GLYCINE-ASSISTED REGENERATION OF THE ANTIOXIDANT ACTIVITY OF (+)-CATECHIN AND ITS DERIVATIVES

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ABSTRACT

The oxidation of food components such as polyunsaturated fatty acids, proteins, polyphenols, and vitamins is a major cause of deterioration, leading to food waste. Synthetic antioxidants have long been used as food additives due to their superior performance but have come under scrutiny by consumers and experts alike after evidence of potential carcinogenicity surfaced. Naturally occurring antioxidants such as polyphenols constitute a safe and healthy alternative; however, their lower antioxidant capacity hinders their widespread use. Although the oxidation of (+)-catechin impairs its antioxidant properties, the resulting *o*-quinone is available to react with nucleophiles, such as amino acids. This has been proposed to regenerate its protective function.

In this thesis, different approaches were examined to assess the glycine-assisted regeneration of the antioxidant capacity of (+)-catechin-related structures. Hydroquinone (HQ) reacted with glycine in aqueous media subjected to both heated and ambient temperature treatments representative of cooking and storage of foods. The products were analyzed using isotope-labeling techniques by electrospray ionization coupled to quantitative time-of-flight high-resolution mass spectrometry (HRMS) as well as tandem mass spectrometry (MS/MS). The identified adducts indicated an antioxidant activity regeneration process involving a Michael addition mechanism with the possibility of extending HQ's antioxidant capacity up to three-fold. Investigation of under alkaline mixtures of (+)-catechin and glycine by visible spectrophotometry confirmed their interaction, altering the color profile and thus the chemical makeup of the flavonoid's oxidation products. The presence of glycine significantly decreased the generation of yellow compounds while slightly increasing the red compounds during the oxidation of (+)-catechin. Cyclic voltammetry (CV) was performed on (+)-catechin, 4-methylcatechol (4-MC), and HQ to probe the impact of glycine on their redox behaviors. No significant effect was found with 4-MC or HQ, but glycine lowered the oxidation potential of (+)-catechin, indicating a rapid reaction between the two.

The antioxidant properties resulting from glycine/phenolic interactions were also studied in food media. The rate of oxygen consumption of polyphenol-rich red wine decreased in the presence of glycine, therefore increasing its oxidative stability. The same method was applied to

flaxseed oil (FSO) containing 4-methylcatechol and glycine, where glycine showed an unanticipated antioxidant effect on FSO that disappeared when stirred. The p-anisidine value (pAV) assay was also attempted to determine the impact of HQ and glycine additives on the generation of secondary oxidation products in FSO. Again, the method was judged inappropriate for several reasons including the interference of additives and FSO's oxidative behavior.

All methods were evaluated to assess their potential and shortcomings in the study of the reaction of flavonoid and amino acids. HRMS, visible spectrometry, and direct oxygen consumption measurement were considered suitable methods, whereas CV and the pAV assays were unsuccessful.

RÉSUMÉ

L'oxydation des composantes alimentaires telles que les acides gras polyinsaturés, les protéines, les polyphénols et les vitamines est une cause majeure de détérioration, entraînant le gaspillage alimentaire. Les antioxydants synthétiques sont utilisés depuis longtemps comme additifs alimentaires en raison de leurs performances supérieures, mais ont été critiqués par les consommateurs et les experts suite à la publication d'études indiquant leur potentiel cancérigène. Les antioxydants naturels tels que les polyphénols constituent une alternative sûre et saine ; cependant, leur plus faible capacité antioxydante constitue une entrave à leur utilisation. Bien que l'oxydation de la (+)-catéchine anéantis ses propriétés antioxydantes, la structure o-quinone résultante peut alors réagir avec les nucléophiles, tels que les acides aminés. La régénération de sa fonction protectrice a été proposée comme conséquence possible de cette réaction.

Dans ce mémoire, différentes approches ont été examinées pour évaluer la régénération assistée par la glycine de la capacité antioxydante des structures liées à la (+)-catéchine. L'hydroquinone (HQ) en présence de glycine en milieu aqueux a été soumis à des traitements de température élevée et ambiante représentatifs de la cuisson et du stockage des aliments. Les produits ont été analysés à l'aide de techniques de marquage isotopique par ionisation par électrospray couplée à la spectrométrie de masse à haute résolution (HRMS) ainsi qu'à la spectrométrie de masse en tandem (MS/MS). Les adduits identifiés indiquent un processus de régénération de l'activité antioxydante impliquant un mécanisme d'addition de Michael avec la possibilité de multiplier par trois la capacité antioxydante de l'HQ. L'étude de mélanges de (+)-catéchine et de glycine en milieu alcalin par spectrométrie visible a confirmé leur interaction, modifiant le profil de couleur et donc la composition chimique des produits d'oxydation du flavonoïde. La présence de glycine a significativement diminué la génération de composés jaunes tout en augmentant légèrement les composés rouges lors de l'oxydation de la (+)-catéchine. La voltamétrie cyclique (CV) a été réalisée sur la (+)-catéchine, le 4-méthylcatéchol (4-MC) et l'HQ pour sonder l'impact de la glycine sur leurs comportements redox. Aucun effet significatif n'a été trouvé avec le 4-MC ou le HQ, alors que la glycine a abaissé le potentiel d'oxydation de la (+)-catéchine, indiquant une réaction rapide entre les deux.

Les propriétés antioxydantes résultant des interactions entre la glycine et les composés phénoliques ont également été étudiées dans des modèles alimentaires. Le rythme de consommation d'oxygène de vin rouge riche en polyphénols a diminué en présence de glycine, augmentant ainsi sa stabilité oxydative. La même méthode a été appliquée à l'huile de lin contenant du 4-méthylcatéchol et de la glycine : cette dernière démontre un effet antioxydant non-anticipé qui disparaît lorsque l'huile est remuée. La détermination de la valeur de p-anisidine (pAV) a également été tentée pour évaluer l'impact de l'HQ et de la glycine sur la génération de produits d'oxydation secondaires dans l'huile de lin. Encore une fois, la méthode a été jugée inappropriée, notamment en raison de l'interférence des additifs et du comportement oxydatif de l'huile de lin.

Toutes les méthodes employées ont été évaluées selon leur potentiel d'application et leurs lacunes dans l'étude de la réaction des flavonoïdes et des acides aminés. Le HRMS, la spectrométrie visible et la mesure directe de la consommation d'oxygène ont été considérés comme des méthodes appropriées, tandis que le CV et les tests pAV ont échoué.

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LIST OF ABBREVIATIONS

4-MC	4-Methylcatechol
4-MBQ	4-Methyl-1,2-benzoquinone
ABTS•+	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADI	Acceptable daily intake
pAV	*para*-Anisidine value
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CID	Collision-induced dissociation
CML	N_{ε}-carboxymethyl lysine
CV	Cyclic voltammetry
DhC_2	Dehydrodicatechin
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Epicatechin
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ESI	Electrospray ionization
FRAP	Ferric reducing antioxidant power
FSO	Flaxseed oil
FTIR	Fourier-transform infrared spectrometry
GCE	Glassy carbon electrode
HAT	Hydrogen atom transfer
HQ	Hydroquinone
K_{eq}	Reaction equilibrium constant
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge ratio
p-BQ	*para*-Benzoquinone
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
PV	Peroxide value

ROS	Reactive oxygen species
SPME	Solid-phase microextraction
TBHQ	*tert*-Butyl hydroquinone
UV	Ultraviolet

CHAPTER 1. GENERAL INTRODUCTION

Despite ongoing technological advances to improve the quality and stability of foods, food loss and waste remains a considerable issue. The quantities of food waste have reached 242 kg per capita per year in Canada, when considering the processing to the consumer stages[1]. This implies needless land, energy, and resource use, as well as the emission of greenhouse gases and economic loss[1]. The causes of such waste are diverse and complex, but usually have one common aspect: quality deterioration.

One of the causes of food quality loss is oxidation, a process during which susceptible compounds lose electrons to other components, resulting in structural modifications and functional impairments[2]. This type of reaction affects major and minor food components, notably polyunsaturated fatty acids (PUFA)[3], vitamins[4], and proteins[5], and polyphenols[6]. Oxidation is promoted by pro-oxidants, which induce oxidative stress. These include ultraviolet (UV) radiation, heat exposure, contact with transition metal ions, the presence of oxygen, most importantly reactive oxygen species (ROS), and more[7]. In foods, oxidation can lead to losses in nutritional properties, the generation of off-flavours and odours, or changes in appearance and texture[8-9]. In some cases, oxidation products exhibit toxic properties. For example, products of edible oil oxidation have numerous detrimental effects on human health, including inflammation of various organs[10-12], genotoxicity[13], and mutagenicity synthesis[14]. These effects can lead to diseases such as age-related neurodegenerative disorders, cardiovascular disease, metabolic syndrome and cancer[15].

Oxidative stress can be prevented or alleviated by controlling pro-oxidant factors. Another strategy involves mitigating oxidation by countering its early effects, preventing cascading oxidative reactions. Antioxidant compounds can exhibit different mechanisms to achieve this goal, whether they chelate metal ions, scavenge free radical species, or interact with relevant enzymes[16]. The addition of flavonoids, a class of naturally occurring polyphenols, as antioxidants to food products constitutes a mild, health-promoting strategy to protect the structural integrity of their components. This type of antioxidant generally receives the public's praise, as they are perceived as "natural". However, naturally occurring antioxidants are generally less efficient than the synthetic antioxidants used commercially and produce less dramatic results. For example, the

addition of mixed tocopherols at 200 parts-per-million (ppm) to conventional and high-oleic acid vegetable oils resulted in oils having less than half the oil stability index compared to the same oils to which *tert*-butyl hydroxyquinone was added at the same concentration[17]. Therefore, there is a need for more efficient natural antioxidants in the food industry.

The objective of the present research was to investigate the potential regeneration of (+)-catechin's antioxidant activity through its reaction with glycine, and in doing so, adapt and evaluate selected analysis methods. To do so, the reaction mechanism must first be tentatively identified via the structural determination of products formed in a model system composed of an aqueous hydroquinone (HQ) and glycine mixture. To ensure the model used reflects (+)-catechin's behavior, the flavonoid's interaction with glycine must also be demonstrated. Because oxidation affects compounds in a variety of ways, multiple strategies are employed to assess the antioxidant effect of the interaction of glycine with phenolic compounds, including the monitoring of oxygen consumption, color evolution, redox properties, and secondary oxidation products, both in simple solutions and real food media.

CHAPTER 2. LITERATURE REVIEW

2.1 Oxidation in food systems

Oxidation, or the chemical process involving the removal of an electron from an atom or molecule, is an important reaction that affects a vast array of substances, both organic and inorganic. The oxidation of a compound is always coupled with the reduction of another. In food, oxidation is often vilified due to its role in the development of rancid flavours in delicate oils or the browning of fresh fruit. Controlling the many contributing factors to oxidative stress is paramount to preserving the quality of many food systems. These enhancing factors include the exposure to UV rays, heat, ROS, free radicals, oxidases, and more. Because many oxidation reactions in foods are part of cascading pathways catalyzed by miniscule amounts of ROS or metal ions, for example, the deleterious associated effects are difficult to prevent in products destined for later consumption. Certain lipids, proteins, vitamins, and polyphenols are omni-present in the human diet and are among the molecules that are most susceptible to oxidation.

2.1.1 Oxidation of fatty acids

Unsaturated fatty acids and their glycerol-bound counterparts are particularly prone to oxidation due to the lowering of the dissociation energy of carbon-hydrogen covalent bonds adjacent to stabilizing double bonds[18]. This effect is most remarkable in PUFAs with methylene-interrupted double bonds[19]. The abstraction of such hydrogens initiates lipid autooxidation reactions (Figure 2.1). Reactions with oxygen species cause propagation of oxidation, as the peroxyl radicals formed easily abstract hydrogen atoms from neighbouring fatty acids. The resulting hydroperoxide fatty acids may decompose by β-scission into a variety of products with low molecular weights which affect food's flavour and odour profiles[18]. These secondary oxidation products include aldehydes, carboxylic acids, alcohols, and hydrocarbons[20-22] (Appendix 7.1). Hydroperoxides and conjugated dienes, on the other hand, are considered primary oxidation products[23]. Radicals may also react with one another, effectively terminating the oxidation reaction[18].

