on the rise, contributing to a tremendous interest in substances that assist in the treatment of these diseases (Gasmi et al., 2022).

Both the antioxidant properties and the overriding of epigenetic changes constitute the two leading sources of disease prevention by polyphenols, with their antioxidant activity being tied to their bioaccessibility, to their content in the diet, to possible interactions after ingestion and to their reducing capacity against both reactive oxygen and nitrogen species (de Araújo et al., 2021; Magalhães et al., 2010; Wu et al., 2016), with oxidative stress contributing to the development of the alluded cardiovascular, metabolic and neurodegenerative diseases (Martín & Ramos, 2016).

Reactive species are an umbrella term for an array of compounds that can be categorized into three groups: reactive nitrogen species (RNS) constituted by nitrogen atoms, reactive oxygen species (ROS) constituted by oxygen atoms and reactive sulfur species (RSS) constituted by sulfur atoms (Gulcin & Alwasel, 2023).

Antioxidants counteract ROS which are very unstable oxygen derivatives existent within cells with one or more unpaired electrons, also known as oxidants, that became very reactive, namely superoxide anion radical ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$). Its unrestricted formation can be tied to factors such as the use of alcohol and nicotine, chronic stress, food contamination and pollution of the environment, that within normal quantities are just metabolic by-products.(Agraharam et al., 2022; Chensom et al., 2019; Chlubek & Poland, 2003; Kozlov et al., 2024). Metal ions like iron and copper can aid the

formation of hydroxyl radicals, particularly in the presence of oxygen, according to the Fenton reaction the hydroxyl radical is obtained through the action of catalyst Fe^{2+} ion leading to the decomposition of hydrogen peroxide (H_2O_2); by Haber–Weiss reaction, with the reduction of the ferric ion which then proceeds to react with hydrogen peroxide to originate hydroxyl radicals (Gulcin & Alwasel, 2023; Gupta et al., 2016; Lloyd et al., 1997). Reactive species like $HO^{\bullet}$ can also be produced thanks to other processes namely UV photolysis, water radiolysis in which these radicals are produced by the action of ionization radiation that decomposes water molecules and due to the decomposition of ozone as well (Hoigne & Bader, 1975; Le Caër, 2011; Zheng et al., 2023).

Oxidative stress intracellularly can be described as an unbalance between the level of protection mechanisms, antioxidants, in comparison to the overproduction by the cellular mitochondrion of ROS, which upsets the REDOX balance, high intracellular levels of ROS and a lack of antioxidants leads to the formation of toxic reactive products. ROS interactions with components of the cells lead to the disruption of lipids, proteins and genetic material, damaging or fragmenting membranes, causing random DNA crosslinking and even destroying cells (Kıran et al., 2023; Piao et al., 2023; Schieber & Chandel, 2014; Sies, 2015). Antioxidants can prevent those damages by minimizing oxidative stress through neutralizing said free radicals, granting them electrons consequently making them harmless (Shantabia et al., 2014).

This disruption is pathological, it´s linked to a multitude of illnesses like Alzheimer, Parkinson's, hypertension, lung fibrosis, rheumatism, preeclampsia, carcinogenesis, diabetes and also to psychiatric illnesses like bipolar disorder and schizophrenia (Jomova et al., 2023; Kang et al., 2004; Lambeth, 2007).

Disparity between levels of antioxidants and ROS are a hallmark of pathological events , in spite of that, a quantity of ROS within normal is valuable for the cell response in the face of environmental alterations, operating in the stimulation of cellular signaling pathways, because in those, the pace of the formation of oxidants is evened out by the pace of oxidant remotion (Tohma et al., 2019; Vladimir-Knežević et al., 2012).

Out of the possible mechanisms of phenolic antioxidants' action against ROS, radical scavenging via hydrogen atom donation stands out as the most prevalent one. Operating by transferring H-atoms from their OH groups to the free ROS allowing the elimination of the excess of ROS, recuperating the balance (Di Meo et al., 2013; Joorabloo & Liu, 2024; Kasprzak-Drozd et al., 2022). Thus, antioxidants act by reducing the peroxidation of lipids by free radicals which damages polyunsaturated fatty acids (PUFAs) while also attenuating low-density lipoprotein (LDL) oxidation, which are responsible for instigating a number of pathological events (Kruger et al., 2014; Sroka & Cisowski, 2003; Vuolo et al., 2019). Another mechanism relevant for the antioxidant

activity is quenching that operates by electron transfer in which molecules named quenchers act by donating electrons to ROS, so as to put a stop to the chain reaction of lipid peroxidation (Guo et al., 2022; Kumar & Goel, 2019; Nimse & Pal, 2015). Antioxidant activity can also act by weaker singlet oxygen quenching or by chelation of transition metals which makes them redox inactive (Markovic & Trajkovic, 2008; Rasineni et al., 2008), the generation of ROS is halted by metal chelation because antioxidants captures metals that catalyze reactions of ROS formation, thus operating a preventive effect (Allen, 2015; Potì et al., 2019).

An elevated supply of antioxidants coming from external sources, dietary antioxidants, are then important to preserve the balance between antioxidants and ROS and subsequently stopping oxidative stress (Jakubczyk et al., 2020).

The scavenging capacity of phenolic acids is contingent on their structure, with both the quantity and position of hydroxyl and methoxy groups directly attached to the aromatic ring, conditioning this ability. Gallic acid is the phenolic acid with the most intense antioxidant capacity since it contains three hydroxyl groups directly attached while caffeic acid only has two and coumaric acid and vanillic acid only have one (Birková et al., 2020; Domínguez Avila et al., 2018; Kowczyk-Sadowy et al., 2015; Pérez et al., 2023). Regarding flavonoids' antioxidant capacity, it is also heavily influenced by the quantity and position of hydroxyl groups particularly in the B ring and the glycosylation, with the flavonoids with no hydroxyl groups not possessing any antioxidant activity and flavonols and flava-3-ols standing out among the rest of the flavonoids as the strongest scavengers (Pérez et al., 2023).

Total antioxidant capacity (TAC) of foodstuff is quantified via analytical biochemical assays, with a heightened surge of interest in recent years in reliably developing and improving said assays (Magalhães et al., 2010).

FRAP assay is a colorimetric method initially developed to allow the measurement of the ferric reducing power of human plasma in 1996 by Benzie and Strain, based on the reduction of ferric tripyridyltriazine complex (Fe^{III}-TPTZ) into ferrous tripyridyltriazine (Fe^{II}-TPTZ) due to presence of antioxidants which alters the absorbance of the compound acquiring an intense blue hue, with maximum absorption at 593 nm. This method was subsequently adapted in 2000 to quantify plants' ferric reducing antioxidant power by Pulido, Bravo, and Saura-Calixto (Amarowicz & Pegg, 2019; Malta & Liu, 2014). The total antioxidant activity is dictated by a rise of absorbance at 593 nm. FRAP assay is particularly important to study radical quenching activity of foodstuff (Mukherjee et al., 2011).

Radical DPPH, 1,1-diphenyl-2-picrylhydrazil; was firstly discovered in 1922 by the researchers Goldschmidt and Renn, and the DPPH assay used for the determination of the antioxidant activity, specially radical-scavenging activity, was initially formulated by Blois and then developed by Brand-Williams (Blois, 1958). DPPH assay is a commercially viable colorimetric method in which a stable free radical, DPPH, through suffering reduction by a hydrogen atom source, which reacts with its divalent nitrogen atom or by electron donation in which radicals attack the phenyl ring, becomes decolorated or its color is altered from violet to yellow, forming DPPHH. This drastic change in hue is caused by the transformation of DPPH in its corresponding hydrazine by reduction of its single electron of the nitrogen atom by action of a hydrogen atom of the antioxidant compounds. The elevated stability of DPPH is caused by the delocalization of the spare electron in the molecule (Baliyan et al., 2022; Bondet et al., 1997; Erdoğan et al., 2021; Gonçalves et al., 2018). DPPH displays greater solubility in organic solvents such as methanol (Bondet et al., 1997) and showcases a visible absorption band at 517 nm which is accountable for the intense and stable violet shade whilst the absorbance of DPPHH is lower with the disappearance of this band, provoking the disappearance of the color (Xie & Schaich, 2014).

DPPH assay is particularly relevant to study radical scavenging ability of various compounds thus evaluating the antioxidant activity of foodstuff (Prakash et al., 2001).

2. Materials and methods

Materials

The edible flowers selected for this project: *Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus* were all supplied by MicroGreens (Amora, Portugal). All reagents used were of analytical grade. Ethanol (96%) was purchased from LABCHEM (Santo Antão do Tojal, Portugal) and methanol (CHROMASOLV™, capillary GC grade) (≥99.9%) was purchased from Honeywell (Portugal). HPLC gradient grade solvent acetonitrile (99.9% CH_3CN- 0.2 µm filtrated) and formic acid (99.0%) were purchased from Chemlab (Belgium). Folin-Ciocalteu reagent and the gallic acid standard were from Sigma-Aldrich.The deionized water used with The Milli-Q EQ 7000 ultrapure water purification system was from Merck Millipore with a Millipak 0.22 µm hydrophilic membrane filter.

2.1 Production of polyphenol rich edible flower infused vinegars ‘extracts

Preparation of edible flower infused vinegars

1-month incorporation of three replicas of three different varieties of edible flower infused vinegars were made, each glass jar of 150 mL of commercial store-bought white wine vinegar (acidity of 6%) was infused with 3 g of the whole flower, with the ratio being 1 g of flowers for 50 mL of vinegar. They were stored at room temperature.

Extraction of polyphenol-rich vinegar extracts

After the remotion of the flowers from the enriched vinegars with a strainer, a crude extraction in ultrasounds was performed for 10 min, organic solvent ethanol at 20% (v/v %) was added to the sample to enhance the yield of extraction, then agitated for 15 min and the solution was filtrated with CHROMAFIL® PET 0.22 µm filters (Germany), lastly the organic solvent ethanol is eliminated by evaporation using a rotary evaporator. A reverse phase C18-bounded silica column solid phase extraction (SPE) is a polarity dependent technique that was performed to further remove unwanted non-phenolic substances. The column used was a Millipore LiChroprep® RP-18 (Germany) with a volume of 10g / 75 mL and a particle size of 40 to 63 µm. Initially, 2 column volumes of methanol were added to condition and activate the column and then washed with several column volumes of deionized acid water, acidified with HCl 2M, with a pH ~ 2. Afterwards,

the enriched vinegar extract was slowly passed through the reverse phase C_{18}-bounded silica column, diluted in deionized acid water, a colored "band" will begin to form corresponding to the phenolic fraction that remains retained in the column. The phenolic fraction was later recovered by elution with acid methanol, acidified with HCl 2M, with a pH ~ 2. All compounds that have a greater affinity for water than for C_{18} (stationary phase) are first eluted, such as hydrophilic compounds namely sugars, amino acids, small acids, or water-soluble proteins and then polyphenols are subsequently eluted after the addition of acidified methanol because it has a greater affinity for polyphenols. To the polyphenol rich extracts obtained at the end, it was added 20 mL of Milli-Q water and the extracts were again evaporated using a rotary evaporator to remove the methanol. The extracts are then freeze dried. Successive C_{18} SPEs were performed to increase the purity of the polyphenol rich extracts obtained.

2.2 Physicochemical characterization of the edible flower infused vinegars

Colorimetric analysis

The color of the vinegars was first monitored one hour after edible flower infusion and periodically assessed during 30 days. Measurements of three replicates of each sample were made on day 0, on the 6th day, 11th day, 14th day, 17th day, 21th day and 30th day. A colorimetric technique was used to study the color progression throughout a month using a Lovibond tintometer Ltd and Lovibond TRA 520 Spectrophotometer and accessory kit (Lovibond House, Sun Rise Way, Amesbury SP4 7GR, United Kingdom). The color evaluation was performed using CIELAB color space, it was constructed a graph assessing delta E (numeric expression of the change in how the human eye perceives color difference) throughout the days.

pH analysis

The measurement of pH was performed using an electrode on day 0, on the 7th day after vinegar's infusion of edible flowers, on the 15th day and on the 30th day, to determine pH stability at a temperature of around 20 ºC during one month.

2.3.1 Quantification of the total phenolic content (TPC) of edible flower infused vinegars

To assess the total polyphenol content (TPC) in edible flower infused vinegars, it was chosen the use of the colorimetric reference method F-C. Several FC assays were performed throughout one month to study the effect that the incorporation of edible flowers has on the TPC of vinegars: on the day of the infusion, on the 7th day after, on the 15th day, on the 21th day and on the 30th day. This method requires a reference standard, it was prepared a gallic acid calibration curve in Milli-Q water.

Aliquots of each 15 µL edible flower infused vinegar prepared in triplicate and blanks (aliquots of 15 µL of water) were mixed in microtubes (2 mL) with 75 µL of FC reagent (FCR) and 500 µL of Milli-Q water, followed up by vortexing. Then, it was added 300 µL of 20% sodium carbonate solution (Na_2CO_3) and 610 µL of Milli-Q water, and the mixture was vortexed again. The resulting colorimetric reaction was measured after a period of 30 min of incubation sheltered from the light at room temperature. The samples were transferred to a 96-well plate, 350 µL in each well, the absorbance of the 96 well-plate was read in a Molecular Devices FlexStation 3 Multi-Mode Microplate Reader at a wavelength of 750 nm and at a temperature of 25 ºC. The results were expressed as mg (gallic acid) per mL of extract. A standard gallic acid curve was made to determine the unknown polyphenol content by reference (0-1mg/mL, y= 0.8170x + 0.006308, R^2= 0.9945).

2.3.2 Quantification of the TPC of polyphenol-rich vinegar extracts made from vinegars infused with flowers

For the determination of the total polyphenol content (TPC) in freeze dried polyphenol-rich extracts made from one-month infusions of edible flowers in vinegars, the same FC protocol as before was employed but using as reference a gallic acid calibration curve in ethanol (0-1 mg/mL, y= 0,5340x - 0,01707, R^2= 0,9977).

Firstly, solutions of the polyphenol-rich vinegar extracts with a concentration of 2 mg/mL were prepared, with ethanol filtered with CHROMAFIL® PET 0.22 µm filters (Germany) as the solvent. Next, a FC assay was performed in conformity with the same conditions

and reagents as the ones used previously, with the only difference being that the temperature was taken in consideration, therefore the 30-minute-long incubation away from the light was at 40 ºC. The absorbance was also read at the microplate reader at a wavelength of 750 nm and at a temperature of 25 ºC. Then, consecutive dilutions were perfomed: 2mg/mL 4X (0.5 mg/mL) and 2mg/mL 8X (0.25 mg/mL) to attempt to improve the purity of the extracts.

2.4 Statistical analysis

The interpretation of the results was performed using statistical analysis software GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, EUA). Simple linear regressions were performed for the two gallic acid calibration curves. It was chosen a mixed effects analysis of the TPC of vinegars throughout the 30 days with comparison between all the groups with Tukey's multiple comparison test. For the assessment of TPC content after one month, an ordinary one-way ANOVA with Tukey's multiple comparison test was performed to determine if the difference between the means of the 4 independent groups (Control vinegar + 3 edible flower infused vinegars) is statistically significant. One-way ANOVA with Tukey´s multiple comparison test were performed for the assessment of the statistical difference of the TPC content of the groups of polyphenol-rich edible flower infused vinegar extracts and for the percentage of purity of said extracts. One-way ANOVA with Tukey´s multiple comparison test were also chosen for the assessment of the statistical difference of the antioxidant power of the different groups of vinegar extracts and edible flower extracts in FRAP and DPPH assays. A p-value lower than 0.05 is considered to be statistically significant, and the null hypothesis of the difference between the means of the 4 independent groups being due to random chance can be rejected. A p-value higher than 0.05 and the null hypothesis can´t be rejected. *p<0.05 **p< 0.01 ***p< 0.001 ****p<0.0001

2.5 LC-DAD/ESI-MS analysis

To determine the phenolic profile of the edible flower infused vinegars by detecting and tentatively identifying the polyphenols present, an aliquot of 2 µl of each edible flower infused vinegar was injected and analysed by Liquid Chromatography coupled Mass Spectrometry (LC-MS) analysis in a Thermo Scientific Dionex UltiMate 3000 UHPLC system equipped with Thermo Scientific Hypersil Gold™ VANQUISH C18 column (150

cm × 2.1 mm, 1.9 µm) purchased from Lithuania. All solvents used were previously filtered using a filtration system connected to a vacuum pump. The solvents used for this chromatographic separation were solvent A comprised of 1% of formic acid in water, $HCOOH/H_2O$ (1:99), and solvent B comprised 1% of formic acid in acetonitrile, $HCOOH/C_2H_3N$ (1:99). First the column was stabilized at the initial conditions (37ºC), 99% of A and 1% of B, for 10 minutes. The gradient consisted of 99%-0% A and 1%-100% for 50 minutes at a flow rate of 0.3 mL/min: from 0-10 minutes, 99% of A and 1% of B; from 10-20 minutes 94% of A and 6% of B; from 20-50 minutes,82% of A and 18% of B; from 50-50.10 minutes; 50% of A and B. The column was then washed with 100% B for 5 more minutes and then stabilized at the initial conditions for another 5 minutes. Subsequently electrospray ionization mass spectrometry (ESI) was performed. The mass detector was a ThermoFisher LTQ XL iontrap quadrupole equipped with an atmospheric pressure ionization source, using a electrospray ionization interface (collision force= 60eV). Spectra was recorded in the positive and negative modes. It was monitored at 280 nm in the negative mode and at 280 nm and 520 nm in the positive mode. The chromatograms were then analyzed with the Thermo Scientific™ Xcalibur software, to tentatively identify the compounds corresponding to the different peaks.

2.6 Antioxidant Power of the extracts

2.6.1 FRAP (Ferric Reducing Antioxidant Power) Assay

The FRAP assay was performed according to a previously described procedure in literature with some modifications (Oliveira et al., 2015), it was used for the determination of the total antioxidant activity of polyphenols from the extracts in study. This method was performed for both edible flower infused vinegar extracts and edible flower extracts. The reaction was performed in a microplate reader of 96 well plates (8 replicates of each sample of edible flower infused vinegar extract and 12 replicates of each sample of edible flower extract). The reaction was carried out on the plate wells with a temperature of 37ºC. To perform this method, 270 µL of the FRAP reagent (10 vol of 300 mM acetate buffer, pH 3.6 + 1 vol of 10 mM TPTZ in 40 mM HCl + 1 vol of 20 mM $FeCl_3$) were added in the wells and 30 µL of the 6 mg/mL extracts were added and mixed with the reagent. The absorbance was measured at 593 nm at time 0 and 4 min. This same protocol was repeated after normalizing the polyphenol content to the same concentration (0.6 mg/mL;

n=19). The blank assay was performed using 270 μL of FRAP reagent and 30 μL of dH_2O. The calibration curve (y= 0.002786x+ 0.03348) was prepared using solutions of 100 to 1000 μmol Trolox. The antioxidant capacity was expressed as the μM Trolox equivalents/g and μM for a final concentration of 0.6 mg/mL. The results were expressed in Trolox, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, equivalent units. This antioxidant is utilized as a standard , so antioxidant strength is measured based on Trolox (Fernández-Marín et al., 2020).

2.6.2 DPPH (Diphenyl-1-Picrylhydrazyl) Assay

Following the method described in the literature with some modifications (Oliveira et al., 2015), radical activities were determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a free radical. The tested extracts reacted with DPPH and the decrease in the absorbance at 517 nm was measured, which indicated the scavenging potential of the extracts. This method was performed for both edible flower infused vinegar extracts and edible flower extracts. As all samples tested exhibit absorbance at 517 nm, previous control assays were performed with all the samples in order to subtract their contribution. The reaction for scavenging DPPH radicals was performed in a microplate reader (16 replicates of each sample of edible flower infused vinegar extract and 12 replicates of each sample of edible flower extract). The reaction was carried out on the plate wells with a temperature of 25ºC. A solution of 60 μM DPPH was prepared in methanol. To perform this method, 270 μL of the DPPH+ methanol solution was added in the wells together with 30 μL of the 6 mg/mL extracts. The decrease in absorbance was measured at 517 nm, at t=0 and t=20 min. The antiradical activity was expressed as μM Trolox equivalents/g and μM for a final concentration of 0.6 mg/mL. The calibration curve (y = -0.0016x + 0.6409) was prepared using solutions of 10 to 500 μmol Trolox. The results were expressed in Trolox equivalent units as well.

3. Results and discussion

3.1 Colorimetric analysis

The color evaluation was performed using CIELAB color space, also referred to as L*a*b*, it expresses color as these three values: L* (perceptual luminosity), a* (green to red) and b* (blue to yellow). L* represents lightness from black to white on a scale of 0 to 100, negative a* corresponds to the color green while positive a* corresponds to the color red. Negative b* corresponds to the blue color while positive b* corresponds to the color yellow, with the differences in chromaticity being more perceptible than the differences in lightness (MacDougall, 2010; Pecho et al., 2016).