The lipid profile of edible oils depends on their source and processing[24]. As such, olives from a same cultivar will produce extra virgin oils with different proportions of fatty acids when grown in different environments[25], flaxseed oil (FSO) contains considerably higher levels of

PUFAs than palm oil[26-27], and so forth. The lipid compositions of selected edible oils are listed in Appendix 7.2. An oil's stability can be estimated based on its lipid profile: for example, FSO, which is oxidized under mild conditions, contains high levels of triacylglycerols composed of PUFA[28]. In addition to lipids, edible oils contain other compounds that may affect their properties, including antioxidants[24] and pro-oxidants[29].

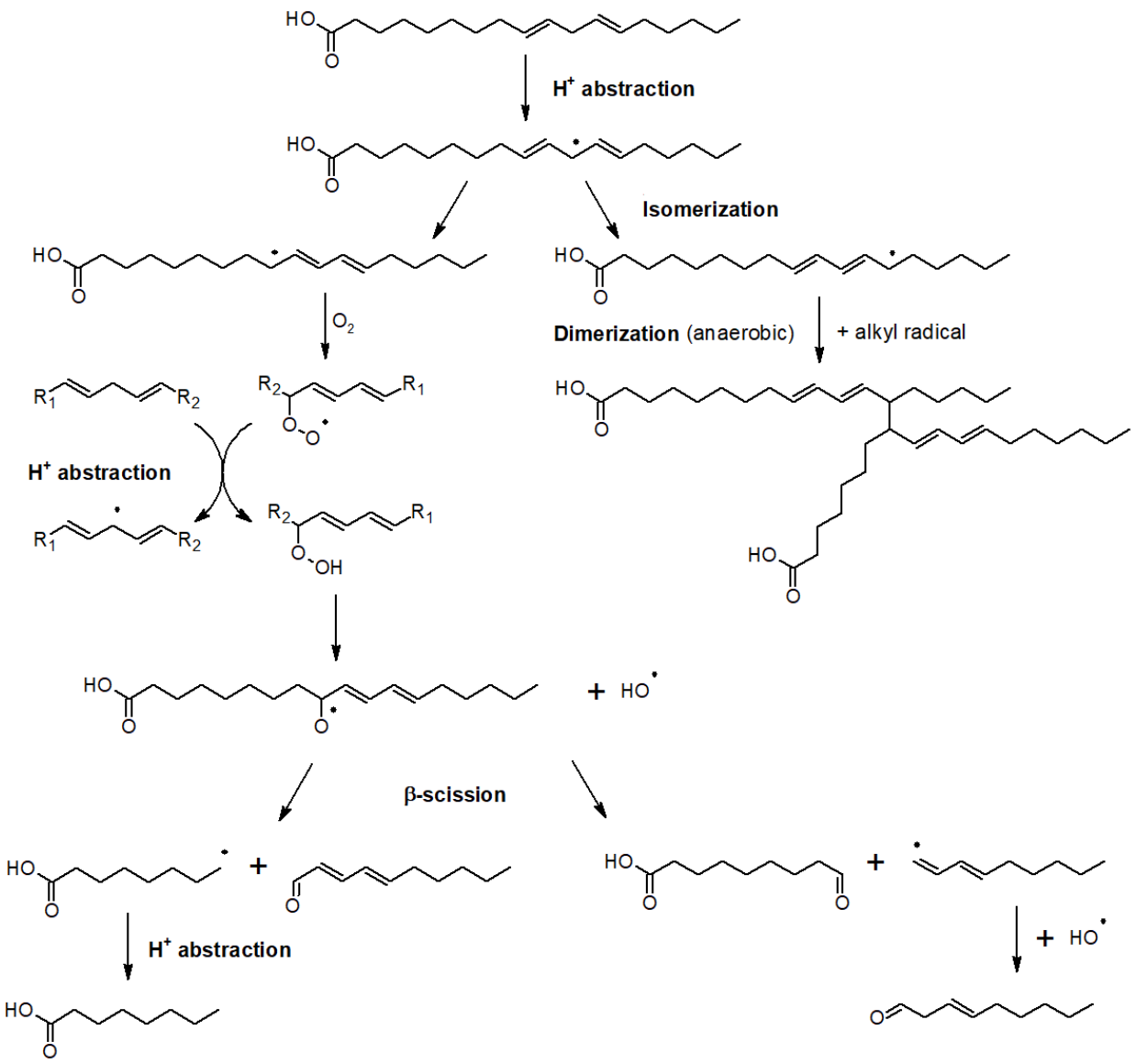


Figure 2.1 Examples of linolenic acid autoxidation pathways[18, 21].

Some products with high lipid contents Because of their antioxidant nature, phenolics also play a role in lipid oxidation and therefore reduce lipid-derived off-flavours[30]. Finally, *m*-diphenols can scavenge lipid-derivatives containing a carbonyl moiety such as 2-alkenal, which generate flavour, therefore modulating the aroma[31].

2.1.2 Oxidation of amino acids, peptides, and proteins

Certain amino acid sidechains are also subject to oxidation, whether they are free or bound within a peptide or protein's structure. ROS such as hydroxyl radicals and superoxide anions may abstract hydrogen atoms from an amino acid's α-carbon, as well as its aliphatic or aromatic sidechain, whether the amino acid is free or protein-bound. The hydrogen abstraction initiates a series of propagating radical reactions[32]. Metal ions can also take part in amino acid and peptide oxidation[33]. These oxidative reactions can lead to peptide bond cleavage, protein carbonylation, intermolecular cross-linkage, and more, affecting protein functionalities[32]. On the other hand, recent studies point towards methionine and cysteine residues acting as internal antioxidants in proteins, stabilizing a variety of enzymes and other proteins[34].

Protein oxidation may be desirable for some applications. The moderate oxidation of whey protein can stabilize emulsions, although excessive oxidation has the opposite effect by causing droplets to aggregate[35]. Protein cross-linking can be increased through oxidation, improving the viscoelasticity of gluten networks in dough[36] and the heat stability of cow milk[37]. On the other hand, protein cross-linking can reduce the water-retention capacity of meat, making it tough and negatively affecting its quality[38]. The oxidation of milk, meat, beer and coffee proteins can also be detrimental to their aroma[39]. One of the most widely studied topics in protein oxidation is the color change associated with the oxidation of red meats. Over time, meat loses its vibrant red color and turns brown as myoglobin's heme iron is oxidized from a ferrous to a ferric state[40]. Both the color stability and/or the tenderness of red meats, including beef[38] and lamb[41], can be improved by supplementing the animal's feed with polyphenols prior to slaughter.

2.1.3 Oxidation of vitamins

Some vitamins are active as antioxidants due to their ability to easily donate hydrogen atoms. However, they may also be susceptible to oxidative degradation. During the extrusion process involved in the production of many foods and animal feeds, vitamins A, C, D, and E can be destroyed under pro-oxidant conditions[42]. Although the structures of vitamins are quite

different, they often react to oxidation in similar ways. For example, α-tocopherol, a vitamin E, produces tocopherylquinone during thermal oxidation but produces α-tocopherol hydroperoxide stereoisomers when photo-irradiated in extra virgin olive oil[43]. On the other hand, ascorbic acid is believed to mainly form monodehydroascorbate, but can also react with ROS to form hydroperoxide adducts[44]. The degradation of vitamins in foods destroys their nutritional value. Wheat flour, which is fortified in vitamin A to aid in the prevention of its deficiency, is prone to oxidation during processing and storage, limiting the flour's health benefits[45].

2.1.4 Oxidation of phenolic compounds

The oxidation of phenolic compounds, often referred to as polyphenols, mainly occurs due to their antioxidant activity, during which they scavenge highly reactive free radicals, or by enzymatic oxidation[46]. Just like fatty acids and proteins, some phenolic compounds have been shown to undergo an autoxidation reaction, generating oxidation products at increasing rates[47-48]; however, semiquinone radicals are stabilized by electron delocalization, making them less reactive than other radicals[49]. Oxidized phenols have been known to oligomerize and polymerize as well as react with other components in a system, such as solvents and nucleophiles[46].

The changes in structure and chemical profile cause a range of organoleptic changes, including textural, color, and aroma modifications. Oxidized polyphenols can form complexes with proteins, granting them the astringency perceived in products such as tea and red wine[50-51]. In many fruits, such as apples, the oxidation of phenolics with an *o*-dihydroxyl group to the corresponding quinone leads to rapid browning due to the activity of polyphenol oxidase[52]. Polyphenols and their oxidation also impact the quality properties of coffee, beer, cocoa, cereals, tree nuts, and, as discussed in the next section, red wine[51, 53-55]. They can also play a role in the Maillard reaction, which generates a variety of compounds that impart desirable aroma as well as off-flavours[56-57].

2.1.4.1 Impact of oxidation on wine quality

Red wine constitutes a polyphenol-rich beverage that is susceptible to oxidation. During its production, ageing and storage, condensed tannins are generated following the oxidation and subsequent condensation of flavonoids, reaching high degrees of polymerization[58]. The formation of tannins shifts the solution's colour from vibrant yellow, red, and orange hues to deep brick-red

tones. Intermediate-sized tannins (molecular mass < 3000 Da) can cause flocculation by interacting with colloids, such as proteins and colloidal iron[59]. This leads to visible deposits in bottles.

Due to their susceptibility to oxidation and microbial contamination, most wines contain added sulfites as preservatives; their reactions with H_2O_2 and polyphenol-derived quinones help control wine's stability and quality[60]. On the other hand, one study proposed that sulfites may accelerate flavonoid oxidation in the presence of trace metal ions in wine[61]. Sulfite additives can cause adverse health effects in sulfite-sensitive people[62], and many others believe they are to blame for red wine-related headaches, although this evidence remains anecdotal and unproven[63]. Consumers show a preference for wine with no added sulfites[64], motivating the search for alternative solutions to retain long-term quality.

Although sealed bottles limit contact between wine and oxygen, oxygen is introduced into wine during its processing[65]. Oxygen can also slowly flow through corks during ageing and storage[66-67]. Oxidation of red wine is "associated with sensory and/or microbiological degradation"[65]. On the other hand, micro-oxygenation, the process of exposing wine to small quantities of oxygen post-bottling, helps simulate barrel aging by stabilizing the color, reducing off-odors and improving mouthfeel[68]. Catalytic amounts of oxygen suffice to initiate autooxidation of monomeric (+)-catechin[47], which imparts bitterness and astringency[69]. Therefore, minimal oxygen and the oxidation processes it leads to are essential to the achievement of high-quality red wine.

2.2 Antioxidants

Antioxidants are compounds that enhance the oxidative stability of other components present in their environment, including in food matrices and biological systems[18]. A wide variety of antioxidants, both enzymatic and non-enzymatic, are naturally produced by plants in response to certain oxidative stressors as an endogenous defense mechanism[70]. Humans, on the other hand, can both consume and produce antioxidants to compose with oxidative stress; in fact, vitamin D, synthesized in cutaneous tissue exposed to sunlight[71], and vitamin C (ascorbic acid), ubiquitously present in fruits and vegetables, both act as essential antioxidants[72-74]. Antioxidants can take on different forms and are categorized accordingly. Some synthetic compounds, metals, vitamins, enzymes, polyphenols, etc. can have antioxidant effects[75-76]. Antioxidants follow various

mechanisms of action such as metal ion chelation and free radical scavenging, among others[16, 77]. However, specific conditions may be required for the antioxidant to be effective[78-79].

2.2.1 Antioxidant mechanisms

Metal ion chelation refers to a compound's ability to bind metal ions and form complexes, stabilizing them in the process[80]. These metal ions could otherwise act as pro-oxidants via the Fenton reaction, during which metal ions catalyze the conversion of hydrogen peroxide to a hydroxyl free radical and result in the metal's oxidation[81]. By sequestering metal ions, their oxidative effects are controlled. However, metal chelation can also confer pro-oxidant activity to phenolic compounds; some flavonoids (myricetin, quercetin, kaempferol, taxifolin, etc.) may reduce Cu^{2+} to Cu^{+} in the process, activating its pro-oxidant ability[82].

Some antioxidants can also stabilize oxidation-prone amino acids and amino acid residues in proteins: the most effective being methionine and cysteine[83]. Polyphenols can interact with amino acid residues in proteins by creating hydrogen bonds with their sidechains, and thus inhibit the action of oxidative enzymes such as xanthine oxidase[84].

Radical scavenging involves the quenching of free radicals of different kinds, including alkyl, hydroxyl, and superoxide radicals as well as other ROS[16]. Radical scavenging reduces or reverts the damage exerted on oxidation-prone targets by preventing the activation of oxygen to ROS or terminating chain reactions, such as lipid autoxidation[79]. Radical scavenging antioxidants can attack unstable radicals through various isolated, concerted, or sequential electron and proton transfers[77, 85-86] or radical adduct formation, characterized by the formation of an adduct between the antioxidant and the unstable radical[87]. The mechanism(s) used by the antioxidant depend on the conditions, which in turn affects the radical quenching efficiency for different free radicals[85]. Some of these mechanisms result in the formation of a new radical, which must be relatively stable to avoid further oxidation reactions. Structures capable of resonance, conjugation and hydrogen bonding typically increase a radical's stability and hence, its antioxidant effect[77]. Antioxidants with *p*- and *o*-dihydroxybenzyl moieties are capable of hydrogen atom transfer (HAT), a mechanism during which a hydroxyl group's hydrogen is donated in a concerted proton and electron transfer step to quench a free radical. Although the phenolic compound becomes a radical itself in the process, the lone electron is stabilized by resonance throughout the phenol moiety,

yielding a more stable and therefore less reactive compound[88-89]. After a further HAT, a stable quinonoid structure is formed.

Antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase or glutathione reductase are found is plants and protect cells from oxidation and oxidation products[90-91]. These can catalyse the reduction of their substrate but often rearrange the structure of this substrate or a co-factor as well, as is the case for glutathione peroxidase[92]. Because enzyme reactions are substrate-specific and often need a co-factor, they do not act as effectively as other types of antioxidants in complex mixtures formed during oxidative reactions due to their limited applications. Antioxidant enzymes are affected during storage of fruits and vegetables but can be protected by coatings[91]. Agricultural practices can be employed to ensure suitable levels of antioxidant enzymes are produced in plants[93]. Although the use of antioxidant enzymes as food additives has been proposed they are not currently added to processed foods due to their unproven efficiency in food systems[94].

The chemical structure of polyphenols influences both their antioxidant mechanism and efficiency. For example, the location of functional groups such as hydroxyl groups on a benzene ring affects a compound's metal chelation properties. Glycosylation of flavonoids can decrease antioxidant activity, compared to aglycone equivalents[95]. The environment to which antioxidants are exposed also affects their efficiency. In the case of phenolic antioxidants, the bond dissociation energy for the hydroxyl group's hydrogen atom depends on the solvent. In polar solvents like acetonitrile or *t*-butyl alcohol, the bond dissociation energy is higher than in non-polar solvents such as benzene[96-97]. Therefore, the intermolecular hydrogen bonding that occurs in polar solvents decreases the antioxidant's radical scavenging ability[98]. However, antioxidants must be in contact with target compounds to be effective, so the antioxidant and the target compound must be properly combined. These must either be solubilized in the same solvent or present in an emulsion to maximize antioxidant activity[99-100].

2.2.2 Activity of synthetic and natural antioxidants in foods

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butyl hydroquinone (TBHQ), and propyl gallate are the main synthetic antioxidants used in foods, mainly those with high lipid contents such as margarines and oils[101] or powdered foods such as cake mix and dried potatoes[102]. Although synthetic antioxidants are generally considered as more

potent than their natural counterparts, studies on the effects of natural antioxidants in oils have provided variable results, demonstrating that the environmental conditions, concentration, analytical method and other factors can influence an antioxidant's efficiency. For example, the addition of 0.5 to 1 % of antioxidant red pepper oil resulted in soybean and sunflower oils with a high peroxide value (PV) but delayed light-induced rancidity, whereas 0.02 % BHT had more pronounced effects[103]. In seal blubber and menhaden oils, dechlorophyllized green tea extract with a high flavonoid content used in concentrations of 500 and 1000 ppm was found to have better antioxidant activity than 200 ppm of BHA and BHT but was not as efficient as 200 ppm of TBHQ[104]. FSO, which is very sensitive to oxidation, was found to be more effectively stabilized by a (+)-catechin (555 µmol/kg)/ascorbyl palmitate (241 µmol/kg) mixture than BHA (555 µmol/kg), based on hydroperoxide content, fatty acid profile and residual antioxidant after heating[105].

Because they are permitted in foods in greater quantities (see section 2.2.3), naturally sourced antioxidants may offer advantages over synthetic ones in certain cases. On the other hand, synthetic and naturally occurring antioxidants can act synergistically. For example, diludine, a synthetic antioxidant, exhibits synergistic antioxidant activity with BHT and α-tocopherol[106]. Overall, synthetic antioxidants generally outperform natural antioxidants in reducing oil oxidation.

2.2.3 Regulation of antioxidant food additives in Canada

In Canada, the use of synthetic antioxidants in oils is regulated under the *Food and Drug Regulations*, alongside other food additives. The imposed limits depend on the specific antioxidant and the food product they are added to. The upper limit for BHA, TBHQ and BHT in fats and oils other than olive oil, suet, essential oils (except TBHQ) and milk fat is a mass fraction of 0.02 %, and if combined, must not exceed this concentration in total[107]. The use of flavanols and catechins is not regulated and is therefore subject to principles of Good Manufacturing Practices[108]. Therefore, if any reasonable concentration of natural antioxidants could prove to be as effective as synthetic ones at their 0.02 % limit, they could provide a suitable alternative.

2.2.4 Health effects of synthetic and naturally occurring antioxidants

Many studies have evaluated the potential health effects of synthetic antioxidants. Although BHA and BHT may induce tumors in rats, epidemiological studies suggest they are not a cancer hazard in humans and may have anticarcinogenic effects at levels at which consumers are

exposed to in a normal diet [109-110]. In fact, a cohort study of over 120,000 Dutch aged between 1986 and 1992 determined average BHA and BHT intake based on chemical analysis of reported frequently consumed foods (105 and 351 µg/day, respectively) and correlated these with the incidence of stomach cancers within the cohort. They did not find a significant association between stomach cancer and normal intakes of BHA or BHT [110]. On the other hand, animal studies continue to raise concerns about other adverse health effects[111]. Both synthetic phenolics are known to affect thyroid function and the development of a grouped acceptable daily intake (ADI) rather than individual ADIs has been suggested[112]. It is also reported that European children may have daily intakes above the current ADIs[112]. Therefore, although low doses of BHA and BHT may be considered safe, it would be advisable to limit their use.

As for TBHQ, the molecule shows potential effects on the immune system, based on high-throughput screening and toxicological data, as well as laboratory animal and epidemiologic studies[113]. TBHQ may also interact with *Lactobacillus rhamnosus* probiotic bacteria and enhance their anti-cancer properties on human colorectal adenocarcinoma cells[114] or reduce the neurotoxicity associated with amphetamine intake in rats[115].

Natural antioxidants are linked to many health benefits. In the case of bioactive polyphenols, these include the prevention of several types of cancer, the alleviation of metabolic syndrome symptoms, and the reduction of risk factors for coronary heart disease; however, further studies must be conducted to recommend an appropriate daily intake for public health[116]. Consumers tend to distrust synthetic food additives[117], but associate natural food antioxidants with health[118]; natural antioxidants are therefore highly sought out in a variety of foods. The health effects of flavonoids will be further discussed in section 2.3.2.1.

2.3 Phenolic compounds

One of the categories of antioxidants has gained a lot of interest due to the diversity of its bioactive properties: phenolic compounds, also known as polyphenols. These are compounds containing one or more aromatic rings with hydroxyl groups and other substituents[46]. Polyphenols can be further classified into different classes, according to their structure, as indicated in Figure 2.2.

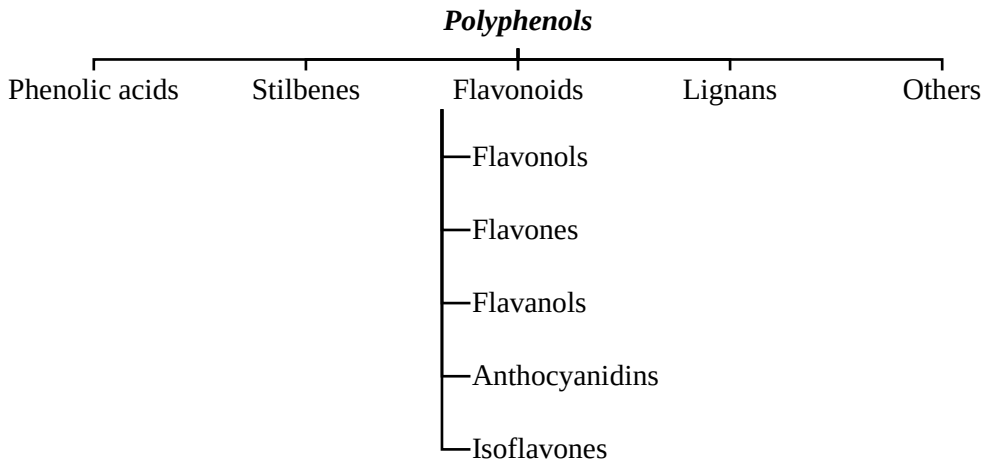


Figure 2.2 Classification of polyphenols[46].

2.3.1 Occurrence

Most polyphenols are formed in plants via the shikimate pathway, which generates phenylalanine, tyrosine and tryptophan, followed by the phenylpropanoid pathway, in which phenylalanine undergoes different reactions to form flavonoids, lignans and coumarins[119]. Animals cannot produce polyphenols, and they are uncommon in bacteria, fungi and algae[46].

Many fruits, vegetables and herbs along with wine, coffee, tea and dark chocolate figure amongst the 100 foods richest in polyphenols [120]. Daily phenolic intake is heavily diet-dependant, but some estimates suggest around 1g per day. Coffee, tea, wine, soy products, chocolate drinking and consumption habits have a large influence on total phenolic intake. When plants are transformed into food products, some polyphenol-rich parts are discarded, including the pomace, seeds, and peels. These provide the industry with a potent source of nutraceuticals and food additives to be extracted and incorporated into other food products, providing them with an added value [121]. The polyphenol content of fruits, vegetables, and other foods has been vastly studied and has been compiled in reviews[122] and books[123-124].

2.3.2 Flavonoids

Flavonoids are a class of low molecular weight polyphenols with the same basic structure: a flavan. Based on additional functional groups, flavonoids can be further categorized as flavan-3-ols, anthocyanidins, flavonols, flavones, flavanones or isoflavones[125] (Figures 2.2 and 2.3). They exhibit a variety of biological properties in plants, animals, and bacteria: they can act as UV filters,

signal molecules, phytoalexins, cryoprotectants, colorant, etc.[126-128] Flavonoids can be extracted from onion discards, pomegranate peel and macadamia skin rejected during processing[129].