Delta E is measured using the formula described below, the greater the value of delta E, the larger the colour difference exhibited (Salueña et al., 2019).The perception scale of Delta E (ΔE) fluctuates from 0 to 100, according to Zachary Schuessler if the value of Delta E is below 1, the color difference is imperceptible to the human eye, if the value is from 1 to 2 it is only perceptible under close observation, 2 to 10 the difference is noticeable with a glance, 11 to 49 the colors are substantially different and 100 represents the observation of opposite colors in the color wheel (Schuessler, Z. (n.d.)) Another perception scale by Adekunte more broadly indicates that any value of Delta E above 3 is undoubtedly different, the values between 1.5 and 3 can potentially be considered different and below 1.5 the difference is considered very slight (Adekunte et al., 2010).

We performed this method to determinate the numerical difference between shades exhibited by the edible flower infused vinegars via delta E: it was quantified the difference between colors of each specific edible flower infused vinegar from day 0 to day 6, day 6 to day 11, day 11 to day 14, day 14 to day 17, day 17 to day 21 and day 21 to day 30.

$$\Delta E = \sqrt{(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2}$$

L1 - the CIE L* value of the reference color

a1 - the CIE a* value of the reference color

b1 - the CIE b* value of the reference color

L2 - the CIE L* value of the sample color

a2 - the CIE a* value of the sample color

b2 - the CIE b* value of the sample color

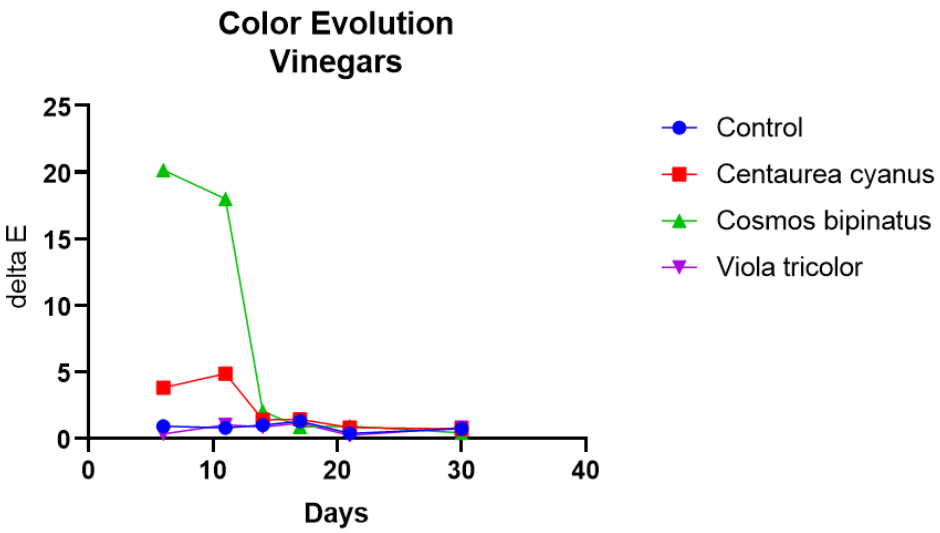

Figure 5: Graph representing the values of ΔE measured over time, throughout 30 days, of vinegar control (vinegar in the absence of flowers) and of each edible flower infused vinegar.

Table 1: Values of delta E from day 0 to day 6, day 6 to day 11, day 11 to day 14, day 14 to day 17, day 17 to day 21 and day 21 to day 30.

ΔE	0 a 6	6 to 11	11 to 14	14 to 17	17 to 21	21 to 30
Control vinegar	0,881	0,761	0,960	1,250	0,350	0,699
Viola vinegar	0,297	0,982	0,817	1,12	0,207	0,775
Centaurea vinegar	3,78	4,84	1,37	1,40	0,789	0,671
Cosmos vinegar	20,3	17,9	2,01	0,827	0,890	0,392

Control vinegar is the vinegar in the absence of edible flower infusion so it represents the control. Until the 14th day, delta E is below 1, as evidenced in the table 2 so any alteration in color is so minimal to be imperceptible to the human eye. From the 14th to the 17th day a very slight difference in color occurs because delta E is above 1 and after that, the color remains constant. On the graph (Figure 5), it´s apparent how constant the trendline of the Control vinegar remains.

Viola vinegar only exhibits a very slight color difference on the 17th day in comparison to the 14th day with the value of delta E being marginally above 1, after that, the color also remains constant. Visually, as it is shown in Figure 6 Viola vinegar doesn't possess a color perceived significantly different than the one observed in Control vinegar at the end of the month. On Figure 5, it´s visible the almost complete overlap between the trendlines of Control vinegar and Viola vinegar.

Centaurea vinegar exhibits a color shift that´s perceptible with just a glance (delta E above 2), gaining a tone leaning more towards an orange hue after the first 6 days of infusion, which is illustrated in Figure 8. From the 6th to the 11th day, the most significant color shift of Centaurea vinegar occurs. As observed on the graph, after three weeks (21 days) the color stabilizes. It can be noted that the final color perceived after a one-month long infusion is a brownish orange.

Cosmos vinegar´s color after a week of infusion of edible flowers is red, the value of delta E is higher than 20 hence the color witnessed is completely different than the color seen on the first day. From the 6th to the 11th day, the color is continuously altered in a significant way, being clearly depicted a steep decline in the graph until the 17thday. The final color observed is a burgundy hue.

Figure 6: Visual representation of the colors of edible flower infused vinegars over time (30 days).

Cosmos vinegar is the vinegar that presents the most dramatic color change by far, reaching values of delta E superior to 20 whilst the other edible flower infused vinegars don't even surpass a value of 5. The color showcased by Cosmos vinegar imparts it with a greater visual appeal than the others, potentially contributing to this vinegar becoming more sough after commercially for its new-found striking appearance. These findings can be related to the presence of different pigments in each edible flower, tentatively suggesting that cosmos flowers present a greater abundance of anthocyanins in comparison to the other two species of edible flowers studied. It´s also important to consider the pH environment of vinegar, with the fully protonated red flavylium ion (AH^+) of anthocyanins being preferably favoured in an acidic environment over a basic one, as is the case of the environment presented in vinegar. It's clearly established the stabilization of color of all the vinegars after a period of three weeks (21 days). Showing that, after the period of one month the color doesn't continue to change because it has a achieved a stable color. For the commercialization of these vinegars ascertaining that the color don't continuously change for an undetermined period of time is a key piece of data.

3.2 pH analysis

The measurement of pH was performed to determine if the presence of edible flowers alters the pH of vinegar. As shown in the graph, pH stays constant throughout the month, therefore the results indicate that the inclusion of any of the three species of edible flowers doesn't change the pH of vinegar, which remains stable, staying around values of 2.68-2.89. There´s no observable difference in pH between vinegars, it´s possible to observe an overlap of the pH values of all 4.

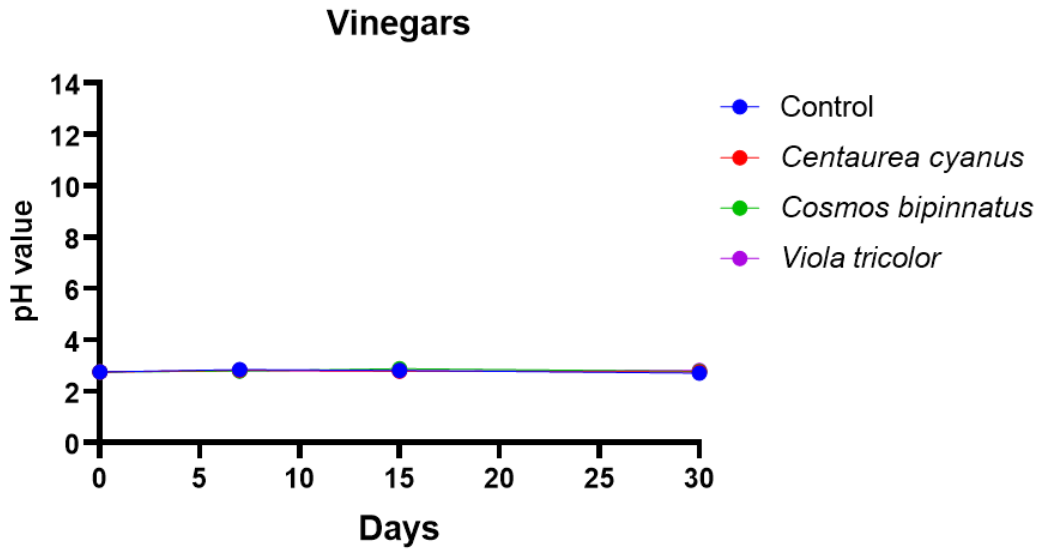

Figure 7: Evolution of the values of pH of the vinegars over time (30 days).

3.3.1 Quantification of the total polyphenols content (TPC) of the edible flower infused vinegars through a F–C assay

Using FC assays, we assessed, as illustrated in Figure 10, the content in polyphenols of vinegars infused with *Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus* on the day of the infusion (blue), 7 days after (red), 15 days after (green), 21 days after (purple) and 30 days after (orange) to evaluate this progression of TPC due to the presence of polyphenol-rich flowers over time. It was intended to determine if the presence of these flowers is capable of transmitting polyphenols to the vinegars.

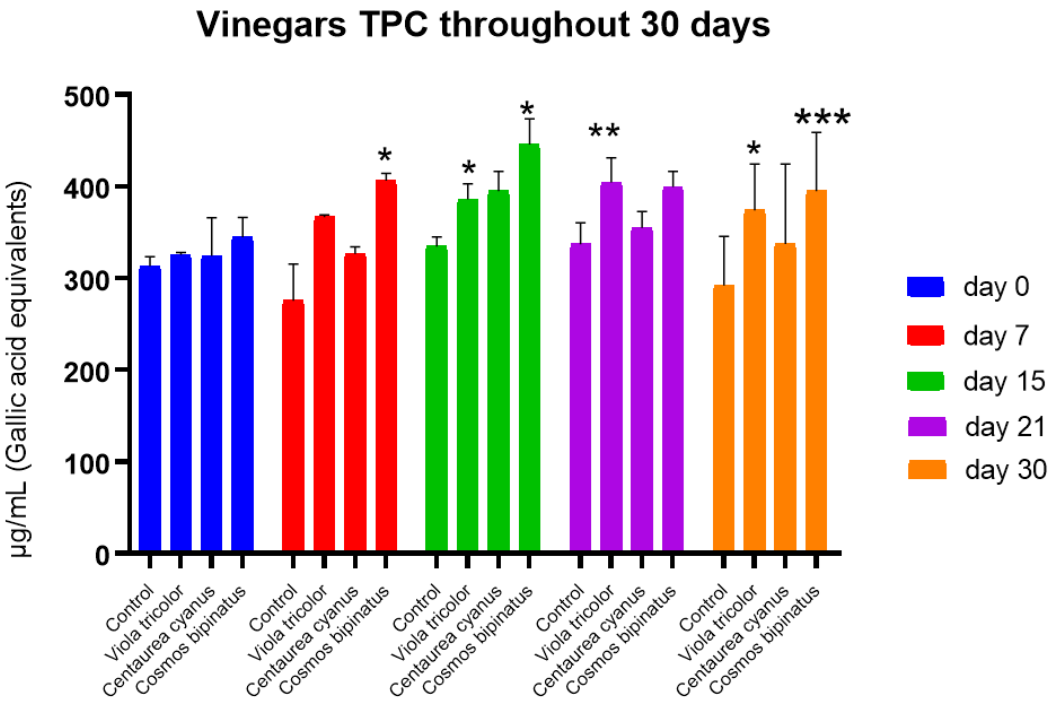

Figure 8: Progression expressed as (µg/mL) gallic acid equivalents of the phenolic content of vinegars throughout the month (day 0, day 7, day 15, day 21 and day 30. The data presented were expressed as mean ± standard deviation (SD). * means that the flower infused vinegar and Control Vinegar are statistically different $p<0.05$; ** means that the flower infused vinegar and Control Vinegar are statistically different $p < 0.01$; *** means that the flower infused vinegar and Control Vinegar are statistically different $p< 0.001$.

Vinegar in the absence of edible flowers represents the control, with all the vinegars having approximately the same TPC at day 0, with the vinegar infused with cosmos flowers presenting a slightly larger TPC than the others. By the end of month, as it was expected there´s no increase in phenolic content in Control vinegar because no polyphenol replete foodstuff was added to it, furthermore a slight decline is even observed. At the start of the 30 days, it possessed a TPC of 313.3 µg/mL and at the end a TPC of 292 µg/mL. An explanation for this slight decrease may be related to the degradation of some of the polyphenols initially present, considering polyphenols are

compounds characterized by a high sensitivity to a number of factors such as light, temperature, oxygen and low pH (Enaru, Socaci, et al., 2021). With the exception of the Control vinegar, figure 8 displays the general trend of the content of polyphenols increasing over time, showing that the TPC of Viola, Centaurea and Cosmos vinegars is higher at the end of the month than it was at the beginning, indicating that the edible flower infusion has an overall effect of boosting vinegar´s phenolic content.

Looking at Figure 8, it's clear that Cosmos vinegar presents the highest TPC during most of the measurements except on the 21th day in which it's slightly lower than the TPC presented by Viola vinegar, pointing towards *Cosmos bipinnatus* being the richest in polyphenols of the three edible flowers tested. Seemingly suggesting, that the vinegars infused with cosmos flowers are the most beneficial to human health of the ones studied. The TPC of the Cosmos vinegar on the day of the infusion is 344.7 µg/mL with its content raising to 394.7 µg/mL on the 30th day. Cosmos vinegar is the one with the highest TPC, with the vinegar infused with *Viola tricolor* possessing the second highest TPC (373.8 µg/mL) and the vinegar infused with *Centaurea cyanus* showcasing the lowest increment (337.2 µg/mL). Pointing towards cornflowers having a lower concentration of polyphenols than the other two species. Another possible explanation for the lower TPC observed is that cornflowers possess polyphenols with a higher sensitivity than the ones contained in the other two species, and might have suffered greater degradation during this one-month period.

By ANOVA test, it was determined that the difference in mean groups between Control vinegar and Cosmos vinegar is particularly statistically significant with $p < 0.001$, as it is shown in Figure 9.

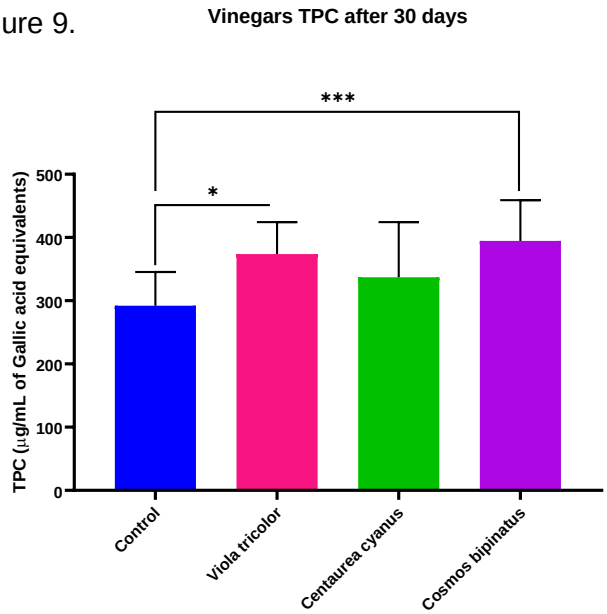

Figure 9: Content in phenolic compounds in edible flower infused vinegars on the 30th day of infusion. The data presented were expressed as mean ± standard deviation (SD). * means $p<0.05$; *** means $p< 0.001$

This method has certain limitations which must be taken into consideration when interpreting the results. It´s crucial to take into account that the F-C method despite being a practical and easily reproducible method only provides a general estimate of the total content of polyphenols present, because not every polyphenol will react with FCR in the same manner (Prior et al., 2005), it´s also pertinent to note the possibility of overestimation because even though this method is a popular method for the detection and quantification of polyphenols, it was not specifically designated for their detection, so there´s the risk that it might detect non-phenolic compounds that also have the ability of reducing FCR (Huang et al., 2005; Munteanu & Apetrei, 2021). Gallic acid was chosen as the standard but it also would have been possible to use for calibration curves other reference molecules: caffeic acid, catechin, tannic acid, chlorogenic acid, and ferulic acid (Pérez et al., 2023). F-C is a reference method so choosing distinct standards means establishing distinct calibration curves , which leads to obtaining different values of TPC (Bastola et al., 2017), thus it´s important to bear in mind that the results obtained are in reference to gallic acid in specific. Another limitation to consider is that we´re comparing polyphenol content from different plant sources (distinct species of edible flowers) whose polyphenols might vary structurally from each other, this diversity of structures can be quite significant. Both the number and position of hydroxyl groups of polyphenols can influence the degree of reduction of FCR thus affecting the absorbance (Appel et al., 2001).

3.3.2 Quantification of the total polyphenols content (TPC) of the polyphenol rich freeze-dried extracts through a F–C assay

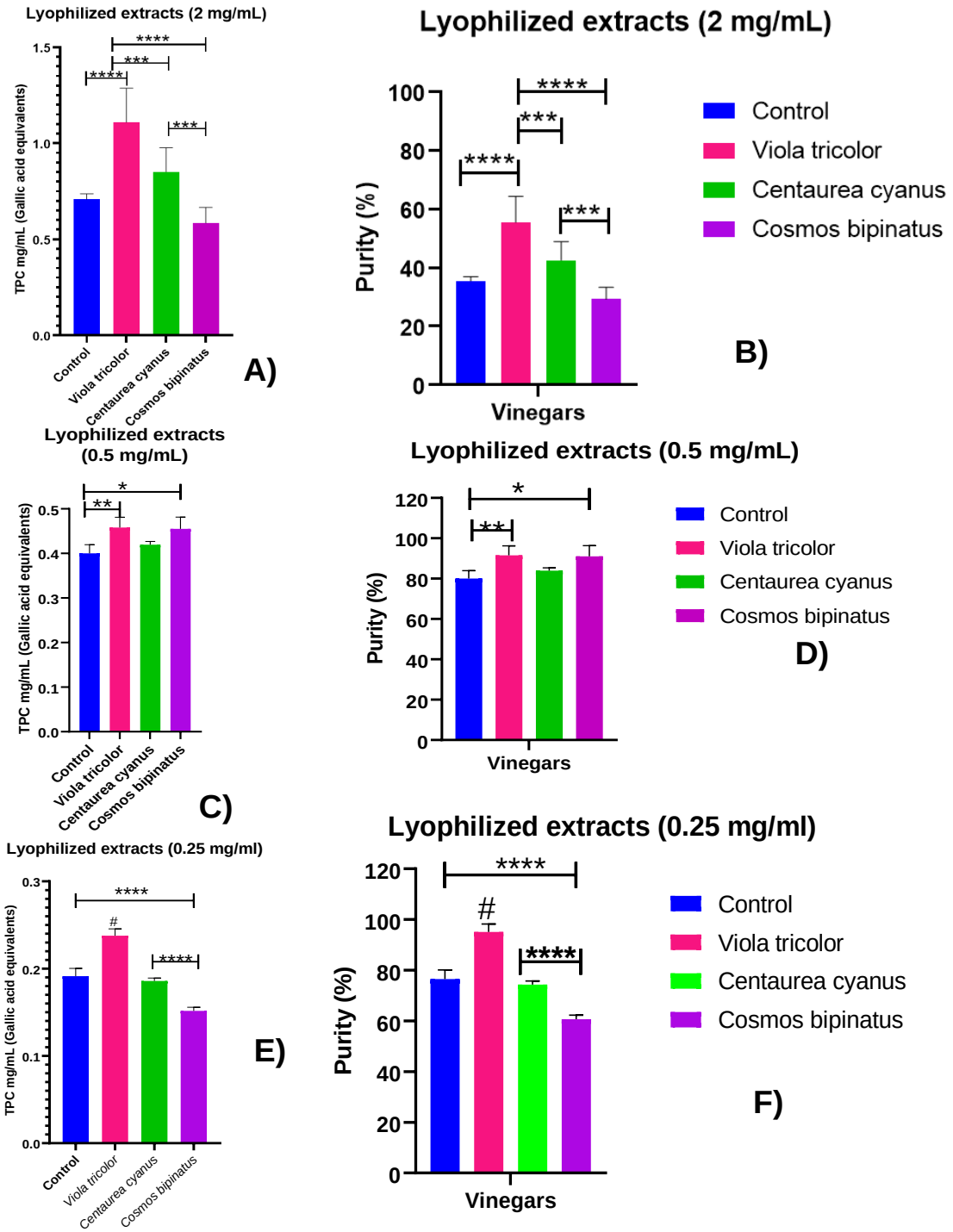

Figure 10: Total phenolic content determined by Folin-Ciocalteu assay expressed as (mg/mL) gallic acid equivalents A) Concentration of polyphenols, mg in mL, in a polyphenol-rich vinegar extract with a concentration of 2mg/mL made after the 30 days. B) Purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 2mg/mL. C) Concentration of polyphenols, mg in mL, in a polyphenol rich extract with a concentration of 0.5 mg/mL. D) Purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 0.5 mg/mL E) Concentration of polyphenols, mg in mL, in a polyphenol rich extract with a concentration of 0.25 mg/mL. F) Purity in percentage (%) of the concentration of polyphenols in a polyphenol rich extract with a concentration of 0.25 mg/mL. The results presented were expressed as mean ± standard deviation (SD). * signifies a statistical difference of p< 0.05; ** signifies a statistical difference of p< 0.01; *** signifies a statistical difference of p< 0.001; **** signifies a statistical difference of p< 0.0001; # signifies that *Viola tricolor* is statistically different from the other vinegars p<0.0001

The results illustrating the relationship between the concentration of edible flower infused vinegar extracts and the concentration of phenolic compounds in these polyphenol-rich extracts are represented in Figure 10. Although these extracts are made up mostly by phenolic compounds, they´re still not exclusively comprised of them. Hence, the quantification of polyphenols in polyphenol-rich extracts required successive dilutions to ascertain which concentration of extracts allowed the obtention of the highest purity of phenolic compounds. We intend for the purity to be as close to 100% as possible and therefore the concentration of polyphenols in the extracts to be the highest possible.