2.3.2.1 Health benefits of flavonoids

An abundance of research has been conducted on the health impacts of flavonoids. They were once classified as vitamin P but were eventually deemed non-essential to the human diet[130]. While epidemiological studies have linked the consumption of flavonoid-containing foods with healthier populations and *in vitro* studies have demonstrated their bioactive effects, the precise effects of flavonoids on human health have yet to be confirmed. This uncertainty is in part caused by their ranging bioavailability as well as sometimes inconsistent or irreproducible results[131].

Although an array of investigated health claims exists, the strongest associations between flavonoids and health have been found in coronary artery disease and strokes[131]. Because there are few antioxidants in artery walls compared to blood plasma, low-density lipoproteins that encounter arterial lesions are subject to oxidation and can then be phagocyted by foam cells and form arterial plaque[132]. Tea catechins have been found to reduce the importance of cardiovascular disease in animal studies by reducing macrophage intake of low-density lipoprotein, which in turn decreases the formation of abnormal plaque in arteries' inner wall[133]. Many other probable mechanisms of atherosclerosis prevention by polyphenols have been identified and are under study[134].

In diabetic rats, tea catechins were also found to help reduce blood plasma glucose levels when ingested prior to starch and sucrose consumption by inhibiting intestinal α-amylase and brush border membrane sucrase[135]. Flavonoids have also been found to help counteract diabetic complications, such as retinopathy, nephropathy and neuropathy[136]. These results suggest an alleviation in diabetic symptoms and complications associated with flavonoid consumption. Higher intakes of flavonoids at midlife were associated with healthy aging, defined as living past 70 years of age with "no major chronic diseases or major impairments in cognitive or physical function or mental health" in a clinical cohort study including over 13,000 women[137]. Other studies have indicated anti-inflammatory, antimicrobial, anti-angiogenic as well as other effects associated with flavonoids and green tea extracts[125, 138].

Various flavonoid supplements are available on the market. Although many studies are available on the benefits of a high-flavonoid whole food diet, such as consumption of fruits, tea and cacao, little is known about highly purified flavonoid consumption, their interactions, product

safety and effectiveness[139]. Nutritionists rather recommend that consumers eat fruits and vegetables as a whole to ingest flavonoids along with other healthy plant metabolites such as vitamins, minerals and dietary fibre[140].

2.3.2.2 Uses of flavonoids in the food industry

Flavonoids have been or could be used in the food industry in several ways. Tea catechins have been shown to effectively protect oxidation-prone compounds, mainly lipids, from the effects of hydroxyl, superoxide, peroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, in vitro, in foods such as fresh and frozen chicken[141-142]. Their major source as an additive is from tea leaves because it is abundant, available internationally and relatively inexpensive[143]. These flavonoids can also be used in beef, pork and fish products; however, the concentration of catechins used in fish products must be higher than in meat products due to the higher unsaturation of the fatty acid fish contains[144]. In comparison with BHT, the thermal stability of catechins in oils has been found to be superior[145]. A biodegradable poly(lactic acid)-poly(hydroxybutarate) plasticized by acetyl(tributyl citrate) and rendered active with catechin, giving it thermal stability and antioxidant properties, has been produced as a suitable alternative to other traditional food packaging, based on its physico-chemical properties[146]. The shelf-life of pork sausages can also be extended by the addition of a chitosan-based active film with added green tea extract. This extract contained various polyphenols, including (+)-catechin, epigallocatechin (EGC), epicatechin (EC) and epigallocatechin gallate (EGCG), and was found to decrease the thiobarbituric acid value of the sausage even after 20 days compared to controls[147].

Some challenges to the addition of flavonoids as natural antioxidants in food products persist. Phenolic compounds can react with components in food products, mainly proteins, leading them to aggregate and precipitate, causing adverse effects in the food as well as the loss of the phenolic additive's functionality[148-149]. To counteract this problem, phenolics including flavonoids can be encapsulated. For example, (+)-catechin and EGCG can be added to liposomes and incorporated into low-fat hard cheese effectively. However, researchers found that this technology needs improvements to increase the encapsulation efficiency, improve solvent compatibility, reduce cost, increase capsule stability, and improve sensory properties[150].

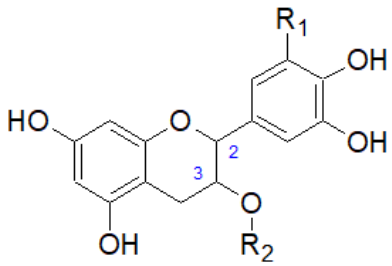
In the case of flavanols, the degradation induced by processing and the destabilization resulting from neutral and alkaline media defeats their purpose as food antioxidants and

nutrients[151]. Different approaches have been attempted to improve their applicability, including structural modifications[152-153] and even their combination with *Saccharomyces cerevisiae* as a protective yeast[154]. Palmitoylated green tea catechins, formulated to improve the oil solubility of the flavanols, is currently used in products such as cookies, mayonnaise, frosting, nut spreads and more[155].

Another obstacle is solubility; chosen antioxidants must be compatible with the solvent or matrix to which they are added. As such, the choice of solvent has been shown to have an effect on genistein's antioxidant mechanism[97]. In the case of quercetin, decreasing solvent polarity increased its radical scavenging capacity[156]. For both epicatechin and quercetin, antioxidant activity is diminished by a hydrogen-bonding solvent compared to a solvent in which this interaction does not take place[157]. Flavonoids have low solubility in fats and oils, and their effect in these is therefore minimal. Their use could be more suitable in emulsions[158]. In fact, phosphatidylcholine has been used to emulsify green tea catechins to dissolve them in oils[159]. This strategy could be useful to dissolve (+)-catechin and other flavanols in edible oils and other fatty foods.

2.4 (+)-Catechin and other flavanols

Along with epicatechin, (+)-catechin is one of the most studied flavanols, a sub-class of flavonoids with a hydroxyl group attached to the third position of their C-ring[160] (Figure 2.3). This sub-class is also often referred to as flavan-3-ols or catechins. The latter may lead to confusion between the compound (+)-catechin and the class of compounds derived from it. For example, the phrase "catechin content" is sometimes used, alluding to the sum of various flavanols.



Common name	R_1	R_2	C-2	C-3
(+)-catechin	H	H	R	S
(-)-epicatechin	H	H	R	R
(-)-epigallocatechin	OH	H	R	R
(-)-epigallocatechin gallate	OH	Gallate	R	R
(-)-epicatechin gallate	H	Gallate	S	S

Figure 2.3 Structure of common flavan-3-ols[161-162].

(+)-Catechin is ubiquitous in a variety of plants, some of which are consumed fresh, processed into foods, or harvested for their medicinal properties[163]. The catechin content of plants varies according to species[164], harvesting season[165], growth environment[166], processing conditions[167], as well as many other factors. Flavanols are the main type of polyphenols in green tea, comprising up to 83 % of its total polyphenol content[168] and up to 30 % of the leaves' dry mass[169]. Their substituents and configuration affect their performance in scavenging activity tests as well as their stability[170]. The total catechin and epicatechin contents of food sources are listed in Table 2.1. The two compounds are diastereoisomers.

Table 2.1. Occurrence of catechin and epicatechin in selected food sources[122].

Source	Content	By serving (mg)
Chocolate	460–610 mg/kg	23–30
Beans	350–550 mg/kg	70–110
Apricot	100–250 mg/kg	20–50
Cherry	50–220 mg/kg	10–44
Grape	30–175 mg/kg	6–35
Peach	50–140 mg/kg	10–28
Blackberry	130 mg/kg	13
Apple	20–120 mg/kg	4–24
Green tea	100–800 mg/L	20–160
Black tea	60–500 mg/L	12–100
Red wine	80–300 mg/L	8–30
Cider	40 mg/L	8

2.4.1 Antioxidant mechanism and activity of (+)-catechin

(+)-Catechin has the ability to donate a hydrogen atom via a HAT, which gives it radical scavenging properties[171]. This is related to their one-electron reduction potential, which is similar to tocopherols but higher than ascorbic acid; they can therefore donate electrons less readily than ascorbic acid[172]. DPPH assays have been performed and show that although the reduction potential of flavanols is greater than that of ascorbic acid, the former are better radical scavengers[173], whereas the latter are more efficient ferric ion chelators[174]. Other studies suggest the electron donation mechanism of (+)-catechin depends on the solvent polarity and would rather involve a sequential proton loss-electron transfer or a single electron transfer-proton transfer in solution[175].

(+)-Catechin can donate up to two hydrogen atoms and their associated electrons, which originate from the hydroxyl groups on catechin's B-ring[176]. This oxidative process converts (+)-catechin to a semiquinone radical upon a first HAT, and finally to a quinone, dehydrocatechin, upon a second HAT[177] (Figure 2.4). The (+)-catechin semiquinone radical formed is highly stable compared to other flavonol or flavone radicals[178].

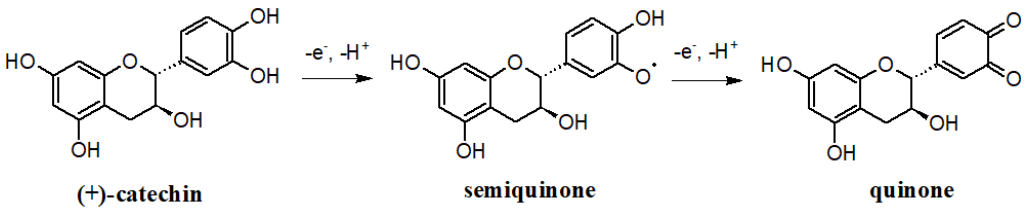


Figure 2.4 Oxidation of (+)-Catechin[176-177].

Catechins can also chelate metals, such as aluminum, copper, iron and zinc, which often have prooxidant properties. For example, they can chelate copper and iron ions, which in turn keeps these transition metal ions form catalyzing the free radical formation of other molecules[171]. Evidence suggests that this could be done via the B-ring and via a gallate moiety, in the case of epicatechin gallate or epigallocatechin gallate, for example[179-180]. It has been shown that metal-flavonoid complexes have a greater antioxidant potential than the flavonoids themselves, probably due to the gain of a new superoxide dismutation center, capable of scavenging superoxide, an ROS[181-183]. Aluminum has been identified as the metal granting the greatest antioxidant efficiency between the four aforementioned metals, and that ascorbic acids plays a synergistic role with the complexes[184]. Flavonoids have been reported to chelate in flavonoid-to-ferrous ion ratios ranging from 1:1 to 3:1 under near-neutral conditions. The higher flavonoid-to-metal ratios involve the adjacent carbonyl and hydroxyl groups on the C-ring; intermediate ratios were found for flavonoids with *o*-dihydroxyl B-rings; and equimolar ratios were found for a flavone with a trihydroxylated A-ring[185]. Therefore, under a pH near 7, two molecules of (+)-catechin can form a complex with a ferrous ion[186] (Figure 2.5). Additional studies confirm the role of B-ring hydroxyl groups in metal chelation and imply that deprotonation caused by radical scavenging could even enhance metal chelation activity[184]. Different conditions have been found to affect (+)-catechin's iron chelation mechanism and kinetics. At a low pH the ligand-metal complexes formed are unstable and break down, forming a catechin quinone and reducing Fe^{3+} to Fe^{2+} in the process. At a pH between one and three, (+)-catechin can reduce twice as many ferric ions. The reaction

mechanism is dependant on whether the flavanol or the metal is present in excess[187]. Iron in the ferrous form is more bioavailable than ferric iron[188] but induces more oxidative damage[189].

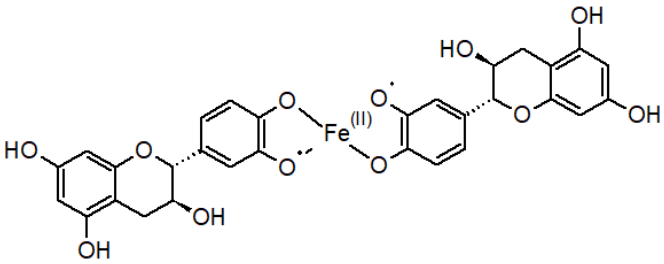


Figure 2.5 Iron (II) chelation by (+)-catechin[186].

(+)-Catechin's antioxidant activity is therefore due, in part, to its ferric ion reducing ability. This property is measured by the ferric reducing antioxidant power (FRAP) assay, which associates a FRAP value to the analyte[190]. (+)-Catechin's FRAP value is greater than resveratrol's, but lower than the determined values for quercetin, myricetin or gallic acid[191].

Studies indicate that (+)-catechin is not the most efficient antioxidant of the flavanol class, which is rather EGCG, based on its hydrogen peroxide and radical scavenging activities[192]. Nonetheless, (+)-catechin can be used as a simpler model in place of other flavanols to help advance the understanding of their reaction with other compounds[193].

2.4.2 Oxidative reactions of (+)-catechin

(+)-Catechin oxidation leads to the formation of dimers, which can further oligomerize, due to the high reactivity of the monomer's quinone form[194]. (+)-Catechin has been observed to epimerize, forming epicatechin, in basic aqueous solutions[195] and under heating. Therefore, resulting dimers may be formed by intermolecular bonds between (+)-catechin and/or epicatechin subunits. Dimers can either be formed by cross-linkage of two catechin units between one unit's A-ring and the other's B- or C-ring. The former (A-C bond) results in the generation of procyanidins, whereas the latter (A-B bond) forms dehydrodicatechins[196]. Dehydrodicatechins (DhC_2) are typically classified as either type A or B, although researchers have suggested more specific notation methods to clarify the structures (Figure 2.6), due to evident confusion between the subunit ring and the type of linkage involved[196]. While DhC_2 B (β_{AB} or ε_{AB} linkage) is formed via a single linkage and remains colorless, like (+)-catechin, the extended conjugation of DhC_2 A

(α_{AB}, γ_{AB}, or δ_{AB} linkage) involving ether and carbon-carbon linkages, making them appear yellow[194, 197].

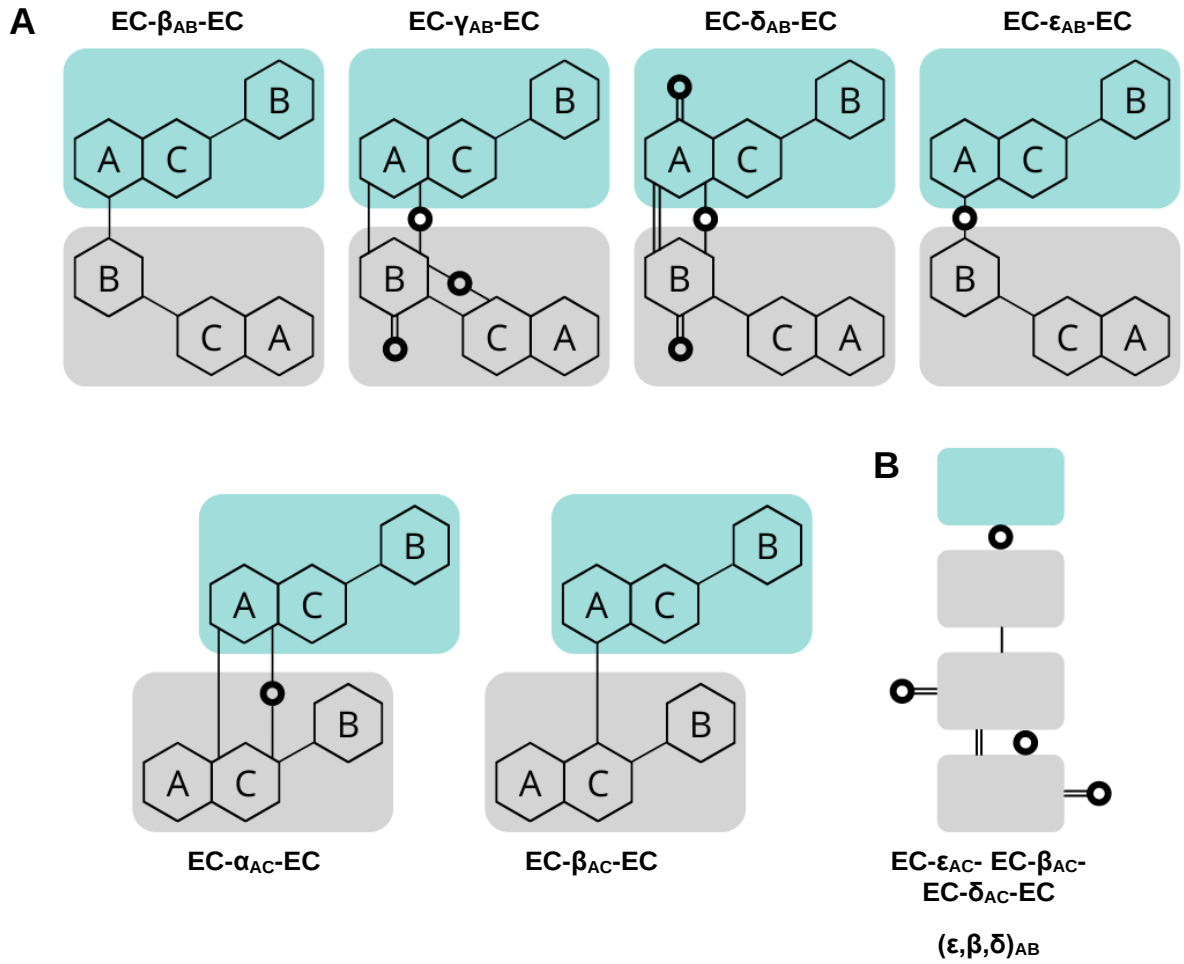


Figure 2.6 Nomenclature of dehydrodicatechins and procyanidins proposed by Verloop, Gruppen and Vincken[196].

(A) Different types of catechin dimer configurations. **(B)** Example of the proposed nomenclature for a catechin oligomer. Circles represent oxygen atoms and lines represent covalent bonds. EC, epicatechin. Teal shade, terminal unit; grey, extension unit.

2.5 Reactions between phenolic compounds and amino acids

Various reactions can take place between amino acids and quinones or flavonoids in foods, biological systems, and model solutions. These depend on the reactants' structures, which in turn can depend on their environment. Phenolic compounds have different reactive functional groups, whereas amino acid side chains have a range of properties which influence the reaction they can undergo. The main reactions known to occur between these types of compounds are the formation of Schiff bases and Michael additions of amines and thiols. These reactions are biologically

important for some organisms, particularly when products polymerize[198]. For example, the polymerization of certain flavanols with protein's amino groups has considered as a key factor in insect exoskeleton sclerotization[199]. They are also involved in the crosslinking of adhesive proteins by marine organisms[200] and the biosynthesis of melanin[201].

Phenolic compounds and amino acids have been found to react in certain food matrices. In honey, polyphenols form high molecular weight complexes with proteins under heated or prolonged storage conditions via covalent bonding. This reduces polyphenol availability as an antioxidant and modifies the affected proteins' functionalities[202]. The reaction mechanism has not been identified, but the reactions discussed below could explain the changes observed in honey. In a cookie model system, spray-dried olive mill wastewater reduces Maillard reaction products due to its high secoiridoids content, which react with asparagine, forming both Schiff bases and Michael addition products[203].

2.5.1 Schiff base formation

Schiff bases are compounds with an imine structure (Figure 2.7). They are formed when a primary amine reacts with an aldehyde or ketone under favoring conditions, in the presence of trace acid[204]. The reaction is due to the carbonyl's electrophilic nature and the amine's nucleophilicity[205]. The reaction has been exploited to create cross-linking in various polymers, such as hydrogels for medical applications[206]. Schiff base formation is also the first step of a complex series of reactions that occur in foods, most commonly between sugars and amino acids, known as the Maillard reaction, which results in browning and the production of a multitude of fragrant compounds[207-208]. Schiff bases are usually unstable, and quickly rearrange or react with other compounds[209].

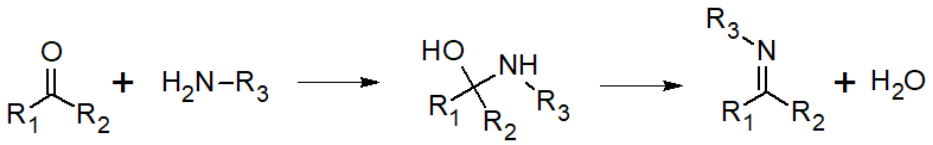


Figure 2.7 General Schiff base formation between an aldehyde or ketone and a primary amine[210-211].

Published literature relating to the formation of Schiff bases derived from quinones is scarce. (+)-Catechin Schiff bases and their degradation products have been reportedly formed

between (+)-catechin and glycine upon heating in water until complete evaporation[176] as well as between (-)-epicatechin and lysine under slightly basic aqueous conditions at 70 °C[212]. Older research suggested that Schiff bases only form concurrently with addition products[213-215]. This theory has been supported by others[216-217].

2.5.2 Strecker degradation

The classic Strecker degradation involves the sequential decarboxylation and hydrolysis of Schiff bases with an α-keto imine structure, releasing carbon dioxide, a 1,2-enaminol and a Strecker aldehyde[218-219] (Figure 2.8). This constitutes an amino acid deamination mechanism. Further reactions may produce ammonia and other compounds from the initial Strecker degradation products[218]. Strecker degradation impacts flavour generation, as the formed aldehydes have low detection thresholds[220] (Table 2.2).

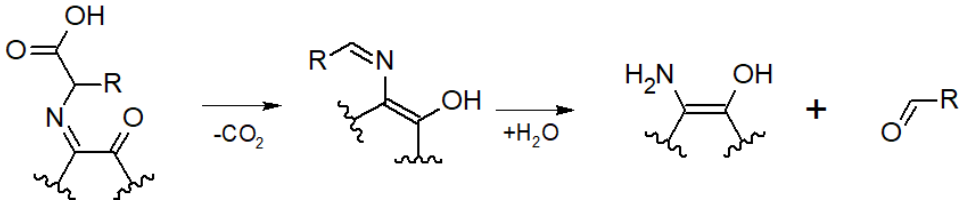


Figure 2.8 Typical Strecker degradation of a Schiff base formed between an *o*-quinone and glycine[218-219].

Strecker degradation reactions of quinone/amino acid mixtures have been reported in the literature. In neutral solutions containing oxidizing potassium hexacyanoferrate (III), phenylacetaldehyde and methional were produced from the reaction between some phenolic and phenylalanine or methionine, respectively, at room temperature. The tested phenolic compounds included (+)-catechin and epicatechin[217]. In another study, phenolic compounds were heated in the presence of phenylalanine. Phenylacetaldehyde, the amino acid's corresponding Strecker aldehyde, was produced in mixtures of phenylalanine with 4-MC, HQ, *para*-benzoquinone (p-BQ) and other simple *ortho*- and *para*-diphenols at a pH of 3. Increasing pH had a decreasing effect on the concentration of Strecker aldehydes. However, (+)-catechin and other complex flavonoids did not produce significant levels of the Strecker aldehydes, suggesting that either Schiff bases are not produced under the experimental conditions, or that these do not undergo a Strecker degradation[221].

Table 2.2 Detection threshold of Strecker aldehydes found in beer, determined by the ascending method of limits test and a performed three alternate forced choice test[220].

Strecker aldehydes	Threshold (ppb)	Aroma
2-Methylpropanal	86*	varnish, grainy, fruity
2-Methylbutanal	45	almond, apple-like, malty
3-Methylbutanal	56*	malty, almond, cherry, chocolate
Benzaldehyde	515	almond, cherry stone
Phenyl acetaldehyde	105	hyacinth, floral, roses
Methional	4.2	cooked potatoes, worty

*Odor detection threshold. All other thresholds are combined odor and flavour detection thresholds. ppb, parts per billion.