At a concentration of 2 mg/mL, the purity of all the extracts is very low, with all of them except the extract of Viola vinegar possessing a purity below 50% and thus the quantity of polyphenols in the extracts at 2 mg/mL is below 1mg/mL, with only the extract of Viola vinegar having a purity of 55.5%. By analysis of the graphs, it´s possible to establish the positive effect that both dilutions, 2mg/mL 4X and 2mg/mL 8X, have on the values of purity obtained, demonstrating that of the three concentrations of extracts studied (2mg/mL, 0.5mg/mL, 0.25 mg/mL), it was at the intermediate concentration that the highest concentration of polyphenols with the highest purity was obtained. At 0.5 mg/mL and 0.25 mg/mL concentrations, the purity of the extracts of Viola vinegar are exceedingly high, 91,6% and 95.1% respectively, despite the purity of this extract being slightly superior at 0.25 mg/mL the other extracts are higher at 0.5 mg/mL, thus indicating that it´s the most suitable concentration. At a concentration of 0.5 mg/mL, all of the extracts exhibit a purity above 80%. The most substantial disparity between the results obtained at a concentration of 0.25 mg/mL and at a concentration of 0.5 mg/mL is the purity of the extracts of Cosmos vinegar that drastically increases from 60.6% to 91%.

Our findings highlight the importance of the concentration of extracts. It´s relevant to consider for this methodology the concept of linearity, in which within a specific range absorbance is directly proportional to the concentration of the samples; therefore, it´s necessary to employ dilutions to obtain values of absorbance of the samples in the linearity range of the prepared calibration curve (Martins et al., 2021). A likely explanation for the low TPC of extracts calculated at the concentration of 2 mg/mL might be due to falling outside of the linear range of the calibration curve we previously calculated (0-1 mg/mL, $y= 0,5340x - 0,01707$, $R^2= 0,9977$), while at a concentration of 0.5 mg/mL and of 0.25 mg/mL, the results fall within the linear range of the calibration curve. In this range, it´s verifiable that the absorbance of a solution of an absorbing substance obtained by spectrometry techniques is directly proportional to this substance's

concentration by Beer-Lambert law, thus explaining why at a concentration of 0.5 mg/mL the highest TPC is showcased (Delgado, 2022).

3.4 Phenolic characterization of the edible flower infused vinegars

The LC-DAD/ESI-MS analysis allowed the tentative identification of the polyphenols in each edible flower infused vinegar. The preceding chromatography is necessary to separate compounds on the basis of their polarity, granting them different retention times (Pitt, 2009; Tellez et al., 2009). When the samples passed through the spectrometer they were ionized, by conversion of molecules into a charged ionized state which are oftentimes fragmented during the analysis in a way that is characteristic to the compound. The ionized molecules and fragments were detected based on their mass to charge ratios (m/z). This method has already been established as a common method for the identification of polyphenols, it possesses two polarity methods: negative ion mode and positive ion mode (Harrison, 2018). In the negative mode, the analytes are deprotonated and in the positive mode the analytes are protonated. The m/z value at which one detects a singly charged ion is, therefore, 1 Dalton (Da) lower in mass in the negative mode than the molecular weight and the *m/z* value at which one detects a singly charged ion is 1 Dalton (Da) higher in mass in the positive mode than the molecular weight (Becker, 2008; Wu et al., 2004).

Polyphenols were detected by mass spectrometry, in both ion modes. The negative mode has already been shown to be better suited for the detection of the majority of polyphenols with the exception of anthocyanins. It´s preferable to detect anthocyanins in the positive mode because in acidic extracts they´re present as their cationic forms, flavylium ions, since unlike the others they´re already in their ionic form they won't be ionized (Jin et al., 2015; Mansour et al., 2021; Volpi & Bergonzini, 2006). Anthocyanins also differ from other polyphenols by showcasing a prominent UV absorbance at 520 nm, which is dissimilar to the other polyphenols whose prominent UV absorbance is at 280nm (Berland & Andersen, 2021).

Based on the chromatograms retrieved, the tentative characterization of edible flower infused vinegars was accomplished by consulting the general literature and by observation of the fragmentation patterns obtained**.** The tables present the tentative assigned identities of the peaks in the extract, reporting their ion mode, retention times (R_t), wavelengths of maximum absorption (λ_{max}) and MS spectra. In the negative mode, chromatograms were monitored at 280 nm whereas in the positive mode chromatograms were monitored at 280nm and at 520 nm, positive mode was predominantly used to allow

the identification of anthocyanins. In each chromatogram, compounds were numbered by their elution order. This tentative identification aims to provide better understanding about the polyphenols detected in the vinegar by itself (control vinegar) and the polyphenols added by the infusion of edible flowers.

Control vinegar

The chromatographic profile of Control vinegar was monitored at 280 nm in the negative ion mode and it was monitored at 280 nm and 520 nm in the positive ion mode, respectively represented in Figures 11 and 12.

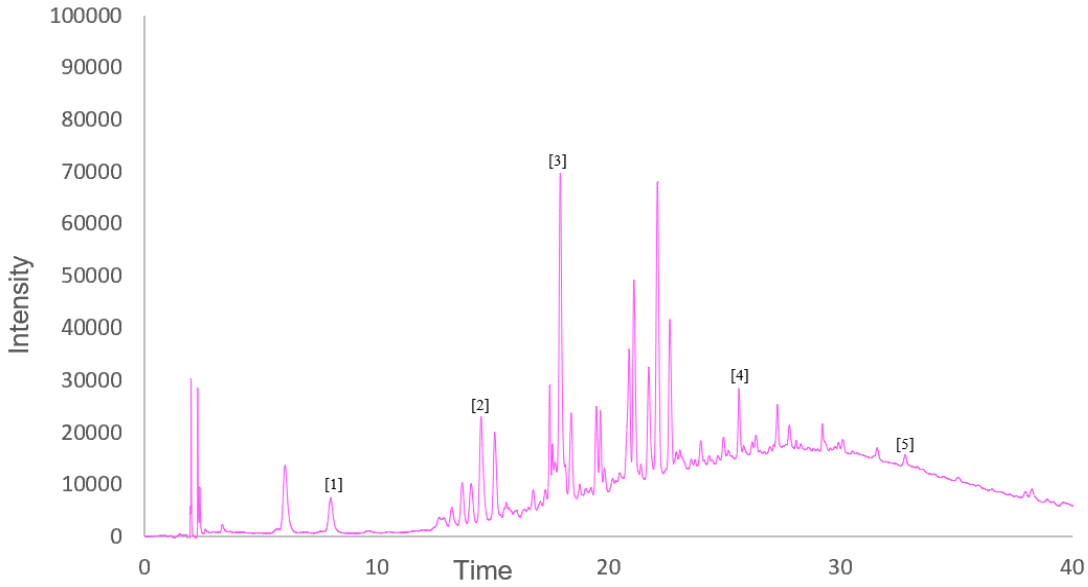

Figure 11: Chromatogram of Control vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS.

Table 2: UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Control vinegar at 280 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]	Negative	8,02	260 296	152.91		3,4-Dihydroxybenzoic acid
[2]		14,50	328	178.99	134.86	Caffeic acid
[3]		17,92	312	162.99	118.87	m-Coumaric acid
[4]		25,62	356	477.20	301.04	Quercetin-3-O-glucuronide
[5]		32,79	344/ 356/ 372	301.13	178.92	Quercetin

LC-DAD/ESI-MS analysis allowed the tentative identification of one flavonoid (quercetin) and one of its derivatives and 3 phenolic acids. Compound 5 on negative mode corresponds to the flavonol quercetin with a m/z 301.13 and a fragment with a m/z=178.92, with peak 4 corresponding to its glycoside conjugate: quercetin-3-O-glucuronide , which is a quercetin attached to a beta-D-glucuronopyranosyl moiety at position C-3 via a glycosidic linkage (Nunes Alves Paim et al., 2022). Quercetin-3-O-glucuronide has a precursor ion with m/z 477.20 that dissociates by result of collision-induced MS to a smaller fragment ion, product ion, with a m/z =301.04 in the MS^2 spectra corresponding to quercetin, meaning it lost its glucuronyl moiety (176 a.m.u). It was also possible to identify two compounds derived from hydroxycinnamic acid (m-coumaric acid and caffeic acid and one derived from hydroxybenzoic acid (3,4-dihydroxybenzoic acid). Caffeic acid, compound 2, has a molecular ion with m/z 178.99 and a fragment with m/z 134.86 due to the loss of CO_2 (44 a.m.u). m-Coumaric acid, compound 3, has a molecular ion with m/z 162.99 and a fragment with a m/z=118.87 due to the loss of CO (28 a.m.u). One of the highest peaks in Control vinegar, as it is shown in Figure 11, has tentatively been identified as m-coumaric acid.

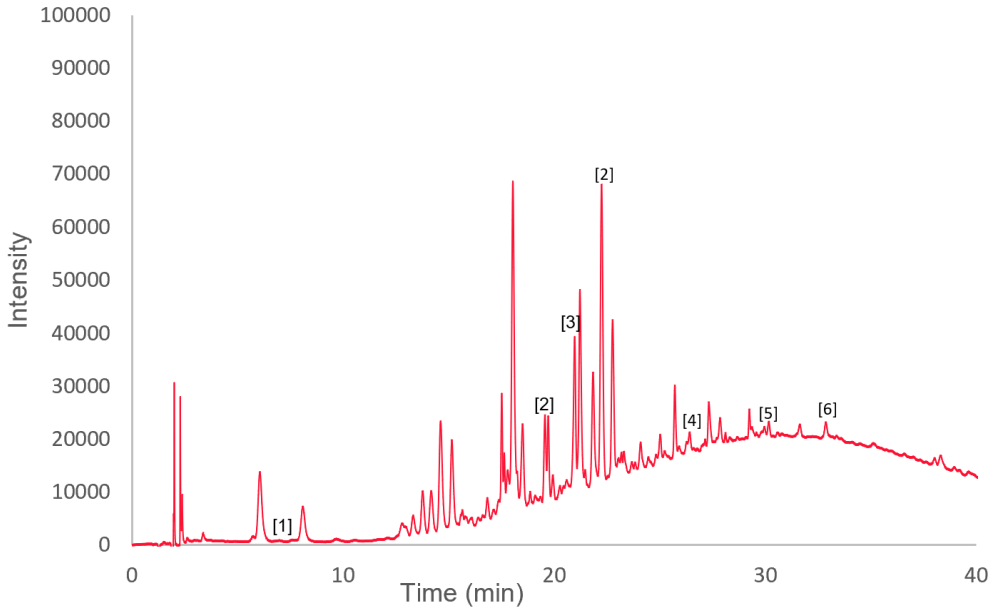

Figure 12: Chromatogram of Control vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS.

Table 3: UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main phenolic compounds identified in Control vinegar at 280 nm and 520 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]	Positive	7,60	328	181.08	163.02 125.05	Caffeic acid
[2]		19,56/22,25	276	147.17	119.02	Coumarin
[3]		20,97	272	463.07	698.63;443.39	Luteolin 7-glucuronide
[4]		26.42	280	213.32	185.15	Pinosylvin
[5]		30.17	284	271.43		Apigenin
[6]		32.87	356 368	303.37	257.14; 229.10	Quercetin

The highest peak was tentatively identified as coumarin, compound 2, in the positive mode of Control vinegar, which is a hydrocinnamic benzopyrone characterized by a vanilla-like aroma, with the basic hydrolysis of coumarin originating coumaric acid. Coumarin is represented as compound 2 with a molecular ion with m/z 147.17 and one

fragment with m/z 119.02. The difference between coumarin and the coumaric acid identified previously in the negative mode as compound 3 is the presence of an additional water group (18 a.m.u) on coumaric acid. Furthermore, two flavones were also identified, apigenin and luteolin 7-glucuronide, with the latter being a metabolite of luteolin with a flavonoid moiety O-glycosidically linked to the glucuronic acid at the C7-position. Stilbene Pinosylvin was also identified within control vinegar. LC-DAD/ESI-MS allowed in total, considering both positive and negative ion mode, the tentative identification of 11 compounds in control vinegar. The commercial white wine vinegar used for this identification is a complex foodstuff which isn't exclusively comprised of polyphenols therefore it´s to be expected that some of the peaks observed will correspond to non-phenolic substances. Not every compound fragments like compound 5, tentatively identified as apigenin, which makes the process of identification more confounding because the fragmentation pattern is so crucial for this process but a tentative identification was still carried out based on the molecular ion and λ_{max}.

No anthocyanins were identified in Control vinegar in the positive mode, so any anthocyanins identified from this point forward are provided by the presence of edible flowers. Other polyphenols not identified in control vinegar but identified in the edible flower infused vinegars will most likely be supplied by said flowers whilst the compounds present in edible flower infused vinegars that were identified in control vinegar as well were probably supplied by the vinegar itself.

Viola Vinegar

The chromatographic profile of vinegar infused with *Viola tricolor* was monitored at 280 nm in the negative ion mode and was monitored at 280 nm and 520 nm in the positive ion mode, respectively represented in Figures 13 and 14.

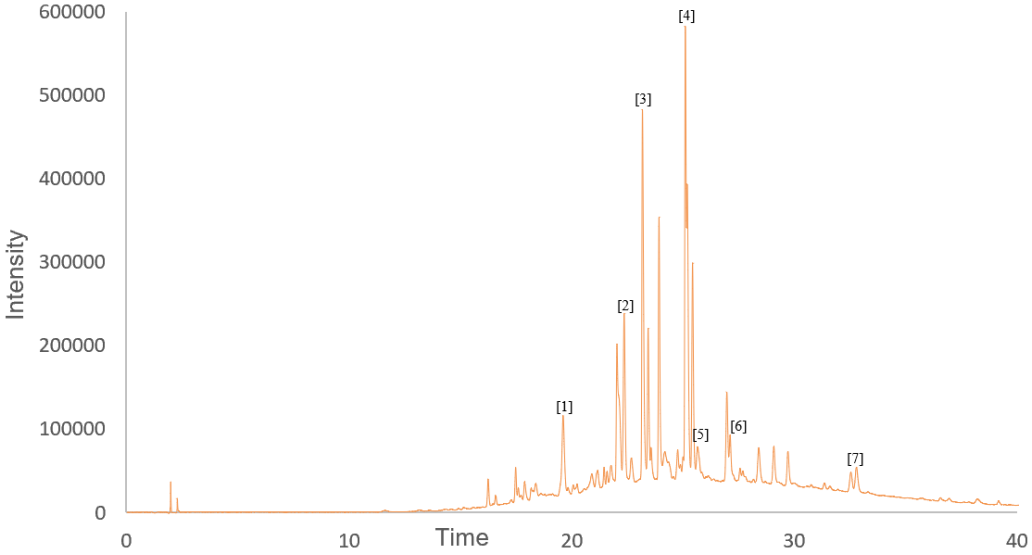

Figure 13: Chromatogram of Viola vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS.

Table 4: UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Viola vinegar at 280 nm. Retention time (Rt), wavelengths of maximum absorption (λmax), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]	Negative	19.61	288	319.08	192.88; 164.91	Dihydromyricetin
[2]		22,35	288	481.26	193.02; 164.93	Isosilybin
[3]		23,17	260/356	625.34	317.03; 178.89	Myricetin 3-robinobioside
[4]		25,10	272/336	577.46	457.29; 353.13	Violanthin (Apigenin-6-C-glucosyl-8-C-rhamnoside)
[5]		25,65	352	477.25	301.06; 178.88	Quercetin-3-glucuronide (Miquelianin)
[6]		27,11	264/344	593.33	285.04; 257.01	Kaempferol-*O*-deoxyhexosyl-hexoside
[7]		32,87	368	301.11	178.98; 150.89	Quercetin

LC-DAD/ESI-MS analysis of vinegar infused with *Viola tricolor* allowed the tentative identification of the flavonol quercetin and its glucuronide derivative, which were also

present in control vinegar, these compounds are most likely supplied by the vinegar itself. Another flavonol identified was dihydromyricetin. The highest peak was violanthin, which is known in literature for being very abundant in *Viola tricolor*. Violanthin is a flavone C-glycoside that is a flavone substituted by hydroxyl groups at the positions C-5, C-7 and C-4'. Violanthin corresponds to compound 4, it has a molecular ion with m/z 577.46 and two fragments with m/z 457.29 and 353.13. The fragment with a m/z 577.46 in MS^2 spectra is obtained through the loss of 7 hydroxyl groups (17 a.m.u each).

The second highest peak corresponds to myricetin 3-robinobioside which is a glycoside derivative of the flavone myricetin with a molecular ion m/z 625.34 and two fragments with m/z 317.03 and 178.89. The fragment with a m/z 317.03 in MS^2 spectra corresponding to myricetin with the loss of robinobiosyl residue (308 a.m.u). Compound 6 has been identified as kaempferol-O-deoxyhexosyl-hexoside, a glycoside of kaempferol, with a molecular ion m/z 593.33 and two fragments m/z 285.04 and m/z 257.01. The fragment with m/z 285.04 in MS^2 spectra corresponds to kaempferol, it´s obtained through the loss of both deoxyhexosyl moieties (146 a.m.u) and hexose moiety (162 a.m.u), later it suffers from the loss of CO (28 a.m.u), obtaining the fragment with m/z 257.01.

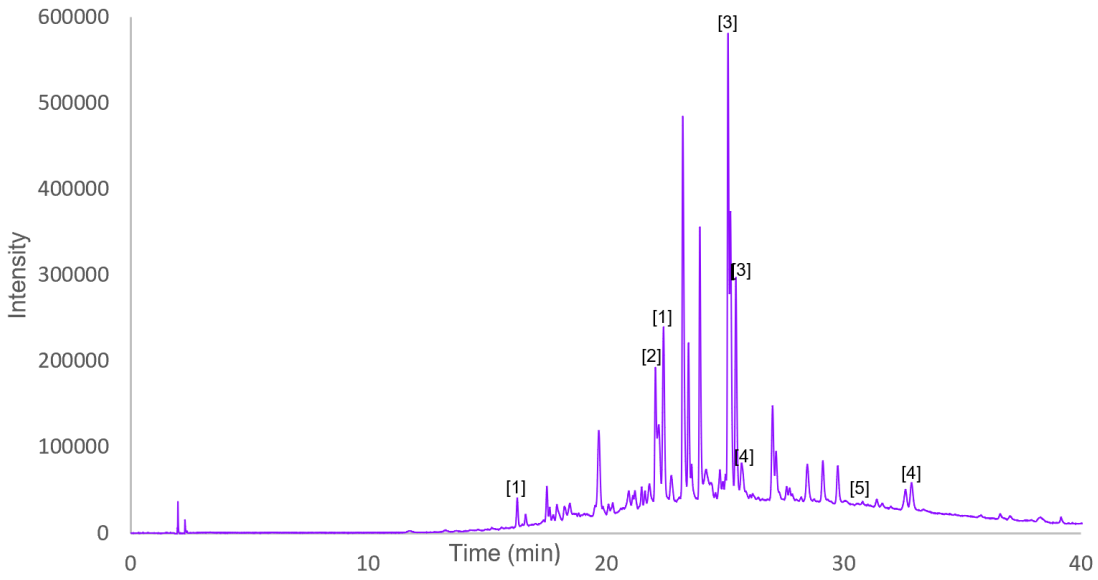

Figure 14: Chromatogram of Viola vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS.

Table 5: UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main compounds identified in Viola vinegar at 280 and 520 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]		16.28/ 19.69/ 22.41	284	321.30	303.13; 275.17	Adipostatin A
[2]		22.07	232/256 356/526	773.33	627.35	Delphinidin-3-O-rutinoside-5-O-glucoside
[3]		25.13/ 25.23	232/272/ 336	579.45	561.35; 457.36	Violanthin (Apigenin-6-C-glucosyl-8-C-rhamnoside)
[4]		25,70/ 32,86	232/356	303.38	257.18; 229.12	Quercetin
[5]		31.40	232/280	235.41	218.16; 146.98	p-Coumaroylputrescine

LC-DAD/ESI-MS in the positive ion mode analysis of vinegar infused with *Viola tricolor* allowed the identification of the hydroxycinnamic acid p-coumaroylputrescine, the resorcinol adipostatin A, the flavone glycoside violanthin and the anthocyanidin-5-o-glycoside delphinidin-3-O-rutinoside-5-O-glucoside. The highest peak in the positive mode also corresponds to violanthin, compound 3, with a molecular ion with m/z 579.45 and two fragments with m/z 561.35 and 457.36. The fragment with a m/z 561.35 in MS^2 spectra results from the loss of water molecule (18 a.m.u) followed by a series of losses of hydroxyl groups (17 a.m.u each).Delphinidin-3-O-rutinoside-5-O-glucoside corresponds to the compound 2 with a m/z 773.33 and one fragment with m/z 627.35, it constitutes the first anthocyanin identified thus far, it´s derived from delphinidin possessing a rutinoside at 3-O-position, and a glucoside at the 5-O-position. This is the only anthocyanin identified in this vinegar, justifying its almost imperceptible color alteration.