The presence of certain compounds has been found to inhibit Strecker degradation. For example, the presence of glucose has been reported to decrease phenylacetaldehyde production when added to a phenylalanine/catechin mixture in a wine model system[222], possibly due to competing reactivity of the amino acid with glucose, leading to the formation of different products. The presence of 2-pentenal decreases phenylacetaldehyde levels resulting from phenylalanine reacted with simple phenolic compounds, such as catechol, HQ, or *p*-BQ. This was found despite the ability of both 2-pentenal and the phenolic compounds to produce phenylacetaldehyde upon reaction with the amino acid, suggesting an antagonist effect. This may be due to phenolic compounds' ability to react with both phenylacetaldehyde, resulting in its destruction, as well as 2-pentenal, creating competition between the 2-pentenal/phenylalanine and 2-pentenal/phenolic compound reactions[223]. The effect of other lipid-derived products on Strecker aldehyde formation should be further studied, as this could have an impact on the flavor of oxidized fatty acids in the presence of compounds such as phenols and amino acids.

2.5.3 Michael addition

Michael additions are addition reactions of nucleophiles to conjugated compounds, i.e. α,β-unsaturated carbonyls, that occur via an enolate intermediate and produce thioethers, secondary amines, or other adducts depending on the nucleophile[224]. The reaction's mechanism is shown in Figure 2.9 and is the same for the addition of primary amines and thiols, although their reactivities differ[225]. The Michael addition of amines and thiols to *o*-quinones are thought to be similar to their addition to *p*-quinones[215].

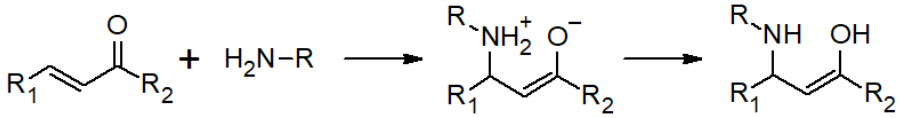


Figure 2.9 Michael addition mechanism between an α,β-unsaturated aldehyde or ketone and a primary amine.

Conjugate additions of thiols and amines to quinones have been demonstrated to occur under a variety of conditions and is postulated to affect foods in different ways. For example, (-)-epicatechin and (-)-epigallocatechin gallate can bind Nε-carboxymethyl lysine (CML), a compound associated with adverse health effects, through Michael addition of the CML's amino groups with the polyphenols' B-ring. It is thought this trapping mechanism could play a role in reducing CML concentrations in foods such as processed coconut milk[226]. The efficiency of amine-quinone addition can be modulated using an immobilized lipase, reaching a 98 % conversion of 2-methyl-1,4-benzoquinone to the target product using butylamine as a nucleophile[227]. Conjugate additions of thiols have also been observed between glutathione and caftaric acid in white wine, inhibiting its browning. The reaction is accompanied by the regeneration of caftaric acid's benzenediol moiety[228]. Cysteine forms adducts with 4-MC through addition reactions, which competitively inhibit polyphenol oxidase due to their higher affinity as the enzyme's substrate. In solutions containing lower cysteine than 4-MC concentrations, the adducts regenerate the 4-methylbenzoquinones, leading to pigmentation as they undergo enzymatic reactions. At higher cysteine concentrations, the adducts are formed without color formation[229].

2.5.3 Influence of reaction parameters

2.5.3.1 Reactants

Michael additions often involve the nucleophilic addition of thiols, thioethers, primary and secondary amines, and hydroxyl groups to quinones[230]. In 3,4-estrone *o*-quinone, Michael addition products were formed with mimics of histidine, cysteine, tyrosine and aspartate/glutamate side-chains, but not with mimics of serine, glutamine/asparagine, arginine and tryptophan side-chains[231]. This indicates different Michael donors have different reactivities. The reaction rates of free thiol- and/or free amine-containing residues with 4-methylbenzoquinone (4-MBQ) have been studied and indicate that cysteine-rich proteins react at least times 10^5 times faster via Michael addition compared to proteins with abundant free amine and guanidine residues[225]. Furthermore,

thiol groups are more likely to undergo Michael addition than Schiff base formation when reacting with polydopamine. Aromatic amines favor the Michael addition whereas aliphatic amines enhance the formation of Schiff bases[232]. Soft nucleophiles such as thiols favor Michael addition, but primary amines, which are considered intermediate nucleophiles, can participate in both reactions[224]. Overall, the reactants are the main determinant for the reaction undergone[232].

2.5.3.2 pH

In wine, Michael additions of phenylalanine and methionine to 4-MC did not occur[233]. However, this conclusion was based solely on the undetected initial adducts, and their possible degradation products were overlooked. Michael additions usually require a neutral or basic medium[234], so a low pH may inhibit the addition reaction. It was found that at neutral pH, 96 % of products formed from the reaction of catechol with aniline were from the Michael addition and 4 % were derived from the Schiff base reaction, whereas at a pH of 11.7, only Michael addition products were observed[232]. This is linked to the accelerated reaction rate due to the protonation state of the amine group[235]. This trend also applies to the Michael addition of thiols to EGCG: at a pH of 7, the cysteine-EGCG Michael addition is nearly complete during its initial phase (first 25 hours), whereas at a pH of 4 the reaction was slower and further from completion even after approximately 95 hours[236]. This study did not assess Schiff base formation, as only thiols were investigated. For amines to serve as nucleophiles, they should not be charged, and therefore the reaction should occur at a pH above their pK_a[237]. Mild acidic media is often used for Schiff base synthesis, as the reaction is acid-base catalyzed but a pH that is too low will increase the amine's protonation and decrease its nucleophilicity as well[238].

The type of base used to catalyze Michael additions has an impact on the reaction's equilibrium constant (K_{eq}). Strong bases result in a large K_{eq} and first-order reaction kinetics, whereas weak bases will produce moderate K_{eq} and second order kinetics[239]. However, strong bases such as hydroxides or methoxides can hydrolyze esters and cause other side-reactions, so non-nucleophilic bases are often preferable[239].

2.5.3.3 Solvent

In terms of solvents, Michael additions are typically performed in methanol, ethanol, diethyl ether, tetrahydrofuran, benzene, xylene, dioxane, or a mixture of the solvents[239]. Within 5 minutes, 1,4-naphthoquinone and *n*-propylamine react in water at room temperature with a 95 %

yield of 2-aminonaphthoquinone. In the same study, similar results were observed with different *p*-quinones and amines, even when sterically hindered[240]. Therefore, aqueous solvents promote high addition reaction rates and yields, thus offering a suitable green alternative to traditional organic solvents[240-241]. Under neat conditions at room temperature, Michael addition products can occur between thiophenol and methyl vinyl ketone with a reaction yield of 76 % after 30 minutes of mixing, which was found to be greater than when several solvents, including water (32 %) and dichloromethane (50 %) were used[242]. The traditional solvents used for Michael additions are methanol, ethanol, diethyl ether, tetrahydrofuran, benzene, xylene and dioxane; special care must be taken in the choice of solvent to avoid side-reactions and dissolve reactants[239].

Schiff bases, on the other hand, may form in aqueous solutions, alcoholic solvents, organic solvents, etc. In aqueous solvents, acid-base catalysis or heat are usually applied[238]. Because water is produced during the condensation reaction, its removal is a common strategy to shift the reaction equilibrium and favor the product formation[243]. In dioxane, acids and peroxides have a strong catalytic effect and the reaction is accelerated by exposure to white light[244]. It is also possible to produce Schiff bases under solvent-free conditions. A P_2O_5 catalyst supported on Al_2O_3 is sufficient to accelerate the reaction between benzaldehyde and aliphatic or aromatic primary amines ground together at room temperature under solvent-free conditions, resulting in a high Schiff base yield in a short amount of time[245]. Schiff bases can also be produced in solvent-free mixtures. The reaction between glucose and different amino acids is accelerated mechanochemically by ball milling at room temperature and produces Schiff bases[246].

2.5.3.4 Temperature

Schiff bases can be formed between aldehydes and primary amines under mild temperatures easily if excess water is removed, but ketone Schiff bases require more reactive conditions, including high temperatures, excess amines, catalysts, and longer reaction times[247]. Michael additions are also favored by high temperatures and long reaction times[224]. Therefore, temperature treatments cannot be exploited to favour one reaction pathway.

2.5.3.5 Catalysts and presence of other compounds

In the presence of a polyphenol oxidase or ferricyanide catalyst, Strecker aldehydes are produced from mixtures of amino acids, catechols, and molecular oxygen under mild conditions[216]. Lewis acids such as boron trifluoride, aluminum trichloride or zinc chloride have also been used

to catalyze Michael additions. These slightly withdraw electrons from the carbonyl, thus activating the alkene[248]. Michael additions are also favored by the presence of copper catalysts[224].

2.5.4 Effect of functional group on antioxidant activity

Replacing a functional group by another, such as replacing a hydroxyl group by an amino group, could theoretically result in a variation of the compound's antioxidant activity due to a structural change. This change could occur during Schiff base formation followed by the Strecker degradation. Additionally, the position of substituents could impact an aromatic compound's reducing ability. A comparative study reported the changes in induction period between catechol, HQ, 1,2-aminophenol, and 1,4-aminophenol, at both 110 and 170 °C. The results suggest that the substitution of a phenolics hydroxyl groups with amines could, at around 110 °C, increase their oxidative stability, whether the two substituents are in the *ortho*- or *para*-position. This could be caused by oxygen and nitrogen's similar electronegativities, as well as the resonance structure of *ortho*- and *para*-substituted benzenes. At a higher temperature, the difference is unclear. In the case of dihydroxy functional groups, the ortho configuration is more stable than the para configuration, but in aminophenols, there is no significant difference between these positions[249] (Table 2.3). As for (+)-catechin, the introduction of a dimethylamino group to its B-ring enhances the compound's antioxidant activity by decreasing the 3'- and 4'-OH bond dissociation energies. Therefore, the modified catechin has a stronger electron-donating ability than its precursor[175].

Table 2.3 Induction time of heated di-substituted benzenes[249].

Compound	**Induction time**	
	110 °C (days)	170 °C (h)
1,2-Dihydroxybenzene (Catechol)	1.5	0.5
1,4-Dihydroxybenzene (HQ)	1	0.25
1,2-Aminophenol	2.5	0.5
1,4-Aminophenol	2.5	0.5

2.6 Potential regeneration of (+)-catechin's antioxidant activity

The regeneration of antioxidants has been studied in the past. Four phenolic antioxidants, including (+)-catechin, converted to radicals by one electron oxidation, were found to be regenerated to different extents by other antioxidants, and most efficiently by all-rac-α-

tocopherol[250]. It has also been found that although the presence of thiols such as glutathione can accelerate the oxidation of EGCG in emulsions, the adducts formed "[do] not appear to compromise the oxidative stability of food lipids"[236]. Additionally, sources propose chemical mechanisms in which the hydroxyl moiety is regenerated when nucleophile is added to *o*- or *p*-quinone structures via Michael addition[215]. Some reports propose that the mono-substituted *o*-benzoquinone produced by 1,4-addition of glycine to HQ can undergo condensation with another glycine molecule and oxidatively deaminate the latter before the regeneration the monosubstituted HQ[214]. If the added group does not inhibit antioxidant activity, the hydroxyl group could potentially participate in antioxidant activities again. In the case of the direct addition of an amino acid, it has been shown that amino acids can react with flavonoids' B-ring to form Schiff bases[176]. Phenolic compounds including catechol, HQ, and p-BQ, produced phenylacetaldehyde when heated in the presence of phenylalanine at acidic pH[221], indicating the occurrence of Strecker degradation.

2.7 Review of analytical methods

2.7.1 Structural identification of phenolic compounds and their amino acid adducts

The detection of products generated during the reaction between amino acids and quinones is generally determined by mass spectrometric methods. Adducts formed between 4-MBQ and amino acids have been successfully using ultra-performance liquid chromatography with a mobile phase gradient composed of 0.1 % ammonium formate and methanol, followed by electrospray ionization (ESI) in the positive mode on a quadrupole time-of-flight tandem mass spectrometer (MS/MS)[251]. Other mobile phase gradients involving 0.1 % formic acid in water and acetonitrile have been used with a similar liquid chromatography coupled to ESI-MS/MS have been used to identify phenylalanine/gallic acid adducts[252]. Rosmarinic acid-derived adducts and dimers formed by its reaction with lysine or cysteine have also been identified using flow injection and omitting chromatographic separation, also followed by ESI-MS/MS, although the negative ionization mode was used in this case[253]. MS/MS provides an accurate mass for the detected ions as well as fragmentation products which can help elucidate both the molecular formula and the structure of adducts. In untargeted analyses, an isotopic labelling technique is useful in discriminating between mass spectrometric peaks formed by adducts and other products, natural isotopes, or sample contaminants[254].

Cysteinyl-catechin adducts can also be detected and their structure determined by combining results of ^{1}H and ^{13}C nuclear magnetic resonance spectrometry by analyzing the chemical shifts of a magnetic field[255]. A similar approach has also been used for the analysis of collagen/(±)-catechin interactions[256] and to identify caffeic acid quinone/*o*-phenylenediamine adducts[216]. The chemical shifts are caused by the electron shielding of an atom's nucleus, which is affected by the other atoms it is bonded to through coupling effects[257].

Fourier-transform infrared spectrometry (FTIR) can also prove useful in the identification of functional groups as was the case for a catechol/arginine derivative, although the full structural elucidation of adducts requires complementary analysis[258]. FTIR has been used to study the effect of phloretin on human serum albumin's secondary structure, providing information on their complex formation[259]. Antioxidants can be quantified in edible plant material and oils by FTIR, based on their structural characteristics[76]. The FTIR absorption regions for many of (+)-catechin's substituents are known[193] (Appendix 7.3). Therefore, structural changes associated with the oxidation of flavonoids could potentially be detected and monitored by FTIR. However, because the oxidation of flavanols entails various cross-linked and carbonyl structures, large sample sets and extensive data processing would be required to build a calibration model[260]. No previous studies monitoring the structural changes during the oxidation of (+)-catechin or other polyphenolic antioxidants using FTIR have been found. However, related methods for the measurement of antioxidant activity in wine by FTIR have been developed[260-262].

2.7.2 Study of oxidative processes and reactions of phenolic compounds via their absorbance of UV and visible light

UV-visible spectrometry is commonly used to obtain preliminary information on flavonol oxidation and their reaction products, due to the UV absorbance of the A-ring around 250-285 nm and the B-ring around 320-385 nm[263], as well as the visible absorbance of pigments formed through oxidation and rearrangements. As such, two absorption bands can be detected when quercetin is analyzed by UV-vis spectrometry, due to its B-ring at λ=369 nm its A-ring at λ=258 nm, caused by $\pi \rightarrow \pi^*$ transitions within the structures[183]. Visible spectrometry is useful not only to study the progress of reaction but also to determine its effect of color, which affects the quality perception of foods.

Solutions of (+)-catechin display an evolving UV-vis spectrum over time when incubated, and this process is accelerated in the presence of Al_2O_3 or a high pH. After approximately 50 days, two peaks appear at 375 and 410 nm whereas the bands initially at 258 and 369 nm become less resolved as a consequence of (+)-catechin's oxidation[193]. Therefore, oxidation of flavonoids, namely (+)-catechin and quercetin, can be monitored by observing the changes in UV-visible spectra. One of the limitations of this methods is the absorbance of different compounds at very similar wavelengths[264], due to their common chromophores. High-performance liquid chromatography is often coupled to a diode array detector to separate compounds before UV-visible absorbance measurements are taken; however, this method cannot scan a range of wavelengths and only provides data for single wavelengths[265].

2.7.3 Analysis of antioxidant activity and reactions of electrochemically active compounds by cyclic voltammetry

Electron-transfer reactions are involved in both reduction and oxidation of flavonoids, making electrochemical methods useful to study their behaviour[266]. One of these methods is cyclic voltammetry (CV), which consists in applying a varying potential to a solution containing a dissolved analyte and measuring the resulting current caused by its redox reactions. This solution must contain a suitable inert electrolyte to minimize the solution's resistance, which could otherwise block the signal[267].

Equation 2.1 Randles-Sevcik equation[266].

$$i_p = 0.446nFAC^0\left(\frac{nF\upsilon D_o}{RT}\right)^{1/2}$$

Where i_p=peak current, n=number of electrons transferred, F=Faraday's constant, A=electrode surface area, C^0= bulk concentration of analyte, υ=potential scan rate, D_o=diffusion coefficient of the oxidized analyte, R=universal gas constant and T=temperature.

The resulting voltammogram reflects the current intensity of the solution against the scanned potential[266]. As the analyte is oxidized at the working electrode, it generates a current following the Randles-Sevcik equation (Equation 2.1). In accordance with this equation, scan rates affect the peak current and must therefore be optimally chosen[268]. Compounds oxidized at the working electrode slow the migration of non-oxidized compounds and therefore reduce the

current; this can be an issue when the electrode is not properly cleaned or when the analyte adsorbs strongly to its surface[177].

CV has long been used to study HQ and *p*-BQ[269-270]. HQ's oxidation mechanism in aqueous systems involves proton-coupled electron transfer[271]. Its redox potential varies with reaction conditions such as pH[272], the working electrode used due to surface adsorption[267], the solvent used [273], etc. These parameters are therefore crucial to the experiment's success and must remain identical to allow reproducibility. For example, in 0.1 M phosphate buffer at pH=7.0 using an Ag/Ag^+ reference electrode, HQ's oxidation occurs around 0.42 V[274]. However, in 0.1 M phosphate buffered saline solution with added KCl at pH=7 against a saturated calomel reference electrode, the oxidation potential of the same compound was approximately 0.1 V[275]. In 40:60 water/acetonitrile with 0.15 M phosphate buffer at pH=7.4 and 25 °C against a saturated calomel electrode, the oxidation peak is around 0.5 V[272]. The previous experiments involved a glassy carbon electrode (GCE) as a working electrode. Factors such as temperature[271], electrolyte, solvent and different reference electrodes[267] may explain these differences, making it difficult to compare results obtained from different studies. HQ is known to undergo reversible oxidation[274], as was the case in all three of the previous experiments. In acetonitrile containing 1.78 M water and 0.1 M tetrabutylammonium hexafluorophosphate as an electrolyte, using a GCE working electrode and platinum electrode as a reference, HQ exhibits an oxidation potential at approximately 0.6 V and is electrochemically irreversible[276], in contrast with the previously mentioned studies.

CV has also shown that *p*-BQ and HQ undergo a one-step two-electron, two-proton reversible redox process in aqueous systems but *p*-BQ rather undergoes two consecutive one-electron reduction steps in non-aqueous systems, which can be observed as two separate cathodic waves in a voltammogram. Under this condition, the first electron transfer is electrochemically reversible whereas the second is quasi-reversible at regular scan rates[273]. This may be explained by the varying level of stability of the semi-quinone in different solvents.

(+)-Catechin, like HQ, can be studied by CV due to its ability to undergo redox reactions. It produces a first pH-dependant reversible peak due to the oxidation of its B-ring hydroxyl groups and a second irreversible peak at higher potentials possibly due to oxidation of less easily oxidized hydroxyl groups, such as the A-ring, or the oxidation of new products formed after the initial oxidation[277]. At higher pH, (+)-catechin is more easily oxidized, as inferred by its lower oxidation

potential, and therefore has greater antioxidant activity[177]. In non-aqueous media, (+)-catechin produces two oxidation peaks but no reduction peaks, pointing to electrochemical irreversibility[278].

In the last decades, methods to assess antioxidant activity using CV have been developed to study human plasma, animal tissue and edible plants[279]. The method is quick and inexpensive while allowing antioxidant activity measurements under a variety of conditions[280]. The oxidation potential indicates a compound's tendency to donate an electron, which is related to its antioxidant activity. Therefore, "the lower the redox potential, the higher is the antioxidant capacity"[281]. The anodic peak potential has been inversely correlated to the FRAP values[282], DPPH radical scavenging activity[280], and total reducing capacity (Folin-Ciocalteu value)[283] of many antioxidants, including flavonoids and wine polyphenols.

Reactions of electrochemically active compounds can be studied by CV; these form voltametric curves typical of a reversible electrochemical step followed by an irreversible homogenous chemical step[266]. By exposing the analyte to a range of concentrations of another reactant and varying reaction conditions, the decrease in the initial anodic and/or cathodic peak as well as arising peaks can provide clues related to the extent of reaction, favoring conditions as well as the reaction mechanism[251, 284].

For example, the CV of 4-MC shows that the addition of CML does not affect the intensity or oxidation potential of the anodic peak, as 4-MC has a low reactivity. However, the 4-MBQ formed may react with CML and therefore the decreased 4-MBQ concentration at the electrode decreases the intensity of the cathodic peak accordingly, and the newly formed adduct produces a second cathodic peak[284]. The reactivity of CML with various o-quinones including catechol is most efficient at neutral and basic pH due to the deprotonation of CML's amino group, thus maintaining its nucleophilicity. Therefore, at pH = 5.0, CML has no influence on *o*-quinones' cathodic peak's intensity[284]. Similar CV experiments showed that the reactivity of *o*-quinones with nucleophiles such as amino acids increased for *o*-quinones with electron-withdrawing groups and decreased for electron-donating groups or sterically hindered *o*-quinones[251]. CV has also shown that gallic acid, caffeic acid and especially (+)-catechin react with nucleophiles, including phenylalanine and methionine, in wine model systems and may act as antioxidants by capturing quinones[285].

2.7.4 Measurement of antioxidant activity by colorimetric assays

The antioxidant activity of compounds is traditionally determined by colorimetric assays. These include tests based the analyte's DPPH, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+), or oxygen radical scavenging activity, as well as its FRAP, and more. Although the mentioned tests are meant to evaluate the total antioxidant capacity of a sample, the determined antioxidant activity depends on the test used, even between those that evaluate the same antioxidant mechanism[286]. The most common colorimetric assays are compared in Table 2.4.

Table 2.4 Comparison between some common spectrometric antioxidant activity assays.

Assay	Basis	Pros	Cons
DPPH radical scavenging[286-287]	Electron transfer, change in A_{517} (or A_{580}) due to DPPH·-quenching	Simple, accessible, similar mechanism to peroxyl radicals, highly reproducible, stable radical	Carotenoids absorb at 517 nm, must be modified to solubilize in water
TEAC[286-288]	Electron transfer, change in $A_{730\text{-}734}$ due to ABTS•+ quenching	Suitable for analysis in water and organic solvents, suitable over a wide pH range, simple, inexpensive	Must be combined with FTIR to increase sensitivity, may be underestimated if analytes react slowly, may be irrelevant to biological systems
ORAC[286-287, 289]	Hydrogen atom transfer, change in fluorescence curve area of E_{514} vs time due to fluorescence probe affected by OH· quenching	Most relevant in biological systems, suitable in aqueous systems	Pyrogallols may generate reactive intermediates that degrade the fluorescence probe, low reproducibility
FRAP[287, 290]	Single electron transfer, change in A_{593} of iron salt according to iron's oxidation state	Thiols and amino acids cause minimal interference, simple, quick, inexpensive, sensitive, can be modified to analyse antioxidants with different polarities	Value may be slightly affected by iron chelation, precipitation/suspension of the indicator, low correlation with radical quenching

Where DPPH, 2,2-diphenyl-1-picrylhydrazyl; TEAC, Trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; A, absorbance at the wavelength(s), in nm, noted in subscript; ABTS•+, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); and E, emission at the wavelength, in nm, noted in subscript.