Centaurea Vinegar

The chromatographic profile of vinegar infused with *Centaurea cyanus* was monitored at 280 nm in the negative ion mode and was monitored at 280 nm and 520 nm in the positive ion mode, respectively represented in Figures 15 and 16.

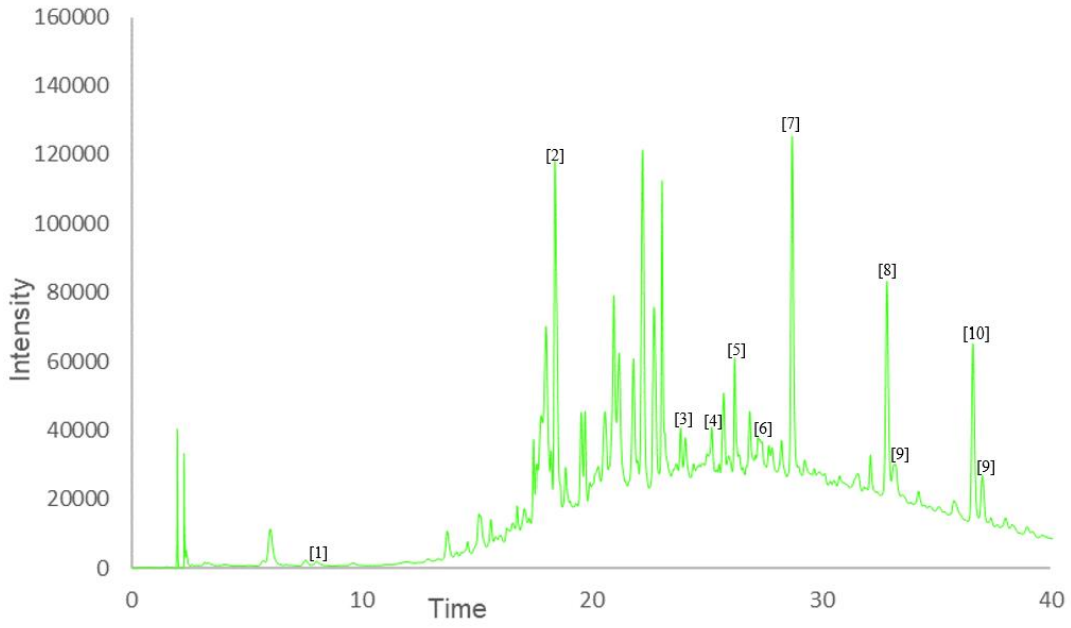

Figure 15: Chromatogram of Centaurea vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS.

Table 6: UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main phenolic compounds identified in Centaurea vinegar at 280 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]	Negative	8.05	264/300 476	433.20		Avicularin
[2]		18.40	324/380	191.09	126.92 172.98; 84.86	Quinic acid
[3]		23.87	284/328	367.31	178.93; 134.85	5-Feruloylquinic acid
[4]		25,22	356/368	463.25	301.04	Quercetin-3-O-galactoside
[5]		25,75	356	477.24	301.03; 178.95	Quercetin-3-glucuronide
[6]		27,25	280/324	353.26	190.96;173.01;126.90	5-O-Caffeoyl-quinic acid (Chlorogenic acid)
[7]		28,73	268/332	431.28	269.05	Isovitexin (Apigenin-6-C-glucoside)
[8]		32,85	368	301.11	178.94; 150.83	Quercetin
[9]		33,22; 37.01; 37.38	268/272/ 280/368	285.17	284.99	Kaempferol
[10]		36,60	268/292 320	269.16	268.98	Apigenin

LC-DAD/ESI-MS analysis of vinegar infused with Centaurea cyanus allowed the tentative identification of quercetin and the quercetin derivative, quercetin-3-glucuronide, that were also identified previously pointing towards their ubiquitous character. While it possible to tentatively identify another 2 quercetin derivatives: quercetin O-glycoside, avicularin, which did not fragment during the analysis, and quercetin-3-O-galactoside, corresponding to peak 4 with a precursor ion of m/z 463.25 that decomposes as product ion quercetin with m/z 301.04 by loss of a sugar group galactose (162 a.m.u). The highest peaks are quinic acid and isovitexin. Quinic acid corresponds to compound 2 eluted at R_t=18.40, with a molecular ion with m/z 191.09 and three fragments at m/z 126.92, at m/z 172.98 and at m/z 84.86. Quinic acid is a natural metabolite of plants and although it is not classified as a polyphenol, it does participate in the biosynthesis of phenolic compounds by processes like esterification with hydroxycinnamic acids forming esters such as chlorogenic acid that is subsequently eluted at R_t=27,25 in the LC-DAD/ESI-MS analysis. Chlorogenic acid is a quinic acid that suffer acylation at positions C-1 and C-5 by caffeoyl and feruloyl groups respectively. Peak 6 was tentatively identified as chlorogenic acid with the precursor ion m/z 353.26, that decomposes to originate a product ion corresponding to quinic acid, m/z 190.96 which was originated by the loss of a caffeoyl group (162 a.m.u) and the further loss of a water molecule (18 a.m.u), originating a fragment with a m/z 173.01. Isovitexin, apigenin-6-C-glucoside, corresponds to compound 7 with a molecular ion with m/z 431.28 and one fragment with m/z 269,05. Isovitexin is a C-glycosyl flavone in which the precursor ion decomposes into product ion, apigenin, with m/z 269.05 by loss of a glucose moiety (162 a.m.u). Apigenin and kaempferol were tentatively identified as well but neither one fragmented during the analysis.

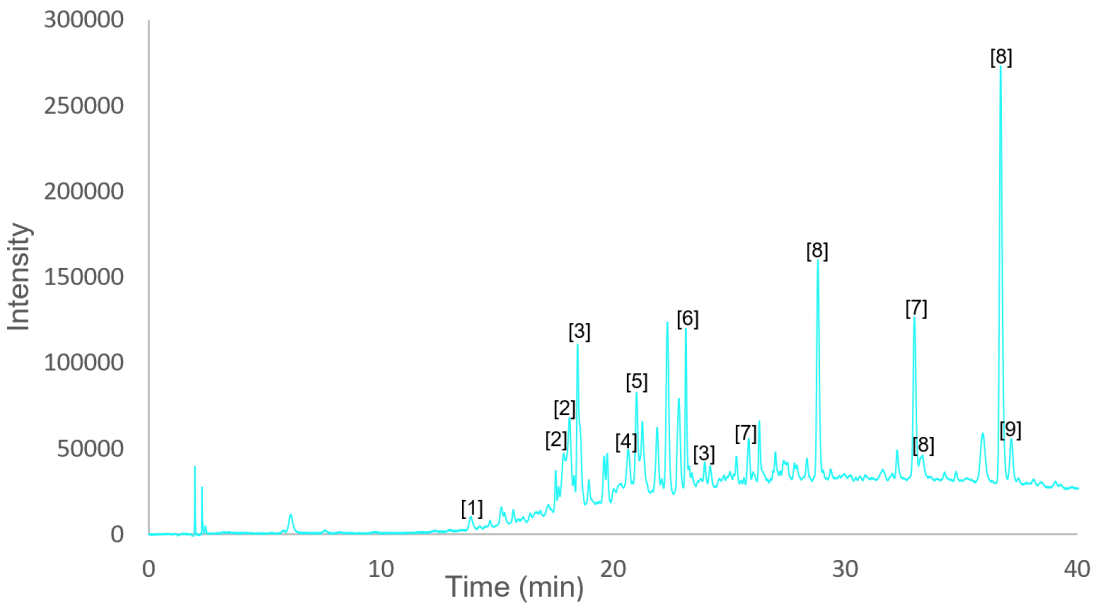

Figure 16: Chromatogram of Centaurea vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS.

Table 7: UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main compounds identified in Centaurea vinegar at 280 and 520 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Compound
[1]	Positive	13.87	328/476/ 564/648	493.10		Oenin (Malvidin-3-O-glucoside)
[2]		17.87/18.12	264/512	611.47	449.26 287.15	Cyanidin-3,5-diglucoside
[3]		18,46/23.95	232/324	163.19	145.00;116.96	7-Hydroxycoumarin
[4]		20.66	232/280/516	449.42	287.16; 287.15	Cyanidin 3-O-glucoside
[5]		21.01	272/516	711.42	549.30, 287.16	Cyanidin-3-O-(6″-succinylglucoside) -5-O-glucoside
[6]		23.13	268/320	609.47	433.30 ;271.15	Apigenin-7-O-glucoside-4'-O-glucuronide

[7]		25.84/32.98	368	303.38	257.18; 229.09	Quercetin
[8]		28.82/33.33/36.65	268	271.34	271.21; 271.16	Apigenin
[9]		37.15	264/368	287.31	287.18; 241.10	Cyanidin

LC-DAD/ESI-MS analysis of vinegar infused with *Centaurea cyanus* in positive ion mode allowed the tentative identification of 4 anthocyanins and 1 anthocyanidin, pointing towards Centaurea vinegar possessing these compounds unlike Control vinegar and at a higher level and with more variety than in Viola vinegar. It was tentatively identified the presence of cyanidin, anthocyanidin cation, three anthocyanins derived from cyanidin and one derived from malvidin. Compound 2, cyanidin-3,5-diglucoside, is cyanidin linked to two glucose moieties at the C-3 position and at the C-5 position, it has a molecular ion with m/z 611.47 which can be decomposed first by the loss of one of the glucose moieties (162 a.m.u) either at C3-position or at C5-position, leading to the formation of cyanidin 3-O-glucoside or cyanidin 5-O-glucoside with a m/z 449.26 and then a further loss of the second glucose moiety (162 a.m.u), corresponding to the aglycone cyanidin with m/z 287.15. Cyanidin 3-O-glucoside is tentatively identified and further eluted at R_t= 20.66 also decomposed by the loss of a glucose moiety (162 a.m.u) at C-3 position, thus leaving the aglycone cyanidin with m/z 285.15. Cyanidin-3-O-(6″-succinylglucoside)-5-O-glucoside has molecular ion with m/z 711.42, with the loss of a glucose moiety (162 a.m.u) leading to a fragment with a m/z 549.30 in the MS^2 spectra, which then loses another glucose moiety (162 a.m.u) and loses a succinyl group (100 a.m.u), with the fragment obtained at the end with m/z 287.16.corresponding to the aglycone cyanidin. Oenin, malvidin-3-O-glucoside, is a malvidin derivative with a glucosyl moiety attached at the 3-hydroxy position. 7-Hydroxycoumarin was also tentatively identified, compound 3, which corresponds to a coumarin substituted by a hydroxyl group at the position C-7, this compound´s precursor ion has a m/z163.19 that dissociates into a product ion with m/z 145.00 that corresponds to coumarin, with a further loss of CO (28 a.m.u), a fragment with m/z 116.96 is obtained. The highest peak corresponds to apigenin, compound 8, with a m/z 271.34. One derivative of apigenin was present as well, apigenin-7-O-glucoside-4'-O-glucuronide, which corresponds to compound 6. It has a precursor ion with m/z 609.47 dissociates to a product ion with a m/z 433.30 in the MS^2 spectra due to the loss of a glucuronyl moiety (176 a.m.u) and then the loss of a glucose moiety (162 a.m.u), obtaining a fragment with m/z 271.16 that matches apigenin.

Cosmos Vinegar

The chromatographic profile of vinegar infused with *Cosmos bipinnatus* was monitored at 280 nm in the negative ion mode and was monitored at 280 nm and 520 nm in the positive ion mode, respectively represented in Figures 17 and 18.

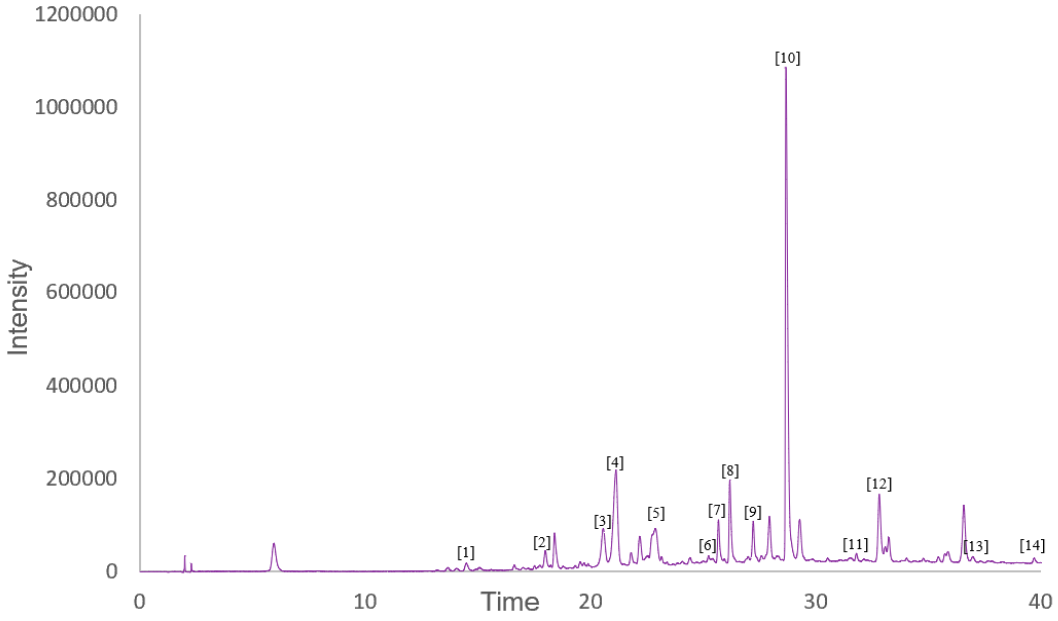

Figure 17: Chromatogram of Cosmos vinegar recorded at 280 nm in the negative mode through LC-DAD/ESI-MS.

Table 8: UPLC-DAD-ESI/MS-MS identification, in negative ion mode, of the main compounds identified in Cosmos vinegar at 280 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]		14,51	328	179.00	134.85	Caffeic acid
[2]		17,98	312	163.00	118.82	m-Coumaric acid
[3]		20.55	280/516	447.21	285.05	(Cyanidin 3-O-beta-D-glucoside)

[4]	Negative	21,12	280/516	593.39	285.11; 256.03	Cyanidin 3-O-(6″-p-coumaroyl-glucoside)
[5]		22.87	280/516	607.36	299.04,284.05	Peonidin 3-O-(6″-p-coumaroyl-glucoside)
[6]		25,23	356	609.29	301.02;178.88	Quercetin 3-rutinoside
[7]		25,67	256 352	477.18	301.03;178.99	Quercetin-3-glucuronide
[8]		26.18	252/348	461.26	285.08	Thermopsoside (Chrysoeriol 7-O-glucoside)
[9]		27,22	328/380	353.17	191.02; 126.84;172.92	5-O-Caffeoyl-quinic acid (Chlorogenic acid)
[10]		28,68	268/336	445.27	269.05;	Apigenin 7-O-glucuronide
[11]		31,54	268/344 356/376 388	269.15	269.05;	Apigenin
[12]		32,82	256/368	301.12	178.93;150,81	Quercetin
[13]		36.98	356/368	285.16	285.03	Kaempferol
[15]		37.67	356/368	315.18	300.03;271.01 150.98	Isorhamnetin

LC-DAD/ESI-MS analysis of Cosmos vinegar allowed the tentative identification of quercetin and quercetin-3-glucuronide, which have identified in all of the vinegars analysed. It was also possible to tentatively identify another quercetin derivative, corresponding to compound 6, quercetin 3-rutinoside, with a molecular ion with m/z 609.29 and two fragments with m/z 301.02 and m/z 178.88. The fragment with a m/z 301.02 in the MS^2 spectra due to the loss of rutinoside (308 a.m.u), corresponds to quercetin. The highest peak corresponds to apigenin7-O-glucuronide that has a molecular ion with m/z 445.27, with the loss of a glucuronyl moiety (176 a.m.u) it´s obtained in the MS^2spectra a fragment with m/z 269.05, apigenin.

It was tentatively identified compound 8 as thermopsoside, chrysoeriol 7-O-glucoside, that has a molecular ion with m/z 461.26, with the loss of a glucose moiety (162 a.m.u) and further loss of the methyl group (15 a.m.u), resulting in luteolin with a m/z 285.08. It was tentatively identified isorhamnetin as compound 15 which is quercetin in which the hydroxyl group at position 3' is replaced by a methoxy group with a molecular ion with m/z 315.18, with three fragments with m/z 300.03, m/z 271.01 and m/z 150.98. The fragment with m/z 300.03 corresponds to quercetin due to the loss of the methyl group (15 a.m.u).

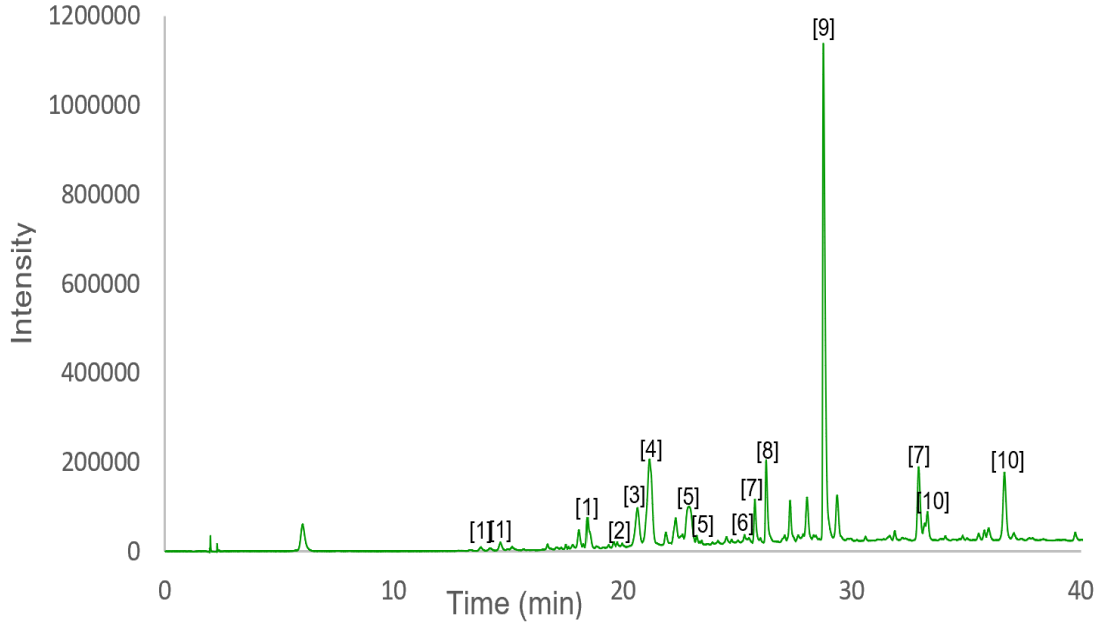

Figure 18: Chromatogram of Cosmos vinegar recorded at 280 nm in the positive mode through LC-DAD/ESI-MS.

Table 9: UPLC-DAD-ESI/MS-MS identification, in positive ion mode, of the main compounds identified in Cosmos vinegar at 280 and 520 nm. Retention time (R_t), wavelengths of maximum absorption (λ_{max}), mass spectra data and tentative identification are represented.

Peak	Ion mode	Rt (min)	λ_{max} (nm)	ESI Full MS (m/z)	ESI Full MS^2 (m/z)	Tentative identification
[1]	Positive	13.81/14.67/18,45	328/380	163.13	144.96; 116.93	7-hydroxycoumarin
[2]		19,59	312/356	147.09	118.94; 90.99	Coumarin
[3]		20,62	280/356/516	449.37	287.15; 287.16	Kuromanin (Cyanidin 3-O-beta-D-glucoside)
[4]		21,14	280/516	595.55	287.19; 287.16	Cyanidin 3-O-(6″-p-Coumaroyl-glucoside)
[5]		22.86¸23.21	280/356/376 516	609.54	301.23; 286.16	Peonidin 3-O-(6″-p-Coumaroyl-glucoside)
[6]		25.30	356/520	611.31	303.18; 257.14	Delphinidin-O-rutinoside
[7]		25.75/32.90	256/368	303.38	257.16; 229.09	Quercetin
[8]		26.24	272	463.42	287.17; 287.15	Luteolin 7-glucuronide
[9]		28.74	236/268/336	447.39	271.17	Apigenin 7-glucuronide
[10]		33.29/36.65	232/268/332	271.37	271.18; 271.15	Apigenin

LC-DAD/ESI-MS analysis of Cosmos in positive ion mode allowed the tentative identification of 4 anthocyanins: 2 derived from cyanidin, 1 derived from delphinidin and 1 from peonidin. The vinegar infused with cosmos flowers showcases the greatest variety of anthocyanins, which can be linked to its most aesthetically striking color change. The

highest peak corresponds to apigenin 7-glucuronide with a precursor ion with m/z 447.39 that dissociates into a product ion with m/z 271.17 with the loss of a glucuronyl moiety (176 a.m.u), corresponding to apigenin. Cyanidin 3-O-beta-D-glucoside has a precursor ion with m/z 449.37, with one fragment with a m/z 287.15 due to the loss of the glucose moiety (162 a.m.u) at C-3 position, with the fragment corresponding to cyanidin. It was also tentatively identified cyanidin 3-O-(6″-p-coumaroyl-glucoside) that has a molecular ion with m/z 595.55. The loss of a glucose moiety (162 a.m.u) and the loss of a coumaroyl group (146 a.m.u), leads to obtention of the fragment with m/z 287.19, that corresponds to the aglycone cyanidin. Peonidin 3-O-(6″-p-coumaroyl-glucoside) has a precursor ion with m/z 609.54 that dissociates into product ions with m/z 301.23 and 286.16. The fragment with a m/z 301.23 in the MS^2 spectra corresponds to the anthocyanin structure with the loss of a glucose moiety (162 a.m.u) and the loss of a coumaroyl group (146 a.m.u), corresponding to the aglycone peonidin. Delphinidin-O-rutinoside has a precursor ion with m/z 611.31 that dissociates into a product ion with m/z 303.18 due to the loss of a rutinoside (308 a.m.u).

Chromatographs were presented with different scales of intensity to better exhibit different phenolic profiles, the peaks of Control vinegar are significantly less intense than the ones of the vinegars infused with edible flowers, with vinegar infused with cosmos flowers showcasing the peaks with the highest intensity.

Quercetin and quercetin-3-glucuronide are present in all the vinegars, alongside apigenin, with derivatives of apigenin corresponding to the highest peaks. Quercetin and different derivatives of quercetin have in general been very prevalent throughout all the vinegars studied. Several other phenolic compounds aren´t identified in all of them however are tentatively in more than one of the vinegars, namely kaempferol or one of its derivatives are present in all of the edible flower infused vinegars. While certain phenolic compounds are only present in vinegar infused with one of the edible flowers in specific suggesting that those polyphenols are characteristic to that species. Many of the polyphenols tentatively identified follow a similar pattern of glycosylation, particularly with glucose, rutinoside or glucuronidation. The prevalence of glycosides can be explained because glycosylation increases the stability of the polyphenols, with glycosides possessing more stability than aglycones (Johnson et al., 2021; Xie et al., 2022). The anthocyanins identified were derived from cyanidin, malvidin, peonidin and delphinidin, with no anthocyanins derived from pelargonidin or petunidin being identified in this analysis. An anthocyanin derived from peonidin was only identified in Cosmos vinegar and an anthocyanin derived from malvidin was only identified in Centaurea vinegar. Anthocyanins derived from cyanidin were the most widespread, with distinct cyanidin

derivatives being found in both Centaurea Vinegar and Cosmos Vinegar. The anthocyanins present are either glycosylated with glucose or rutinoside and/or acylated with coumaric acid, malonic acid or succinic acid.

Due to the technique used it was not possible to identify the identity of all the peaks. The presence of phenolic compounds that didn't suffer fragmentation was one of the obstacle to the total characterization of the phenolic profiles of the edible flower infused vinegars. Another hinderance to the identification by LC-DAD/ESI-MS analysis is related to the obtention of fragments which don't match the fragments expected by consulting the literature. A likely explanation for this phenomenon is related to the co-elution of the precursor ion with another ion, resulting in complex fragmentation patterns.

3.5 Antioxidant studies

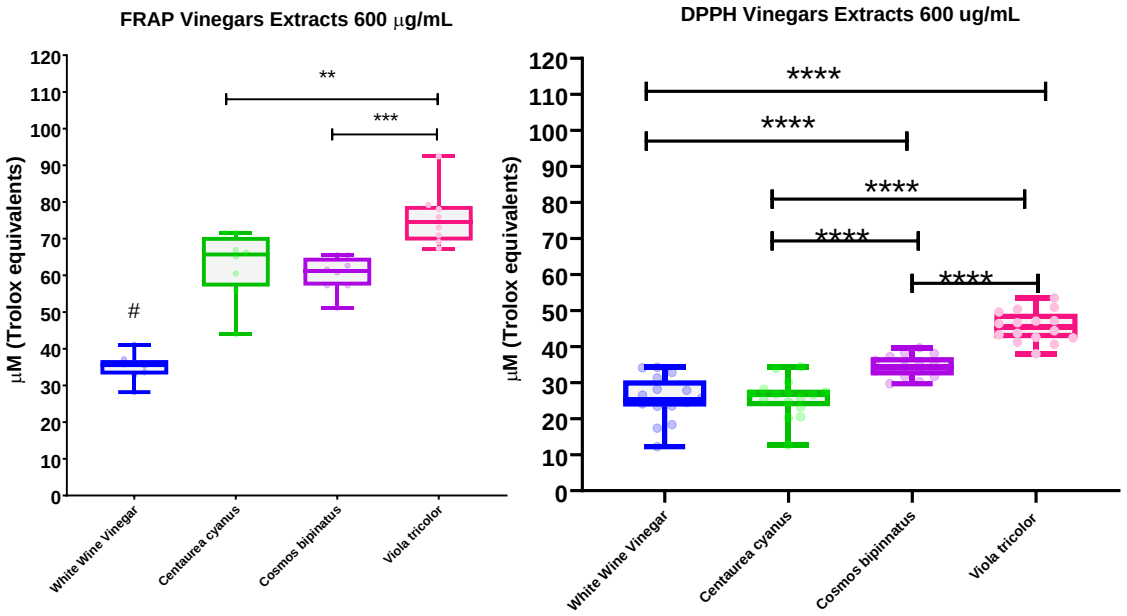

Figure 19. Box plot representation of the antioxidant power; FRAP and DPPH; respectively of polyphenol rich edible flower infused vinegars with a concentration of 0.6 mg/mL. Comparison of the antioxidant activity measured for Control Vinegar, Viola Vinegar, Centaurea Vinegar and Cosmos Vinegar. Data normalized to Trolox equivalents. White wine vinegar (Control Vinegar) is represented in blue; *Centaurea cyanus* is represented in green; *Cosmos bipinnatus* is represented in purple and *Viola tricolor* is represented in pink. * means statistical difference of p<0.05; ** means statistical difference of p< 0.01; *** means statistical difference of p< 0.001; # means statistical difference of p< 0.0001

When analyzing the results of FRAP (Ferric Reducing Antioxidant Power), it´s immediately apparent that the values of antioxidant activity of edible flower infused vinegar extracts have a higher antioxidant activity than the white wine vinegar extract that operates as the Control vinegar, thus showing how the incorporation of edible flowers contributes to a greater antioxidant capacity. In the Control vinegar extract, the whiskers extend from the box to the lowest datapoint of antioxidant activity which corresponds to 28.18 μM (Trolox equivalents) and the farthest one is of 41.11 μM (Trolox equivalents). The median is of 35.36 μM (Trolox equivalents), representing a central tendency of the antioxidant activity. The antioxidant activity of the extract of vinegar infused with *Centaurea cyanus* ranged from 43.98 μM (Trolox equivalents) to 71.62 μM (Trolox equivalents). The lowest datapoint of the Centaurea vinegar extract is higher than the highest one of Control vinegar extract, thus clearly illustrating how this increase in antioxidant activity caused by incorporation of cornflowers is quite significant. The results of vinegar infused with *Cosmos bipinnatus* extract are higher than the results obtained

of Control vinegar extract but not quite as high as the results of the extract of vinegar infused with *Centaurea cyanus*. The central tendency of both vinegar extracts by observation of the second quartile, third quartile and upper limit exceeds the 60 μM (Trolox equivalents). The results of the vinegar infused with *Viola tricolor* extract appear to exhibit the highest antioxidant activity of them all, with the values ranging from 67.31 to 92.43 μM (Trolox equivalents). This data indicates a greater capacity for antioxidant activity in Viola vinegar than in Cosmos vinegar and Centaurea vinegar. These findings allude to the polyphenols contained by this vinegar having the highest radical quenching capacity.

These results indicate that the intensity of antioxidant power increases from White Wine vinegar extract < Cosmos vinegar extract < Centaurea vinegar extract < Viola vinegar extract.

An ordinary one-way ANOVA was performed to determine if the difference between the 4 groups: Control vinegar extract + 3 edible flower infused vinegar extracts infused is statistically significant, with all of them being statistically significant except between Centaurea Vinegar extract and Cosmos Vinegar extract, pointing towards the difference in antioxidant activity between the two vinegars extracts being of little significance.

The results of antioxidant activity obtained by DPPH are not identical which can be explained by them being distinct methods with different underlying mechanisms, with the values of antioxidant activity obtained by DPPH being much lower than the ones obtained by FRAP. While FRAP is associated with evaluating radical quenching, DPPH is associated with evaluating radical scavenging activity, the results point towards the polyphenols tested being stronger quencher than scavengers. In DDPH, the highest antioxidant activity is also measured in the extract of the vinegar infused with *Viola tricolor,* the values range from 37.98 μM (Trolox equivalents) to 53.47 μM (Trolox equivalents). Control vinegar extract and Centaurea vinegar extract exhibit very similar results, suggesting that the polyphenols in Centaurea vinegar have a weak scavenging activity.

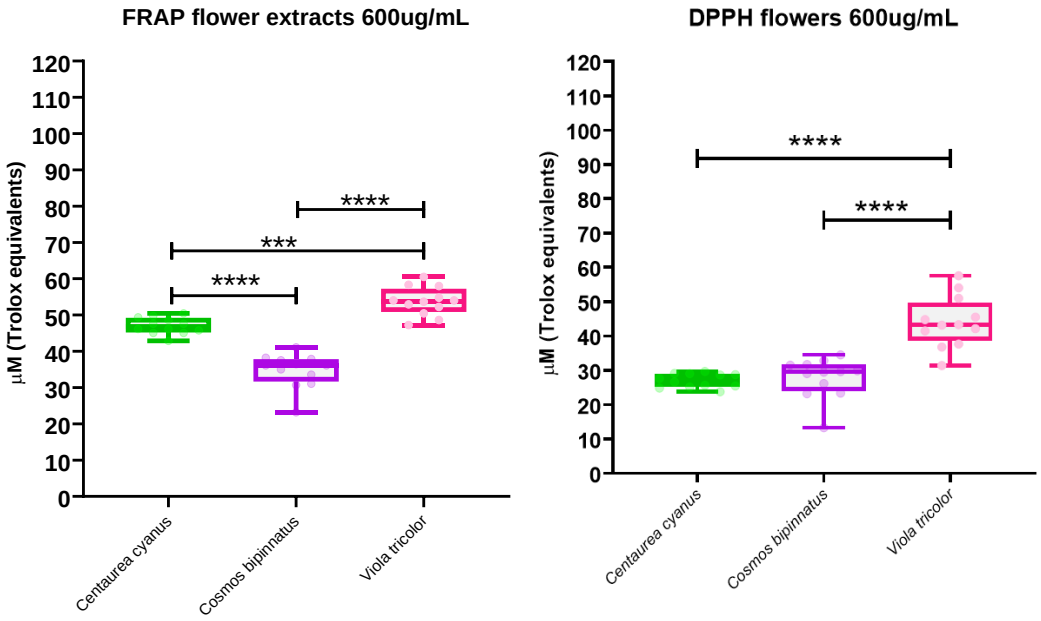

Figure 20: Box plot representation of the antioxidant power; FRAP and DPPH; respectively of edible flower extracts with a concentration of 0.6 mg/mL. Comparison of the antioxidant activity measured between Viola tricolor, Centaurea Cyanus and Cosmos bipinnatus. Data normalized to Trolox equivalents. *Centaurea cyanus* is represented in green; *Cosmos bipinnatus* is represented in purple and *Viola tricolor* is represented in pink. *** means a statistical difference of p< 0.001 and **** means a statistical difference of p < 0.0001

When analysing the values of the antioxidant activity of the flower extracts in comparison with the results of the edible flower infused vinegar extracts, it remains consistent throughout that the highest values of antioxidant activity are obtained thanks to *Viola tricolor*, indicating that they have a phenolic profile richer in polyphenols that are potent antioxidant agents, both in terms of radical scavenging and radical quenching. Therefore, *Viola tricolor* is the most promising from a health perspective out of the three edible flowers. Our findings demonstrate how on the account of possessing different phenolic profiles, edible flowers have a varying degree of importance for the research of antioxidant capacity.

In the FRAP assay, *Cosmos bipinnatus* exhibits the lowest antioxidant activity while in the DPPH assay, its lowest datapoint is lower than the lowest datapoint of *Centaurea cyanus* but its higher datapoint is also higher than the highest datapoint of *Centaurea cyanus*, showing cosmos flowers have a greater variability than cornflowers. By ordinary one-way ANOVA it was determined that the difference between these two species of edible flowers is also not significant.

Chapter 2 - Astringency experiments

1. Introduction

1.1 Astringency

The manner in which consumers perceive food in the oral cavity depends on the qualities of the food whose interactions in the oral cavity are quickly interpreted by the brain, consumers' dietary habits are swayed by their preferences, which are impacted by their sensory responses to the qualities of the food (Engelen & Van Der Bilt, 2008). Polyphenols impact how we perceive food products due to their ability to alter the organoleptic properties of plant based foodstuff such as color, aroma, taste and astringency (Fang & Bhandari, 2010; Gutiérrez-Escobar et al., 2021).

The word astringency comes from the latin *adstringere ,*"to bind", it´s a mouthfeel experienced in the oral cavity marked by a sensation of coating dryness, shrinking, roughness and puckering that can take 20 to 30 seconds to fully develop , it´s not understood as a taste on the grounds of it being felt in areas of the mouth with an absence of taste receptors, it´s currently considered a somatosensation (Habschied et al., 2021; Soares et al., 2020; Wang et al., 2022). Despite not being considered a taste per say, it still is to this day considered a critical component in how the overall flavor is perceived by consumers (Beecher et al., 2008).

Despite astringency being frequently considered undesirable to consumers, with it being for example compared to the unpleasant mouthfeel of fruits that aren't yet ripe, it can also at times be an intentional and coveted characteristic like in beverages such as wine (Allegro et al., 2021; Jiang et al., 2014). In red wine, for example, astringency is credited as being responsible for wines being appraised as rich and velvety (Cosme et al., 2021). Another foodstuff that is known for exhibiting astringency is vinegar (Ho et al., 2017).

Astringency is oftentimes concomitant with bitterness, with the perception of this sensory attribute influencing how astringency is perceived , when a product exhibits both bitterness and astringency, bitterness is the one thought of as the main attribute and astringency as the secondary one (Arnold et al., 1980; Spranger et al., 2008).

1.2 Influence of polyphenols on astringency

Astringents are molecules linked to tissue constricting and protein precipitation, a number of compounds can be counted among astringents like phenolic astringents, metallic astringents, protein astringents, dehydrating astringents and (organic or inorganic) acid astringents (Huang & Xu, 2021; Karolkowski et al., 2023).

Of the phenolic compounds known for being astringents, tannins were among the first to be identified as such. The origin of the word tannins can be traced back to the transformation of animal skin into leather by interaction of the skin proteins with the polyphenols in plant based extracts , which is called tanning practice (Soares et al., 2020).They comprise a complex group of high-weigh phenolic compounds with a molecular weight between 500 to 3000 kDa, derived from flavan-3-ols that are ambiguous to define chemically, since they aren´t part of flavonoids nor of non-flavonoids. Tannins have an elevated solubility in water that notably manifests a powerful binding capacity, originating complexes with proteins, starch and minerals and causing the precipitation of heavy metals, allowing them to assist in treating poisoning and help in the deactivation of microbial extracellular enzymes (Bennick, 2002; Govindarajan et al., 2016; Soares et al., 2020).

They can be divided in hydrolysable tannins or condensed tannins, it´s widely believed by sensory panellists that hydrolysable tannins are greater astringent agents than condensed tannins (Obreque-Slier et al., 2010; Q. Qi et al., 2023; Rauf et al., 2019).

A multitude of studies throughout the years reported that several compounds from other subclasses of polyphenols (namely flavonoids, anthocyanins and phenolic acids) can elicit astringency (Ye et al., 2022). A particularly researched flavonol regarding an association with the perception of astringency is quercetin and its derivatives (Hufnagel & Hofmann, 2008). Glycosides of flavonols have been reported to originate a astringent mouthfeel even if only present in low concentrations (Scharbert et al., 2004).

1.3 The mechanisms behind astringency

The mechanisms responsible for the development of an astringent sensation remain somewhat elusive and not yet totally understood, existing a high probability of multiple mechanisms responsible for astringency happening simultaneously (Gibbins & Carpenter, 2013).

In ancient Greece, astringency was mistakenly thought of as being a taste quality with arguments about how astringency should be classified continuing for centuries (Lyman & Green, 1990). There was a surfacing in literature about astringency in the 1840s, although the investigation around it was immensely limited (Liu et al., 2023).

In 1954 it was originally defended by Bate-Smith that astringency is a tactile sensation elicited in the palate by interaction and subsequent precipitation of glycoproteins with tannins in mucous secretions of salivary glands and the development of a clinging residue through the sticking of the complexes to the oral mucosa (Soares et al., 2020). Bate-Smith was the one that initially stated that astringency should not be understood as a taste and yes as a sensory experience (Bate-Smith, 1954). It´s now known that astringency is a sensation on the grounds of it being felt in areas of the mouth with an absence of taste receptors, as it can be felt on the soft palate, gingiva and on the lips (Bate-Smith, 1954; Cosme et al., 2021; Garcia-Estevez et al., 2017). Joslyn and Goldstein in 1964 defended the tactile aspect of astringency by advocating that protein precipitation co-occurs with the diminishing of tissues' permeability and with the shrinkage of tissues because of water loss (Joslyn & Goldstein, 1964).

The study of astringency has been closely correlated with the study of bitterness. With the researchers Lea and Arnold describing them as operating as twins substances in 1978, with further studies highlighting that many compounds that trigger one of them also trigger the other (Bajec & Pickering, 2008; Lea & Arnold, 1978). More recent studies and sensory analyses better delineate the perception of astringency and of bitterness, distinguishing them by elaboration on their differences (Bertino & Lawless, 1993; Lee & Lawless, 1991).The mechanisms of eliciting astringency are still being heavily researched about, with the central mechanism associated to the induction of astringency being related to the structural change of the oral mucosal pellicle, which is rich in proteins like mucins, cystatins, amylases, immunoglobin A and acid proline rich proteins, this change is attributable to the salivary proteins (SPs) interacting and precipitating by action of astringent compounds such as tannins. Initially, the tannin-SPs interact, resulting in soluble aggregates, as the size of the aggregates increases, the tannin-SPs aggregates become insoluble and thus precipitate due to intramolecular crosslinking. This

precipitation of the proteins removes them from saliva, making them insoluble, leading to a saliva with less viscousness, decreasing mucosal lubrication and thus increasing the friction in the oral mucosa. (Gibbins & Carpenter, 2013; Ployon et al., 2018; M.-Y. Qi et al., 2023; Ye et al., 2022). The diminishing of lubrification leads to the occurrence of increased astringency perception (Pavez et al., 2022).

As it was mentioned previously in section 1.2, a variety of different compounds other than polyphenols can be astringents, with factors like pH and protein isoelectric point having the ability to shape their capacity for evoking astringency: metal salts agents like as iron (III) chloride, sulfate chloride and aluminium (III) chloride, the biopolymer chitosan that operates as a astringent when dissolved in an environment with an acidic pH and proteins with a high isoelectric point like lysozyme (Rehage et al., 2017; Rodriguez et al., 2003). The isoelectric point can be described as the pH in which the net charge of the protein is zero, when the pH is lower than the isoelectric point, the proteins are positively charged and when the pH is higher, they´re negatively charged, proteins with a high isoelectric point are electrically neutral at more basic pH values (Tokmakov et al., 2021)**.** The increase of the intensity of astringency felt can be linked to pH values , with a lowering of pH contributing to the increase of astringency, with this sensation in an acid medium being assigned to either the direct input of H^+ ions or hydrogen bonding abilities of hydroxyl groups present (Cosme et al., 2021).

In an acidic food matrix, the hydrogen acceptor sites of SPs bond with the hydroxyl groups on ring B of polyphenols through hydrogen bonds, this interaction has been posited as responsible for the complex's stabilization. Covalent bonds and nucleophilic addiction are also being established between these groups of compounds as well as hydrophobic interactions between the proline residues of the SPs rich in proline and the aromatic rings of polyphenols, with these interactions also affecting the overall process. The non-covalent bonds between these proteins and astringent polyphenols tend to be reversible although depending on their extent, precipitates can be obtained that are in fact irreversible whilst the covalent bonds between them are generally irreversible (Cosme et al., 2021; Pérez-Gregorio et al., 2020).These interactions are swayed by factors such as the size of the molecules, their steric conformation, polarity and also the pH of the environment and concentration of the compounds (Pérez-Gregorio et al., 2020). This process starts with the mentioned reversible non-covalent hydrophobic bonds, with the complex that is being originated at this point still remaining soluble, if thereafter, the complex formed becomes insoluble, this complex begins after a critical point of aggregation to precipitate (Charlton et al., 2002). It can in general be stated that polyphenols with a more elevated molecular weight and/or a greater number of hydroxyl

or galloyl groups possess more sites for interacting with the SPs (Pérez-Gregorio et al., 2020).

According to recent reports by Zhuang, there´s been the establishment of a connection between subqualities attributed to astringency and the structure of different classes of polyphenols with the degree of phenolic acylation being linked to astringency being perceived as grassy, as well as to the rise of the intensity of the astringency experienced and the degree of hydroxylation being linked to astringency being perceived as coarse (Zhuang et al., 2020). The influence of flavonols in astringency tend to be associated to a silkier and more velvety mouthfeel whilst tannins are associated to a harsher mouthfeel (Huang & Xu, 2021; Schöbel et al., 2014).

Outside of this mechanism of perception of astringency based on the interaction of polyphenols and SPs and subsequent precipitation of the complex formed, it has also been reported that the perception of astringency is related to the interaction between proteins and polyphenols with a low molecular weight that doesn't result in any precipitation occurring at all, with a number of polyphenols that are not involved whatsoever in precipitation nor in complexation being perceived as quite astringent. In this mechanism, astringents can directly bind to oral epithelial cells and can even be absorbed by the lipid bilayer, triggering shrinkage. Thus, it was proposed that in order to evaluate the perception of astringency, it´s not only required to have in consideration the number of SPs complexed, moreover the amount of nonbound astringent stimulus (Schwarz & Hofmann, 2008; Wei et al., 2023). The difference between the two mechanisms described is thought of as being dependent on the different structures that astringent polyphenols may present (Payne et al., 2009).

Over time more and more additional mechanisms associated to astringency have been discovered through a series of different studies. Green in 1993 argued that the oral epithelium could participate in evoking astringency, with more current findings, pointing to the oral cells of the epithelium being capable of directly bonding with tannins causing modifications to the mucosa´s qualities and thus being capable of affecting the production of mucosal pellicle (Green, 1993; Payne et al., 2009). In 2019, Soares effectively managed to develop a new complex cell-based model with the purpose of evaluating how tannin-binding is impacted by the contribution of SPs , oral cells and mucosal pellicles, with the evidence pointing to the the combination of the interactions with all the oral constituents being greater than the sum of the individual interactions (Soares et al., 2019).

Current day theories around astringency involve the study of the possible role of trigeminal nerve, mechanoreceptors, chemoreceptors and G-coupled proteins in the perception of this tactile sensation (Bajec & Pickering, 2008).Trigeminal nerve, which is the largest and most widely distributed in the supra-hyoid neck of the cranial nerves, which possesses sensory and motor components, has been theorized to mediate the perception of astringency, with the anesthesia of the trigeminal nerve eliminating the perception of astringency. (Bathla & Hegde, 2013; Liu et al., 2023; Schöbel et al., 2014). The role of trigeminal nerve has been studied for decades, with Green in 1993 defending that astringency is a diffuse sensation because it's transduced by the free nerve endings of the trigeminal nerve (Green, 1993).

It's been proposed that one of the possible mechanisms responsible for the perception of astringency can be related to the interactions of polyphenols with specific SPs (proline-rich proteins (PRPs), mucins, histatins, cystatins, statherin/P-B peptide), forming a soluble complex that becomes insoluble, when the increase of the content of astringents rises, disarranging both the salivary film and mucosal pellicle. This disarrangement prompts the loss of lubrification and exacerbation of friction, followed by an activation of the calcium signalling that sets off the release of acetylcholine, which in turn bonds with acetylcholine receptors on the trigeminal free nerve endings, stimulating trigeminal neurons, herein leading to the perception of astringency (Wei et al., 2023).

More and more studies for a considerable period of time now, have speculated that a major engine for eliciting an astringent sensation may be the linked to activating trigeminal mechanoreceptors and it has been speculated as well that trigeminal chemoreceptors might also play a role in this mediation, presently there´s still uncertainty surrounding the role of both but it´s proposed that the perception of astringency may depend on one of them or a co-activation of both (Pires et al., 2020; Schöbel et al., 2014). Mechanoreceptors are specialized nerve endings showing selective sensibility for particular extracellular stimuli like vibration and pressure, these somatosensory receptors lead information to the central nervous system via mechanically gated ion channels (Cosme et al., 2021; Garcia-Estevez et al., 2017). Mechanoreceptors are located in the mucosa that surrounds taste buds with their stimulation being posited as prompter of astringency, in histological studies performed in mice, peizo2 was the main mechanoreceptor for the perception of astringency, with the findings of this study suggesting that this mechanoreceptor is the main one in human beings too (G Mouritsen, 2016; Moayedi et al., 2018). As mentioned above, the precipitation of SPs-polyphenols complexes in the mouth causes a lessening of the lubrification and an increase of friction in the mouth. It has been hypothesized that the rise in friction forces at the surface of

epithelial cells incites the activation of mechanoreceptors of somatosensory nerves resulting in the perception of the feeling of “dry mouth” (Lyman & Green, 1990; Schöbel et al., 2014). This activation ushers the opening of the ion channels of the receptor cascade, depolarizing the cells, via action potentials, with the mechanical sensation being transmitted to the nerve center (Liu et al., 2023).

Chemoreceptors of taste may be involved as well by virtue of their capacity for detecting a wide variety of stimuli, considering there´s a widely known association between bitter taste and the feeling of astringency, an astringent compound might activate bitter taste receptors which could explain as to why many astringents are perceived as being bitter (Ramsey et al., 2006; Schöbel et al., 2014).G-coupled proteins influence the induction of tactile sensation too, playing a part in the activation of the signal transduction. After the saliva film is damaged, G protein-coupling pathway is activated, astringency signals are produced leading to an influx of calcium, triggering cell depolarization to complete the action potential and then spreading the astringency signals to the central nervous system (Liu et al., 2023; Schöbel et al., 2014).

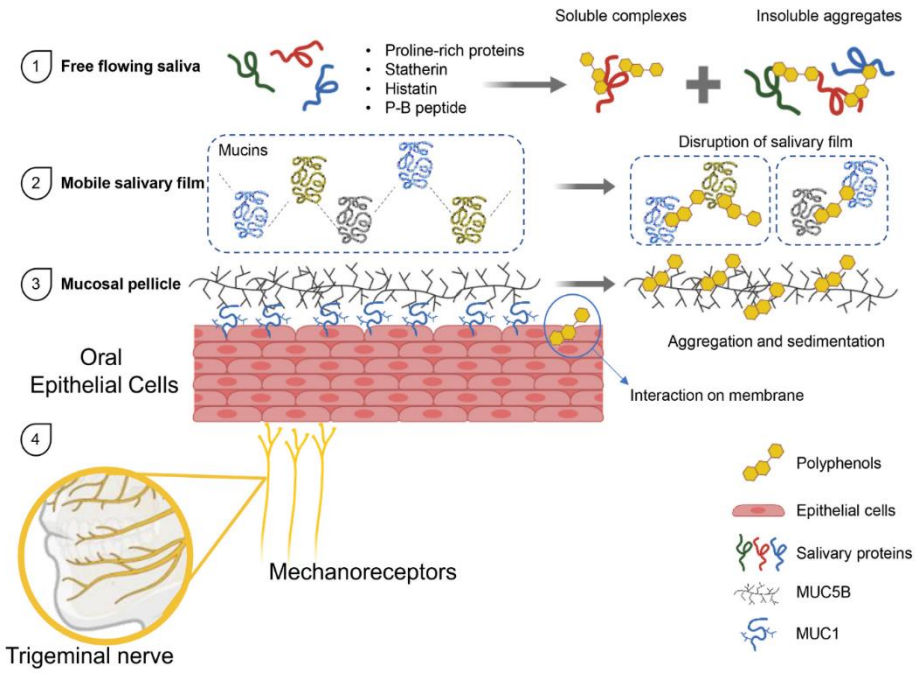

Figure 21: Proposed mechanisms linked to the triggering of astringency. 1- interactions between polyphenols and SPs lead to the formation of soluble complexes, and thereafter, insoluble aggregates. 2- the Interactions between polyphenols and SPs provoke the disarranging of the salivary film. 3- Polyphenols binding directly to oral epithelial cells and interactions between polyphenols and proteins in the mucosal pellicle sparks modifications in the pellicle due to the aggregation and sedimentation observed. 4- The mechanoreceptors present in the trigeminal nerve are stimulated and therefore activated by the astringent polyphenols. All of the mechanisms listed are simultaneously involved in eliciting an astringent sensation. Source: (Guerreiro et al.)

1.4 Saliva

Saliva is a clear liquid which is mostly secreted by the sublingual (SLG), parotid (PG) and submandibular (SMG) glands, being for the most part comprised of water (99%), while also containing a protein filled organic fraction, with the number of proteins identified in it having surpassed 3000. Saliva also possesses electrolytes such as calcium, sodium and potassium that will assist in the start of the process of digestion (Guerreiro et al., 2020; Louro et al., 2023).

The normal pH range for saliva in the mouth is maintained neutral or close to neutrality with the range staying between the values of 6.2 and 7.6, with the pH of resting saliva never decreasing below 6.3 (Baliga et al., 2013).

Saliva is fundamental for the protection of both hard and soft tissues of the oral cavity and upper gastrointestinal tract, it speeds the repair of soft tissues by stimulating wound contraction and decreasing clotting time (Kondo et al., 2015; Mandel, 1987).

During the majority of the time, saliva is present in the oral cavity as a very thin film, named saliva film, constituted by two different layers, one of them is quite dynamic and easy to remove and the other is a pellicle layer covalently linked to the epithelial cells, saliva film has a variable composition dependent on the diffusion of materials into or out of the dental plaque. Saliva´s average volume is 1 mL and the surface area of the oral cavity is coated with a saliva film with an average thickness of 0.1 mm, when the mouth is dry the thickness of this film is lower than 10 μm (Edgar et al., 2004; Pires et al., 2020). The salivary film can be considerably modified by the consumption of food and drinks, with its response to this stimuli, heavily affecting its viscoelasticity, therefore altering oral lubrication (Gibbins & Carpenter, 2013).

The production of saliva by the main three salivary glands is a mechanism reliant on the sympathetic and parasympathetic autonomic nervous systems, with 60-65% of it being generated by the SMG gland, 20-25% by the PG gland and 7/8% from the SLG with the rest coming from several other minor glands, these glands are capable of yielding between 0.5 to 1.5 L of saliva per day (Ghannam & Singh, 2019; Mese & Matsuo, 2007). Salivary glands are made up of duct cells and acinar cells, which can be either serous or mucus, these acinar cells are the ones responsible for the secretion of saliva, initially they produce a isotonic fluid that will become hypotonic saliva through action of the duct cells by reabsorption of the majority of NaCl and secretion of $KHCO_3$,with this process being reliant on the intracellular concentration of ion calcium (Kondo et al., 2015; Sneyd et al., 2021).

Saliva contains a number of proteins and peptides with functions that aren't fully understood yet, ergo the identification of which SPs are involved in originating astringency is of keen scientific importance (Bennick, 2002; Di Pietro et al., 2023). The composition of SPs namely peptides families like statherin, the three groups of proline rich proteins (PRPs) and cystatins determines the involvement of saliva in lubrification, antimicrobial activity, protection of the tissues of the oral cavity and maintenance of the homeostasis of the mouth by preserving the integrity of teeth (de Sousa-Pereira et al., 2013).

1.4.1 Proline-rich proteins

Proline-rich proteins (PRPs) constitute a polymorphic group of SPs with a high prevalence of several amino acids namely proline which constitutes 25 to 40% of the amino acids present, and also glycine, glutamine and glutamate, they are coded by a multi-family of six genes: PRH1, PRH2, PRB1, PRB2, PRB3, and PRB4.) (de Sousa-Pereira et al., 2013; Hajishengallis & Russell, 2015; Wang et al., 2019). The high content in the four amino acids mentioned, in particular proline, is a major feature linked to its singular affinity for tannins and allows proteins´ formation of secondary structures that present the conformation of random coils. permitting proline rich proteins to universally adhere to several subclasses of polyphenols (Hagerman & Butler, 1981; Murray & Williamson, 1994).

Hence, PRPs have a greater ability to connect to phenolic compounds albeit not particularly to the proline residues, by virtue of their conformally open and flexible structures opposed to proteins with tightly coiled conformations which struggle more to connect to the phenolic compounds (Pérez-Gregorio et al., 2020). PRPs represent over 60% of the weight of the total salivary peptidome, PRPs can be divided in three subclasses depending on the molecular charge and the carbohydrates present : acidic PRPs (aPRPs), basic PRPs (bPRPs), and glycosylated PRPs (gPRPs) (Guerreiro et al., 2022; Ramos-Pineda et al., 2019).

1.4.1.1 Acidic proline-rich proteins

Acidic PRPs are encoded by PRH1 and PRH2 in the chromosome 12p13.2, with a C-terminal abundant in proline, glutamine, glutamic acid, and glycine and a N-terminal

comprised of 30 amino acids with a high content in acidic residues like aspartate, glutamate, and also a few serine phosphate residues, with a low isoelectric point, around 4 to 5. (Levine, 2011; McArthur et al., 1995; Messana et al., 2023). There´s secretion of large aPRPs and truncated aPRPs, which showcase distinct biological functions, aPRPs are subject to bacterial proteolysis into becoming low molecular weight peptides (Lindh et al., 2002).

They possess biological importance concerning saliva´s calcium homeostasis because they strongly bind ionic calcium. Suggesting a role in the preservation in saliva of the supersaturation of ionic calcium, regarding the quantity of phosphate ions while also binding to hydroxyapatite thus participating the process of forming teeth pellicles (Hajishengallis & Russell, 2015; Messana et al., 2023; Schenkels et al., 1995). This pellicle formation is a selective process because not all SPs will be found in the pellicle, only certain proteins can be observed in it, specifically phosphoproteins with strong affinity with hydroxyapatite (Hannig & Hannig, 2009).When aPRPs are degraded into peptides, they act as protectors in the oral cavity, showcasing antimicrobial proprieties (Hajishengallis & Russell, 2015).

1.4.1.2 Basic proline-rich proteins

This subclass of proteins is characterized for having a high content in basic amino acids such as glycine, glutamine and proline and by being composed by a sequence of 19 residues that have 5 to 15 repetitions with variations to give bPRPs a length of around 150 amino acids, it´s encoded by the PRB1, PRB2, PRB3 and PRB4 in the chromosome 12p13.2 (Charlton et al., 2002; Messana et al., 2023; Soares et al., 2020). The high quantity of bPRPs in saliva can justified by the fact that each one of the four genes is likely to encode a precursor protein that will afterwards be cleaved, thus bPRPs can be large, medium or small (Amado et al., 2010). Considering that most of the polar amino acids are basics, the isoelectric point of BPRPs is high, above 9, with many of its residues being uncharged at a neutral pH (McArthur et al., 1995).Given that they have a large binding structure and various contact points due to their high content in proline and of glycine, it facilitates their ability to bind and precipitate phenolic compounds (Boze et al., 2010). It has been reported as well for having antiviral properties by bonding to proteins of viruses like HIV-1(MR, 2001).

1.4.1.3 Glycosylated proline-rich proteins

Glycosylated PRPs are derived from basic PRPs , they have carbohydrates linked to the amino acids of the protein backbone and operate as masticatory lubricants (Schenkels et al., 1995; Soares et al., 2020). Beyond that, these proteins also bind oral bacteria and form complexes with tannins, their functions in the mouth are shaped by the conformational geometries that they showcase due to the oligosaccharide moieties that they can present, with different moieties providing specificity for specific bacteria (Boze et al., 2010; McArthur et al., 1995).

1.4.2 Statherin/ P-B peptide

Statherin is an acidic protein with a low molecular weight encoded by the *STATH* gene that is produced by the parotic, submandibular and von Ebner's salivary glands, with a concentration around 10–40 μM in saliva. It comprises a multifunctional phosphor-peptide with a total of 43 amino acids residues with a high quantity of tyrosine, proline and glutamine, which alongside aPRPs acts on the maintenance of the calcium homeostasis of saliva, that operates by inhibiting calcium phosphates and calcium salts from precipitating spontaneously and by modulating the mineralization of enamel (Di Pietro et al., 2023; Messana et al., 2023; Schenkels et al., 1995). P-B peptide is encoded by *PROL3* gene located close to the STATH gene, its role in saliva still requiring further study (Isemura, 2000; Messana et al., 2023). Stat´s structure is composed by a small N-terminal negative tail followed by a long neutral domain rich in proline ,with P-B showcasing a sequence that shares a high similarity with the ionic complement of Stat, comprised of a small N-terminal positive tail followed by a long neutral domain also rich in proline, they interact with each other to form acquired dental pellicles (Inzitari et al., 2006; Jensen et al., 1991).

Dental pellicles, also known as oral pellicles, are formed instantly on the surface of the enamel of teeth when they contact with saliva, comprised of bounded SPs and transmembrane mucin MUC1 expressed by oral epithelial cells. They are proteinaceous biofilms with a thickness ranging from 0.1 to 1 μm, which help with the health of teeth by safeguarding teeth from attrition and abrasion, operating as a lubricant and buffer (Hannig & Hannig, 2009; Rehage et al., 2017). The astringent stimuli modifies the morphology of these pellicles, with astringency being associated with pellicles losing their lubricant quality (Dickinson & Mann, 2006; Rehage et al., 2017). Several reports indicate

that statherin and P-B peptide have the capacity to react with tannins (Guerreiro et al., 2022).

1.4.3 Cystatins

Cystatins are a group comprised of ancestrally related proteins namely type-I cystatins (cystatins A and B), type-II cystatins (C, D, S, SN, SA), and type-III cystatins (Messana et al., 2023). Type-II cystatins are the cystatins present in human saliva, the majority of them are produced by the submandibular and sublingual glands, with a small fraction of them being produced by the parotid gland and they are codified by four linked genes: CST1, CST2, CST4 and CST5, (Baron et al., 1999; Russell et al., 2005).

They´re a group of protease inhibitors ,both intracellular and extracellular, that act on the inhibitory cysteine cathepsins, which are cysteine-cleaving proteases that have functions in the degradation of proteins and post-translational cleavage, while also participating in the innate immune response (Dickinson, 2002; Zhang & Zhan, 2023). Their capacity for inhibiting cysteine proteinases is very important for their role in combating illnesses like cancer because due to the modulation of the proteolytic system (Messana et al., 2023).

2. Materials and methods

Materials

All reagents used were of analytical grade. Acetonitrile (99.8%) was purchased from Chem-Lab whilst bovine serum albumin (BSA) (≥96%) and trifluoroacetic acid (TFA) (99.0%), were both purchased from Sigma-Aldrich. The Milli-Q EQ 7000 ultrapure water purification system was from Merck Millipore with a Millipak 0.22 µm hydrophilic membrane filter. The flower extracts of *Viola tricolor, Centaurea cyanus* and *Cosmos bipinnatus* used herein were obtained from other ongoing projects.

2.1. Collection and treatment of saliva

Unstimulated saliva collection followed a standard protocol. The 15 donors that participated in the collection of saliva had ages comprehended between 20 and 45 years old, they were all healthy, non-smokers and were not taking any medications at the time of the study. The procedure was perfomed after making sure the donors did not eat or drink for at least 1 hour before collection. They were instructed to avoid the consumption of foods rich in polyphenols such as wine, coffee or chocolate in the morning prior to collection. Donors accumulated saliva in the interior of their mouth for 10 minutes before the collection of saliva into falcon tubes. A pool of all of samples was created because we intend on obtaining a general profile of the saliva and not the individual profile of any of the participants. Saliva was pretreated with 10% trifluoroacetic acid (TFA), the ratio was to 900 µl of saliva it was added 25 µl of TFA. This treatment with TFA is necessary in order to reduce protein degradation by oral proteases after collection because TFA can partially inhibit the activity of intrinsic proteases, therefore allowing a better preservation of the protein composition. TFA is also vital for the precipitation of high molecular weight proteins such as mucin and amylases, which can be responsible for the degeneration of HPLC columns. The mixture of saliva + TFA was vortexed and centrifugated for 5 min at 10500 rpm. Only the acidic soluble fraction was considered for the subsequent HPLC investigation, while the pellet was discarded. The saliva samples were then stored under −80°C until further analysis.

2.2. Bicinchoninic acid (BCA) assay

Thermo Scientific Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit is a highly sensitive colorimetric method to detect and quantify total protein concentration, it´s a relative method that is designed to measure the concentration of a protein of interest compared to protein standard Bovine Serum Albumin (BSA). This procedure was performed using the standard protocol of Thermo Scientific. An aliquot of 25 µL of sample was mixed with 200 µL of WR (working reagent) in the ratio 1:8. The WR is prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, Reagent A: B). The calibration curve was made using triplicates of BSA stock (2 mg/mL in water) in the range concentrations of: 0, 0.125, 0.25, 0.5, 0.75, 1, 1.5, 2.0 mg/mL. The saliva sample, whose protein content we intend to determine, was prepared in triplicate. After the incubation time, 30 minutes at 37°C, the samples were transferred to a 96-well plate, it was read at the absorption wavelength of 562 nm in a UV-Vis microplate reader (Biotek 4 PowerWave XS, New England, VT, USA).

2.3. Analysis of the salivary proteins upon interaction with polyphenols through HPLC-DAD analysis

Saliva was treated with NaOH 1M to increase the pH to approximately 7 which is the physiological state of pH (pH 7.4). For this study, the salivary protein content was determined to be 1687 µg/mL.

Three stocks of each edible flower infused vinegars extracts were prepared in triplicate in the concentrations of 1.2, 1.8 and 2.4 mg/mL in acid water, which was acidified by acetic acid (AcOH) to have a pH near to 3, reflecting the pH of vinegar. First, DMSO at 5% was added to each sample, then vortexed and it was submitted to ultrasounds for 10 minutes. After that, acid water at 95% was added and the mixture was vortexed, and again submitted to ultrasounds for 5 minutes and then centrifugated for 5 minutes at 13.500 rpm. For the SPs-polyphenol interactions, 180 µL of saliva were mixed with 60 µL of vinegar extracts and 60 µL of acid water (control condition). The final concentrations of the extracts in the mixture with saliva were 0.30 mg/mL, 0,45 mg/mL and 0.60 mg/mL. The interaction between saliva and vinegar was vortexed and then carried out for 10 minutes at 37°C, body temperature, in the incubator. The Eppendorf tubes were subjected to centrifugation at 13.500 rpm for 10 minutes. Afterwards, we

proceeded to the separation of supernatant and pellet, only the supernatant was analyzed through HPLC.

We also determined the protein profile of the saliva in the presence of freeze-dried edible flower extracts, they were prepared in the same manner as the vinegar extracts, in the same concentrations, aside from them being dissolving in PBS instead of acid water (5% of DMSO and 95% of PBS) with the pH adjusted to 7. The solvent chosen was PBS to attempt to simulate the ingestion of flowers, as PBS is closer to human biological fluids because it mimics its neutral pH, ion concentration and osmolarity. The control consisted of 60 µL of PBS mixed with 180 µL of saliva.

For the purpose of identifying SPs that precipitate through interaction with astringent phenolic compounds, the samples are injected on a JASCO LC-8 BS-4000-1 HPLC system (Tokyo, Japan) equipped with HPLC-SÄULE Vydac C8 column (150 × 2.1mm, 5µm particle diameter) purchased from Avantor®. The compound detection was carried out at 214 nm through a diode array detection (DAD), the injection volume was 120 µL and the method time was 91 minutes. All solvents used were previously filtered. The solvents utilized for this analysis were solvent A and solvent B. Solvent A consisted of 0,2% TFA in water Milli-Q and solvent B consisted of 0.2% TFA in acetonitrile + water Milli-Q (80:20). At the outset, the column was stabilized at the initial conditions, 90% of A and 10% of B, for 10 minutes. The gradient consisted of 90%-0% A and 10%-100% for 80 minutes, followed by 90% of solvent A and 10% of solvent B until the 91 minutes mark at flow rate of 0.5 mL. The eluent program consisted of : 90% of A and 10% of B from 0–10 min ; 83% of A and17% of B from 10-30 min; 73% of A and 27% of B from 30-33 min; 69% of A and 31% of B from 33-50 min; 50% of A and 50% of B from 50-69 min; 30% of A and 70% of B from 69-71 min; followed by a washing step of 100 % of solvent B from 71-80 min; and lastly there´s a stabilization of the column and a return to the initial conditions 90% of A and 10% of B from 81-91 min.The use of the software ChromNAV allowed the observation of the chromatograms obtained for each edible flower infused vinegar extract and each edible flower extract in order to permit the measurement of the areas of the peaks of the SPs of interest. The identification of the families of SPs in the chromatograms stems from research from 2011 conducted by Soares and colleagues (Soares et al., 2011), with the protein profile of saliva being comprised by bPRPs, gPRPs and aPRPs, staterin/peptide P-B and cystatins.

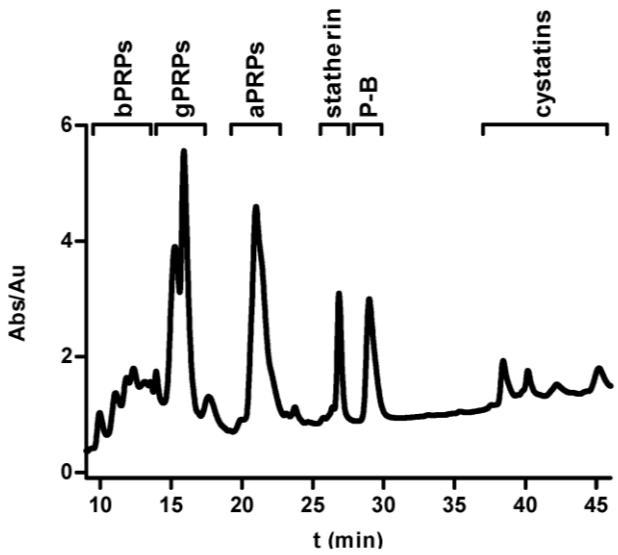

Figure 22: Chromatogram of the protein profile of saliva detected at 214 nm, with identification of the principal families of SPs. The chromatogram is divided into six zones, with each zone corresponding to a protein family. Adapted from (Soares et al., 2011).

3. Results and discussion

3.1. Bicinchoninic acid (BCA) assay

The BCA method combines the biuret reaction of reducing Cu^{2+} to Cu^{+} in a basic environment with the detection of Cu^{+} by weak acid bicinchoninic acid (BCA), which is selectively colorimetric, provoking the emergence of a purple hue by chelation of BCA and Cu^{+}, with an increase in the amount of protein present leading to more BCA-Cu^{+} complex which in turns corresponds to a growing intensity of the purple shade seen. A calibration curve of BSA was made (0-2 mg/mL, y= 0.7875x + 0.0992 R^2= 0.9932)

Therefore, the concentrations of the sample obtained fall between the range of the calibration curve, between the 1mg/mL and 1.7 mg/mL concentrations of the reference standards BSA.

3.2. Quantification of the precipitation of salivary proteins upon interaction with polyphenols

The determination of the proteins that suffer precipitation due to interaction with polyphenols is indirect and it was performed by comparing the initial areas of the peaks of specific families of SPs in the supernatant in the absence of polyphenols and the areas of the peaks of those same families after interaction. By measurement of the areas of the peaks, we proceeded to obtain the percentage of SPs of interest that remained soluble after the interaction, in order to calculate the percentage of precipitated SPs after interaction, represented by the graphs in Figure 23.

The final concentrations chosen for the extracts (0.30 mg/mL, 0.45 mg/mL and 0.60 mg/mL) were selected having in consideration the results of the F–C method, with the highest purity having been obtained at a concentration of 0.5 mg/mL. So, we evaluated the interaction of the polyphenols with saliva at a concentration close to 0.5 mg/mL and another concentration slightly above it (0.60 mg/mL) and another slightly below it (0.30 mg/mL).

Saliva control represents the maximum content of proteins in saliva (100%) and the proteins that remained soluble were in the supernatant while the proteins that precipitated remained in the pellet. There was an overlap between the chromatographic peaks of one of the families of SPs (bPRPs) and the peaks of polyphenols in the vinegar extracts and in the flower extracts, since at this retention time there´s a co-elution of both bPRPs and the polyphenols, therefore a correction was made. The area corresponding to these chromatographic peaks in the condition of interaction with saliva was subtracted here. This correction aimed to remove the effect of the polyphenols in those peaks. It was also necessary the removal of several outliers, despite this removal a high variability can still be in the graphs of Figure 23 below. Experimental error may be responsible for the high variability of the data. It´s imperative to consider that despite the precautions taken when handling saliva (working on ice), the low stability of SPs at room temperature might be an important factor to take in consideration when explaining the results, because it accelerates protein degradation, influencing the variability measured.

High variability may also be due to the effect of the complex food matrix on the phenolic compounds. Considering that the variability of the results of Control vinegar extracts observed in Figure 23 are in general lower than the ones of edible flower infused vinegar extracts, thus implying that this infusion of edible flowers in the vinegar, by constituting a more complex and more unstable food product than the vinegar by itself contributes to a higher variability.

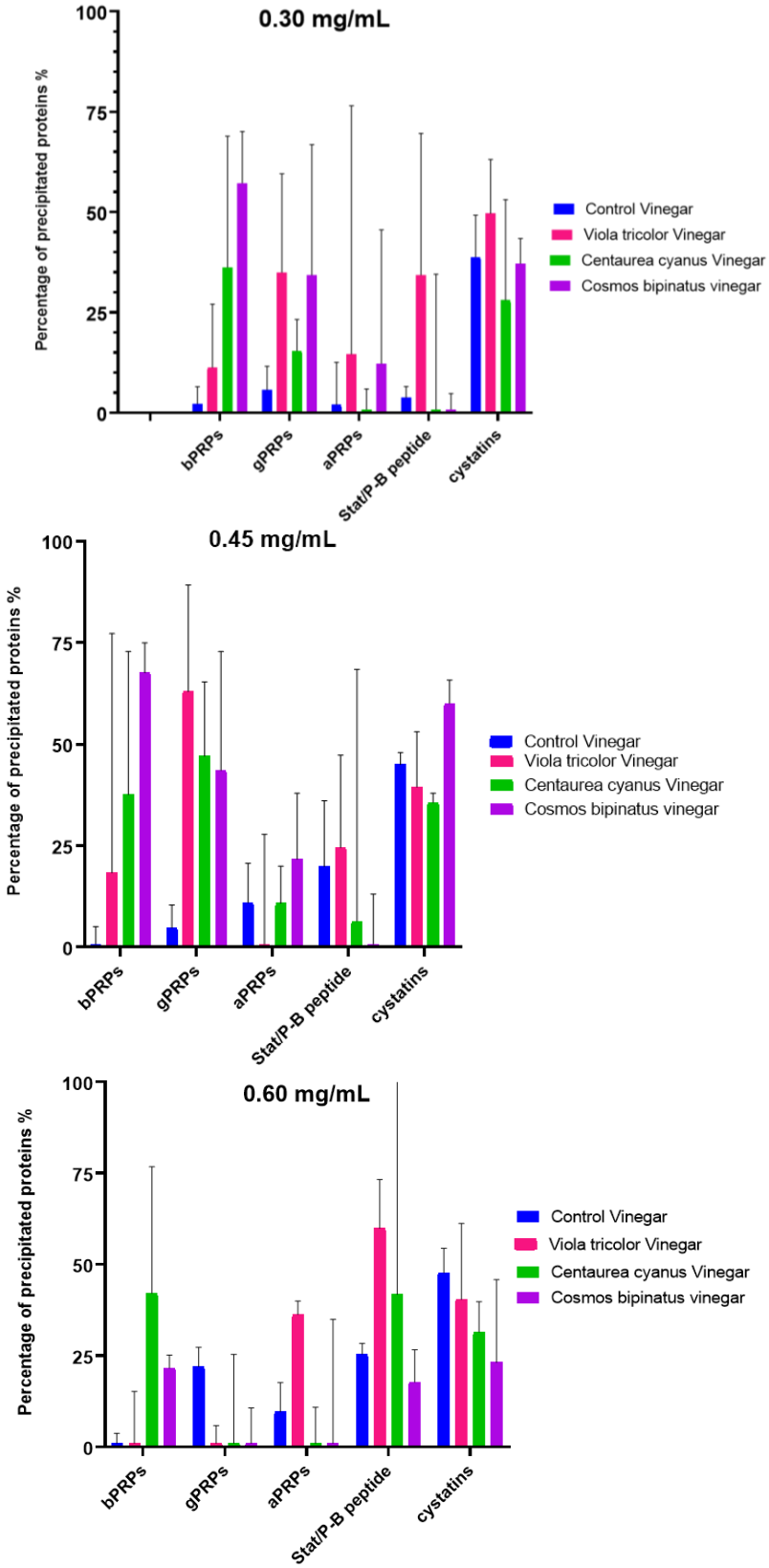

Figure 23: Influence of the interaction with different vinegars on the percentage of precipitated proteins in saliva (%) at the concentrations 0.30; 0.45; 0.60 mg/mL. *Viola tricolor* is represented in pink, *Centaurea cyanus* represented in green and *Cosmos bipinatus* in purple and when it comes into contact with Control Vinegar which does not possess edible flowers. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard deviation (SD).

Analyzing the graphical representations in Figure 23 there´s an overall tendency for the SPs families studied to be affected by the presence of polyphenols, although they´re not all equally impacted.

Control saliva represents the maximum of proteins, because protein precipitation is not expected, unlike saliva in the presence of extracts where the interaction between polyphenols and proteins leads to precipitation. Thus, the interaction with vinegar extracts leads to the decrease in the areas of their chromatographic peaks.

Control vinegar doesn't have any added polyphenols stemming from the presence of edible flowers, only containing the polyphenols naturally found in vinegar therefore allowing to better grasp by comparison the effect of the addition of edible flowers in the interactions SPs-polyphenols. At a 0.30 mg/mL concentration, the interaction of Control vinegar extract with saliva exhibits little precipitation of polyphenols (below 6%) with the exception of cystatins, in which the percentage of precipitated proteins is considerable, 38,8%, cystatins showcase a pretty substantial precipitation after interacting with Control vinegar extract and after interaction with edible flower infused vinegar extracts, it presents values of precipitation exceeding 25% in the presence of all of them. The highest precipitation of cystatins obtained at a concentration of 0.30 mg/mL is in interaction with Viola vinegar extract. bPRPs are mostly precipitated in the presence of Centaurea and Cosmos vinegar extracts, with Viola vinegar extract having a lesser effect in the interaction with these proteins. These findings point towards different species of edible flowers infused vinegars having distinct capacities to cause the precipitation of SP families. Precipitation of aPRPs is mostly observed in the presence of Viola and Cosmos vinegar extracts, 14.5% and 12.2%, respectively but it´s relatively low. Stat/P-B peptide is at this concentration only precipitated by the Viola vinegar extract, suggesting a higher affinity with the polyphenols in this flower than in others.

Precipitation of bPRPs continues to be the highest in the presence of Centaurea vinegar extract and Cosmos vinegar extract but it´s not possible to establish a linear correlation between the concentration of the extracts and an increase in precipitation because the precipitation of bPRPs tends to be greater in concentrations of 0.30 mg/mL and 0.45 mg/mL than in 0.60 mg/mL. Showing the highest precipitation percentage due to Cosmos vinegar extract when extracts are in a concentration of 0.30 and of 0.45 mg/mL and due to Centaurea vinegar at a concentration of 0.60 mg/mL. Suggesting that the precipitation caused by the extracts of vinegars infused with these edible flowers is conditioned by their concentration because the concentration of the extracts does impact the results but with no general tendency that can be clearly pinpointed.

Despite all being proline rich proteins it´s easily observable that the precipitation rate of the three families (bPRPs, aPRPs, gPRPs) is quite different, implying that the differences in structure between those families influences precipitation. As it was analyzed previously the phenolic profiles of all these vinegars differ considerably from each other, suggesting that a different affinity between the SPs families and determined polyphenols as a possible explanation for the differences observed. Control vinegar extract doesn't have any added polyphenols like the edible flower infused vinegar extracts, thus having a lower phenolic content than the others which might explain why it causes considerably less precipitation than the other vinegars extracts, with the exception of its interaction with cystatins, which can be explained by the polyphenols that it does contain demonstrating a high affinity with that protein family.

By observation of all the graphs in Figure 23, it can be suggested that cystatins have a higher affinity to bind to polyphenols than the other protein families, pointing towards being them being involved in causing astringency due to their high percentage of precipitation whilst aPRPs have an overall lower precipitation rate, which does seem to increase in the highest concentration.

Of the vinegar extracts tested, Cosmos vinegar extracts seem like the most involved in precipitation particularly of bPRPs causing the biggest precipitation rates at 0.30 mg/mL, of 57.2%, and at 0.45 mg/mL, of 67.6%, pointing towards a stronger binding ability with bPRPs. While also causing, at 0.45 mg/mL, a precipitation of 60% of cystatins. Viola vinegar extracts can also cause considerable precipitation, depending on both the SP family and the concentration. Viola vinegar extract at a concentration of 0.45 mg/mL leads to a precipitation of gPRPs, of 62.9%, and at a concentration of 0.60 mg/mL it leads to a precipitation, of 59.9%, of Stat/P-B peptide.

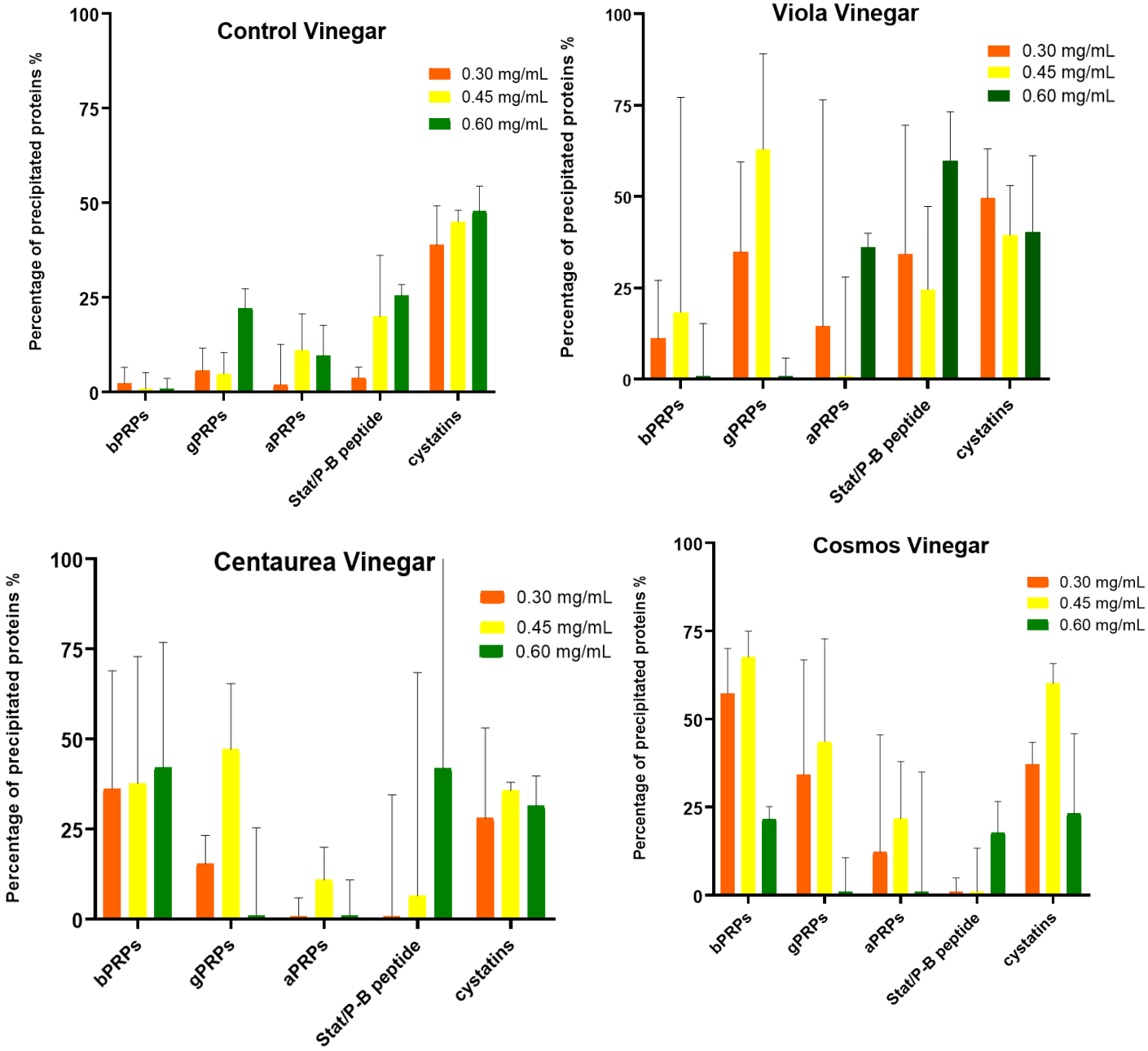

Figure 24: Influence of different concentrations (0.30; 0.45: 0.60 mg/mL) on the percentage of precipitated proteins in saliva (%). 0.30 mg/mL is represented in orange, 0.45 mg/mL is represented in yellow and 0.60 mg/mL represented in green. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard

To complement the information retrieved from the graphs illustrating the precipitation at a concentration of: 0.30 mg/mL; 0.45 mg/mL; 0.60 mg/mL after interacting with different vinegar extracts, graphs were also made representing the percentage of protein precipitation after interaction with a specific vinegar extract (Control Vinegar, Viola Vinegar, Centaurea Vinegar, Cosmos Vinegar) in the three concentrations.

Observing the results obtained from the interaction saliva + Control vinegar extract, there´s a clear increase in the precipitation of several of the protein families like gPRPs, Stat/P-B peptide and cystatins as the concentration of the extract increases, with an increase in precipitation of aPRPs at the concentrations of 0.45 mg/mL and of 0.60 mg/mL in comparison to 0.30 mg/mL. The most pronounced increases are in gPRPs that rises from a precipitation of 5.6% at a concentration of 0.30 mg/mL to 22% at a concentration of 0.60 mg/mL and Stat/P-B that increases from 3.7% to 25.4%.

Unexpectedly the trend observed in the graph above concerning Viola vinegar extracts is that the precipitation of the protein families bPRPs and gPRPs, when in contact with Viola vinegar extracts only occurs at the concentrations of 0.30 mg/mL and 0.45 mg/mL and not in the presence of the extracts at the highest concentration of 0.60 mg/mL. In the other three families, precipitation does occur at this higher concentration, with the highest precipitation being observed at this concentration in aPRPs (36.3%) and in Stat/P-B peptide (59.9%). This data speaks to the unpredictable effect of the concentration on the results. The highest precipitation observed was at the intermediate concentration, with the family most likely to have a higher binding affinity towards the polyphenols present in Viola vinegar: gPRPs.

Unlike Control vinegar extracts and Viola vinegar extracts, Centaurea vinegar extracts impacts the precipitation of bPRPs considerably pointing towards the phenolic compounds present in Centaurea vinegar having a higher affinity with this protein group. Cosmos vinegar extracts showcase an even higher precipitation rate of bPRPs, with the highest precipitation occurring when Cosmos vinegar extracts is at the intermediate concentration. The precipitation of gPRPs after coming into contact with the polyphenols from the Centaurea vinegar extracts is the highest at the intermediate concentration and the precipitation of aPRPs only occurs at all in this concentration as well.

At the intermediate concentration of Cosmos vinegar extract, 0.45 mg/mL, it´s observable the highest precipitation of four of the five SPs: bPRPs, aPRPs, gPRPs and cystatins. These findings fall in line with what we were expecting based on the results obtained previously using the method F-C method in which the highest TPC in the

extracts was obtained at the intermediate concentration (0.45 mg/mL) and not at the highest one (0,6 mg/mL). A possible explanation for this phenomenon of lower precipitation at a higher extract concentration can be linked to the formation of soluble complexes between the proteins and polyphenols which do not precipitate (Rinaldi & Moio, 2021). Establishing this correlation between the interaction of the other two edible flower infused vinegar extracts with saliva and the concentration of the extracts is not as clearly observable, even though the highest levels of precipitation of gPRPs are observed in Viola vinegar extracts and Centaurea vinegar extracts at this intermediate concentration. This greater precipitation of SPs after interacting with the polyphenols from the Cosmos vinegar extract at a concentration of 0.45 mg/mL presents one discrepancy: Stat/P-B peptide, which by observation of all four graphs of Figure 24 are unanimously more precipitated at a concentration of 0.60 mg/mL, suggesting that this family requires a greater concentration of polyphenols to be present in order to precipitate.

The precipitation of SPs in the presence of the flower extracts by themselves was studied as well.

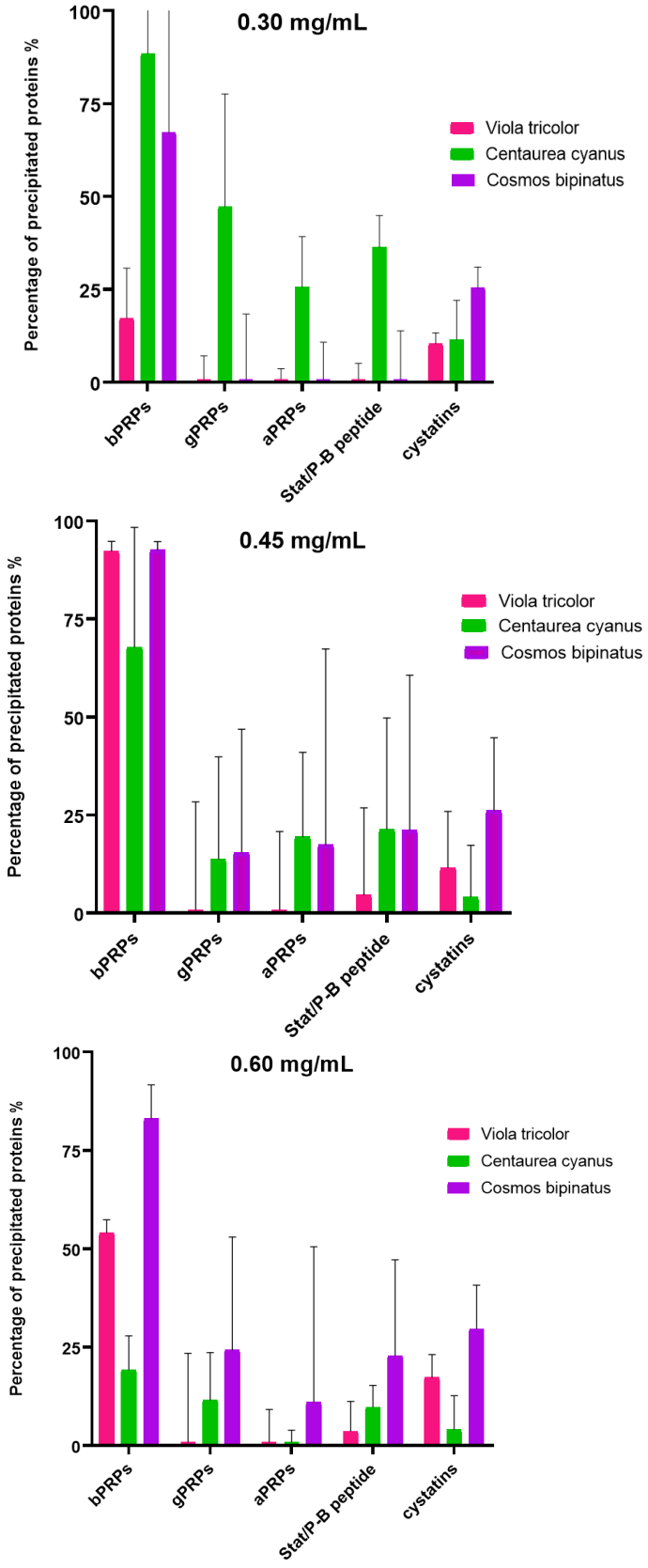

Figure 25: Influence of the interaction with edible flowers extracts on the percentage of precipitated proteins in saliva (%) at the concentrations 0.30; 0.45; 0.60 mg/mL. *Viola tricolor* is represented in pink, *Centaurea cyanus* in green and *Cosmos bipinatus* in purple. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard deviation (SD).

The initial assessment drawn from the observation of the graphs in Figure 25 in comparison with the ones in the Figure 23, is that the precipitation rates of the SPs families when interacting with polyphenol rich edible flower infused vinegar extracts and with the extracts of the flowers by themselves are distinct, suggesting that the phenolic profiles themselves are different. It´s crucial to acknowledge that the polyphenols are naturally unstable and reactive compounds. Therefore, the polyphenols derived from the edible flowers are likely to suffer modifications when inserted in a food matrix such as vinegar like oxidization, glycosylation and reactions of cleavage and addition that might lead to the formation of more complex polyphenols (El Gharras, 2009; Liang et al., 2022). Interactions of polyphenols with components of a store-bought food matrix such as carbohydrates and conservatives can also be linked to the alterations suffered (Ho et al., 2017). Considering that the maximum percentages of protein precipitation obtained in the interaction with vinegar extracts was always below 70%, in the interaction with edible flowers extracts much higher percentages of precipitation are obtained, with the highest ones exceeding 90%. These findings point towards not the totality of polyphenols contained by flowers being passed to the vinegar during the infusion or the partial degradation of some polyphenols taking place in this food matrix. Another key feature important to highlight is that the polyphenols modified by interaction with the food components of vinegar can have a different and potentially higher affinity with the SP families in study.

At a concentration of 0.30 mg/mL, the species of edible flower causing the greatest precipitation of bPRPs is *Centaurea cyanus* (88.5%) and this species is exclusively responsible for causing precipitation of gPRPs and Stat/P-B peptide at this concentration, with the other two species not inducing any precipitation in these families at this concentration, and with *Viola tricolor* not causing any precipitation at all of gPRPs and minimal precipitation of Stat/P-B peptide at a concentration of 0.45 mg/mL and of 0.60 mg/mL.

The two species of edible flowers with significant impact in the precipitation of bPRPs at the concentration of 0.30 mg/mL are *Centaurea cyanus* and *Cosmos bipinnatus*, however as the concentration increases *Viola tricolor* begins to play a bigger role in the precipitation of this protein family. Possessing an extremely high affinity with bPRPs and consequent precipitation at the concentration of 0.45 mg/mL, in which the majority of proteins of this family precipitate in the presence of *Viola tricolor*, 92.2 %, the majority of proteins of this family also precipitate upon interaction with *Cosmos bipinnatus*, 92.6%. These results show that bPRPs is the SP family exhibiting the greatest precipitation and it seem to indicate that in vinegar the polyphenols from the

flower *Viola tricolor* with the highest affinity with bPRPs suffer alterations which decrease their affinity or are degraded.

Cystatins are considerably more precipitated in the presence of the vinegar extracts than in the presence of the edible flower extracts suggesting that the polyphenols contained in the vinegar itself may play a larger role in the precipitation of this family or that the alterations suffered by polyphenols in vinegar provide them a higher capacity for precipitating cystatins. Out of the three species, cystatins are the most precipitated in interaction with *Cosmos bipinnatus.*

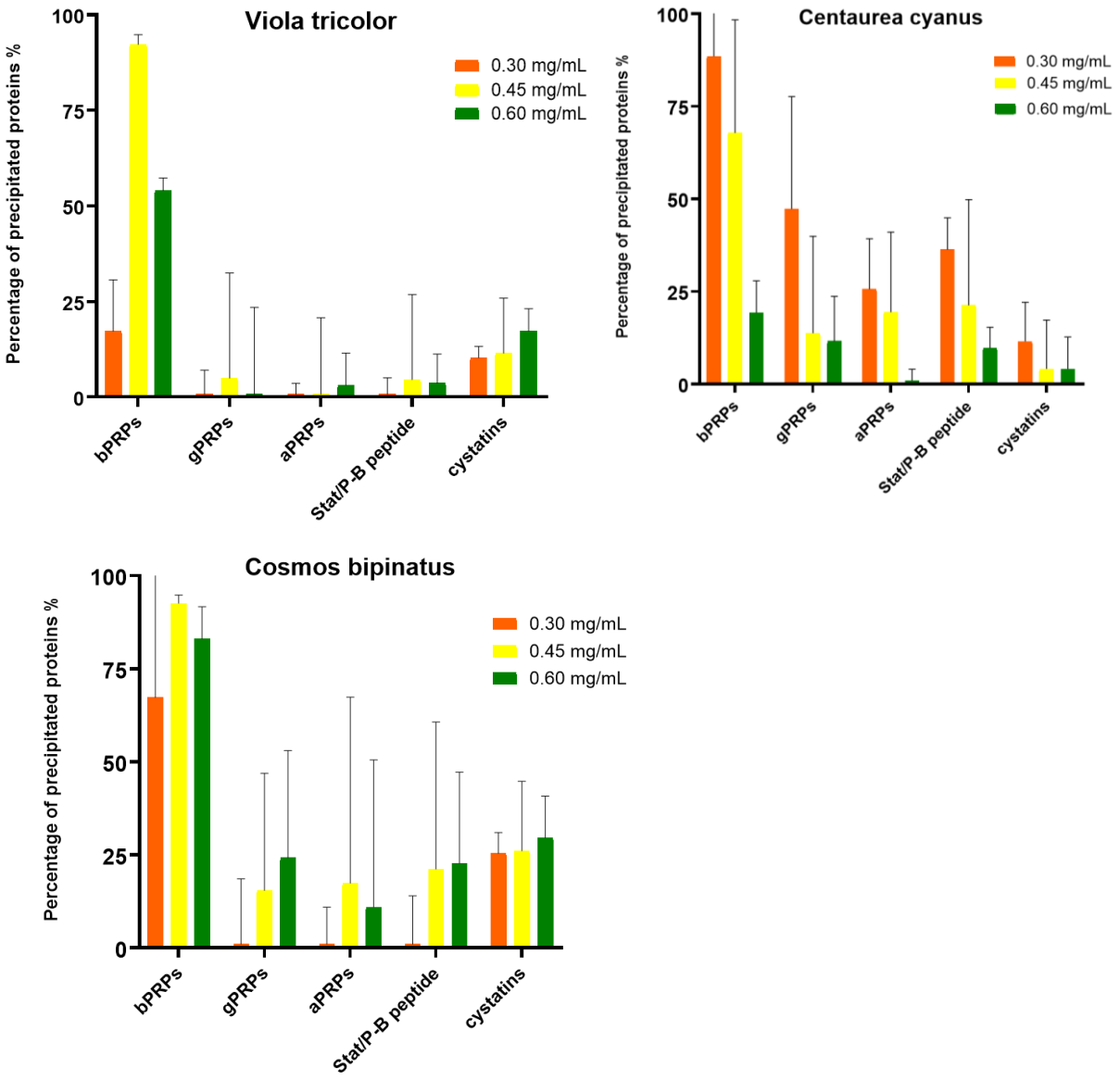

Figure 26: Influence of different concentrations (0.30 mg/mL; 0.45 mg/mL: 0.60 mg/mL) on the percentage of precipitated proteins in saliva (%). 0.30 mg/mL is represented in orange, 0.45 mg/mL is represented in yellow and 0.60 mg/mL represented in green. The results were determined based on the control, saliva control, which corresponded to 0% of precipitated proteins. The results presented were expressed as mean ± standard deviation (SD).

Viola tricolor mostly precipitates bPRPs and has a negligible effect on the precipitation of both gPRPs and Stat/P-B peptide. No completely clear link can be found between the concentration of the extracts of *Viola tricolor* and the precipitation of SPs, although it seems as extracts of these flowers in the lowest concentration have a considerably lower effect on the precipitation of SPs.

Conversely, there appear to be an evident connection between the concentration of *Centaurea cyanus* and SPs' precipitation, with the highest rates of precipitation being unambiguously seen at the lowest concentration, 0.30 mg/mL, and the highest concentration being associated with the lowest precipitation. *Centaurea cyanus* at a concentration of 0.30 mg/mL is associated with the highest precipitation of gPRPs and of Stat/P-B peptide, 47.3% and 36.3% respectively, pointing towards a higher affinity between this flower species and both gPRPs and Stat/P-B peptide and a lower affinity with cystatins, in which the lowest percentage of precipitated proteins are observed.

The interaction with *Cosmos bipinnatus* showed high precipitation rates of bPRPs in all three concentrations (67.3%; 92.6%; 83%), showing no precipitation of gPRPs, aPRPs and of Stat/P-B peptide in the lowest concentration, which suggest that a higher concentration of polyphenols of this species is necessary to cause precipitation of these three families. At the concentration of 0.45 mg/mL and 0.60 mg/mL, the precipitation of these three families exceeds 10% but is below 25%. The highest precipitation of cystatins is observed after interaction with *Cosmos bipinnatus*, increasing slightly with the increase of the concentration*:* 25.4%; 26% and 29.6%.

4. Conclusions and future perspectives

A key purpose of this project´s first chapter was to assess the impact of edible flower infusion on the organoleptic properties and phenolic content of vinegar. The color difference perceived in vinegars after edible flower infusion was dependent on the anthocyanin content, it created a greater visual interest for these vinegars. The color stabilized after three weeks. The vinegar infused with *Cosmos bipinnatus,* which contained the greatest variety of anthocyanins, underwent the most intense color modification, displaying a burgundy hue. Centaurea vinegar also features an easily perceptible alteration in color. Viola Vinegar which had the lowest content of anthocyanins had an almost imperceptible color change. These findings are consistent with the existence of distinct phenolic pigments in the edible flowers studied which was shown by mass spectrometry. It was demonstrated by F-C colorimetric assays, edible flower infusion's role in boosting vinegar´s phenolic content, with *Cosmos bipinnatus* exhibiting the highest phenolic increment.

Another primary focus of this thesis´s first chapter is the characterization of the phenolic profile of all the edible flower infused vinegars by tentatively identifying the polyphenols present, determining the ubiquitous polyphenols present in all extracts like quercetin and apigenin, as well as assessing the heterogeneity of the phenolic compounds associated with a specific edible flower infused vinegar. The highest peaks corresponded to derivatives of apigenin. Most of the polyphenols tentatively identified had suffered processes that increase their stability like glycosylation or acylation. Anthocyanins were detected in Cosmos vinegar and Centaurea vinegar, as well as in Viola vinegar but to a lesser degree. it was only possible to detect anthocyanins derived from cyanidin, delphinidin, peonidin and malvidin. An anthocyanin derived from malvidin was only found in Centaurea vinegar and an anthocyanin derived from peonidin was only found in Cosmos vinegar, with cyanidin presenting the highest variety of derivatives. *Cosmos bipinnatus* is the species that contains a superior content of phenolic compounds, with the anthocyanin derived from peonidin identified in it, contributing to its more heightened color because peonidin provides purplish reddish hues, endowing it the highest potential from a commercial standpoint.

This development of a new food product of added value, thanks to the infusion of polyphenol-rich edible flowers opens doors to similar work being made using different food matrices other than vinegar. For this development of new products, food matrices that are already rich in polyphenols, that can have their TPC further enriched, like olive

oil are particularly noteworthy. This work can equally be performed in other varieties of vinegars in order to determine edible flowers' varying influence on the organoleptic properties of vinegars with distinct characteristics. Elucidating on which varieties of vinegar would benefit the most with the incorporation of edible flowers. To ensure that the new food products developed by infusion of edible flowers in known food matrixes would be accepted by the general public and successful in today´s food market it would be required the conduction of sensorial analyses with trained panelists to evaluate their organoleptic properties.

The antioxidant studies FRAP and DPPH were performed to elucidate on the antioxidant capacity of both edible flower infused vinegar extracts and edible flower extracts, a significant increase in antioxidant activity was observed thanks to the infusion of edible flowers. Throughout the antioxidant tests, *Viola tricolor* steadily presented the highest antioxidant activity and thus greater potential as an antioxidant agent.

The second chapter of this thesis primarily focused on astringency. The study conducted regarding the interactions established between SP families of interest and the polyphenols of edible flower infused vinegar extracts and edible flower extracts aimed to provide insight over the precipitation caused by this interaction, that can be implicated in the development of astringency. Our findings don't allow the drawing of definitive conclusions although some tendencies can be traced based on the graphical representations. SPs seem to suffer the least amount of precipitation upon interacting with Control Vinegar. The families of SPs studied suffered different percentages of precipitation depending whether they were interacting with edible flower infused vinegar extracts or flowers extracts. Cystatins were the most affected upon interacting with edible flower infused vinegar extracts while bPRPs were the most affected upon interacting with the flower extracts. *Cosmos bipinnatus* was responsible for the colossal precipitation of bPRPs. This data points to the phenolic profiles of the edible flower infused vinegar extracts differing from edible flower extracts, with the higher precipitations being observed upon interaction with edible flower extracts. Alluding to the flower extracts being more polyphenol-rich than the vinegar extracts. The concentration of the extracts affects the results however it isn't possible to retrieve a general conclusion regarding its influence.

The ongoing work is currently underway regarding the UPLC-DAD analysis of the precipitation of polyphenols upon interacting with SPs. The analysis of these supernatants is highly relevant because it allows the indirect identification of the polyphenols that precipitate upon interaction and consequently could be related to the

evoking of the sensation of astringency, by measurement of the areas of the chromatographic peaks of the phenolic compounds of the edible flower infused vinegar extracts and edible flower extracts in the absence (control) and presence of saliva. The different phenolic compositions of the edible flowers, that vary in both quantity and classes of polyphenols present, will affect the precipitation of polyphenols observed, which might impact the perception of astringency, since it´s related to the greater or lesser ability of these compounds to interact with SPs.

Astringency is often accompanied by bitterness, with both contributing to the overall oral sensory experience. Therefore, the investigation of the modulation of one is regularly correlated to the investigation of the other, both being necessary to fully comprehend polyphenols' role in the perception of taste and mouthfeel in the oral cavity. In light of this, going forward the screening of phenolic compounds present in the edible flower infused vinegars that may be capable of activating human bitter taste receptors TAS2Rs and of inducing astringency, using cell-based oral models would constitute an important next step for this research.

The research conducted thus far was focused on increasing the ingestion of polyphenols, and on their impact on protein precipitation that may be connected to the perception of astringency, so the research was geared towards polyphenols' behavior in the mouth. It would be valuable to later on study the bioavailability of the polyphenols in the edible flower infused vinegars through the digestion and the intestinal absorption, resorting to simulated digestions and in vitro absorption assays. Increasing polyphenols' bioavailability is of fundamental importance as many polyphenols are known for their low absorption rates.

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