Multiple reviews of the different analytical methods used for the determination of antioxidant activity are available[287, 289, 291]. Care must be taken to avoid interference from compounds that absorb or emit light at the studied wavelength[287].

2.7.5 Determination of antioxidant activity using food-relevant substrates

Antioxidant activity can be measured indirectly through the evaluation of an analyte's effect on the degradation of oxidation-prone substances. DNA strands, RNA strands and lipids are often used as oxidizable biological substrates[289].

The oxidation of lipids can be assessed using several methods. Traditionally, primary oxidation products are measured by the iodometric titration of hydrogen peroxide to determine the PV, and secondary oxidation products are measured by the colorimetric *p*-anisidine assay to determine the anisidine value (pAV)[292]. The PV of oil has been used as an indicator of antioxidant efficacy of phenolic acids in sunflower oil triglycerides[293]. However. hydroperoxides evaporate when heated and decompose as lipid autoxidation progresses, so the PV of late stage oxidized oils may be lower, giving a false sense of oxidative stability[294]. In this case, the pAV becomes a better indicator. This test has also been used to determine evaluate the antioxidant activity of polyphenols in oils. In sardine oil, catechin was associated with a 12 % decrease in pAV after 14 days of storage at 37 °C in the dark[295]. However, in avocado and coconut oils stored at 60 °C for 60 days, caffeic and *p*-coumaric acids both increased the pAV over the studied period, likely due to the reactivity of the oxidized phenolic acids' carbonyl groups with *p*-anisidine[296]. It is therefore unclear if phenolic compounds interfere in the measurement of pAV and if so, under which conditions this occurs. FTIR could be used to determine PV and pAV as an alternative to chemical indicators, by considering the absorbance peaks related to primary and secondary oxidation products, respectively[297-298]. Fatty acid secondary oxidation products, often volatile, may also be extracted by headspace solid-phase microextraction and analyzed by gas chromatography coupled to mass spectrometry, dismissing the need for *p*-anisidine altogether[299].

Finally, oxygen consumption tests have long been used to study oxidation, mainly in oil-based samples. The Rancimat test uses a specific instrument to heat and stream air through samples to cause accelerated oxidation and products are measured electrochemically[300]. New instruments, such as the RapidOxy 100, allow similar analyses using oxygen pressure rather than a gas stream to measure induction period[301]. Because antioxidants slow oxygen consumption rates and therefore

increase induction time[302], oxygen consumption data can be used to gather information pertaining to antioxidant activity. Using a combination of light heat (37 °C) and oxygen pressure to accelerate oxidation, α-tocopherol has been found to greatly prolong the induction time of methyl arachidonate using oxygen consumption measurements[303]. Capsaicinoid from chili pepper has also been shown to be more effective in protecting canola oil at 60 °C than BHT by a different method based on oxygen consumption[302]. Overall, oxygen consumption tests could be a facile method in the analysis of antioxidant activity, as it can be applied to complex samples which could otherwise interfere in common assays.

2.8 Conclusion

Overall, natural antioxidants have shown promising potential in the prevention of quality degradation of food components with both advantages and disadvantages, compared to their synthetic substitutes. Oxidised polyphenolic compounds with a quinonoid structure are known to react with nucleophilic compounds through a Michael addition mechanism and have been reported to form Schiff bases in some studies, although the latter reaction has not been studied as extensively as the former. The reported reactions suggest the regeneration of the molecule's antioxidant activity, as highlighted by some researchers; however, this hypothesis has never been studied, to our knowledge. One of the possible challenges to such an experiment may be the complexity of the interaction, as both the Michael addition reaction and the Schiff base formation are theoretically favored by similar conditions, and other side reactions could take place. Additionally, typical methods for the determination of antioxidant activity have not been evaluated for this application, and interference could cause issues. Therefore, the use of model compounds in lieu of (+)-catechin, as well as the assessment of methods used for antioxidant activity measurements are recommended.

CHAPTER 3. MATERIALS AND METHODS

3.1 Chemicals

Hydroquinone (HQ) (≥99 %), 4-methylcatechol (4-MC) (≥95 %), L-ascorbic acid (≥99 %), flaxseed oil (FSO), *p*-Anisidine (99 %), methanol (≥99.6 %, reagent grade), p-benzoquinone (p-BQ) (≥98 %), anhydrous isooctane (≥99.8 %), potassium chloride (KCl) (reagent grade), potassium hydroxide (KOH) (≥85 %), (+)-catechin hydrate (~98 %) and glycine (≥99 %) were purchased from Sigma-Aldrich (St-Louis, MO, U.S.A.) and its subsidiaries. Glacial acetic acid (≥97 %) was acquired from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Anhydrous sodium sulfate and mono- and di- (99.9 %) basic potassium phosphate were purchased from Fisher Scientific (Hampton, NH, U.S.A) and Thermo Fisher scientific (Waltham, MA, U.S.A), respectively. [^{15}N]-glycine (98 %) was produced by Cambridge Isotope Laboratories, Inc. (St-Laurent, QC, Canada) and was kept at -18 °C during storage. HQ, FSO and *p*-anisidine were refrigerated during storage. A 2020 vintage Beaujolais red wine containing 13 % alcohol per volume (Georges DuBoeuf, Romanèche-Thorins, France) was purchased from a local liquor store and kept sealed until analyzed.

3.2 Analytical methods

3.2.1 HRMS analysis, isotopic labelling studies, and MS/MS of a hydroquinone-glycine model reaction

Solutions containing 0.04 M of HQ and 0.06 M of glycine were prepared using 1.2 mL of 5:1 water/methanol as a solvent, then either heated in Teflon-lined stainless-steel reactors in an oven at 120 °C for 2 hours or left at room temperature in capped glass vials for 24 hours. Samples were then stored at -18 °C until analysis. Prior to analysis, samples were diluted in water and methanol.

The samples were analyzed after syringe infusion into a quadrupole time-of-flight high-resolution mass spectrometer (HRMS) (MaXis Impact, Bruker Daltonics, Germany) operated using electrospray ionization in the negative mode. The ESI source was operated under a nebulizer pressure of 0.6 bar, a drying gas flow rate of 4.0 L/min, a dry heater temperature of 180 °C, and a capillary voltage of 4500 V. Ions were scanned within the mass-to-charge (m/z) ratio range of 50-

500. Preliminary analyses of various samples produced under a range of heat treatments ionized in the positive mode did not reveal ions of interest for this study with sufficiently low errors.

Data was analyzed using the Bruker Compass DataAnalysis 4.2 software (Bruker Daltonics, Germany) and the ChemCalc web-based software (ChemCalc) to determine products' formulae based on accurate mass[304]. Isotopic labelling studies were performed by replacing glycine with an equal concentration of [^{15}N]-glycine in samples subjected to identical treatments with the purpose of confirming the molecular formulae obtained by mass spectral analysis. All sample analyses were performed in duplicates from independent trials. Molecular formulae of adducts of interest were determined based on their low associated errors (below 10 ppm), the correspondence of their number of nitrogen atoms with peak m/z shifts observed in their isotope-labelled counterparts, and their high relative peak intensities compared to the spectral noise.

MS/MS was used to determine the structure of the ions of interest previously identified by HRMS. Fragmentation was achieved by collision-induced dissociation (CID) under collision energies of 5.0 to 15.0 eV. Other MS/MS parameters were identical to those used during the HRMS analyses.

3.2.2 Toxicity prediction of hydroquinone/glycine adducts

The SMILES notation of the three proposed adducts detected by MS/MS were submitted to a toxicity prediction analysis using the Lazar online software (*In silico* toxicology GmbH, Switzerland). All evaluation parameters were selected, which included predictions for acute toxicity, blood-brain barrier penetration, carcinogenicity, lowest observed adverse effect level, maximum recommended daily dose, and mutagenicity.

3.2.3 Cyclic voltametric analysis of different phenol/glycine mixtures

Two stock solutions of phosphate buffers were prepared using K_2HPO_4 and KH_2PO_4 in ultrapure water with concentrations of 0.1 M and 0.2 M of phosphate. The pH was adjusted by adding either HCl or aqueous KOH until a pH of 7 was reached. For the analysis of unheated samples, 10 mM samples were prepared by dissolving 4-MC, HQ or (+)-catechin hydrate with or without 15mM of glycine in the 0.1M phosphate buffer. These solutions were prepared immediately before analysis to limit reaction time before the CV scan. 15 mM glycine and analyte-free blank solutions were also produced in the same manner.

For heated mixtures, 5 mL samples of 20 mM 4-MC or HQ with 30 mM glycine using a 5:1 H_2O/methanol solvent were prepared in 12 mL glass vials with airtight lids. The samples were heated in a sand bath at 110 °C for 2 hours with the lid on, after which the lid was removed to allow evaporation until less than 1 mL of solvent remained. The volume was then completed to 5 mL using ultrapure water and 5 mL of 0.2M phosphate buffer was added, to render final concentrations of 10 mM of the phenols, 15 mM of glycine and 0.1 M of phosphate buffer.

KCl was added to all samples as an electrolyte to a concentration of 0.1 M. Samples were degassed using a stream of nitrogen gas for 10 minutes prior to analysis. A 3 mm diameter glassy carbon disk working electrode (GCE) was sonicated in ultrapure water, wiped with acetone, and scanned in a blank solution in the same potential range as the sample to prepare the surface. Voltammograms were recorded using a cyclic voltammetry analytical device and potentiostat (ElectraSyn 2.0, IKA Works, Inc., USA) using a platinum plated counter electrode and a 3 M KCl Ag/Ag^+ wire reference electrode. The potential scan rate was 50 mV/s.

3.2.4 Oxygen consumption measurements

To study the effect of added glycine on the oxygen consumption of red wine, 2.5 g of the Beaujolais red wine was diluted two-fold with an equal mass of water and analyzed in duplicate by a RapidOxy 100 (Anton Paar, Austria). Corresponding samples with a mass fraction of 1 % added glycine were also tested in duplicates. The instrument parameters were set at 700 kPa of 99 % oxygen and 80 °C, and tests were run for 17.5 hours. The instrument is sensitive to pressure variations of 1 kPa and over.

The same instrument was used to evaluate the effect of an added 4-MC/glycine mixture on FSO's stability. Samples were prepared using pure FSO as a control, or with 0.1 % 4-MC and/or 0.2 % glycine added to FSO. 4 g of each sample was analyzed under an initial oxygen pressure of 700 kPa at a temperature of 120 °C. Analyses were performed until a 60 % pressure drop was achieved and the time was noted. One batch of samples was analyzed with stirring at 100 rpm and the other, without stirring.

3.2.5 *p*-Anisidine value of oxidized flaxseed oil with added hydroquinone and glycine

Samples of FSO were placed in glass vials and left at room temperature (~23 °C) or heated in a sand bath at 70 °C. A 5 mL aliquot was sampled after 24 or 48 hours, capped with an airtight

lid, and stored at -18 °C in the dark until analyzed. Samples of 6 mM HQ or 6 mM HQ/12 mM glycine were also heated at 70 °C for 24 hours.

The pAV of the FSO samples was determined according to ISO 6885:2006(E)[305]. Samples were dried with anhydrous sodium sulfate and vacuum filtered. The mass of FSO samples used for the test varied between approximately 0.5 and 2.5 g, depending on the sample's extent of oxidation, to produce reacted test solutions within the necessary absorbance range. Absorbance was measured at 350 nm using an Evolution 300 UV-visible spectrophotometer (Thermo Scientific, USA) and data was processed using the VISION Pro software (Thermo Scientific, USA).

3.2.6 Extent of reaction between *p*-anisidine and the selected additives

The ISO 6885:2006 method was followed, substituting the oil test sample for 3 mL of 6 mM HQ, p-BQ, or 12 mM glycine in glacial acetic acid rather than isooctane, due to solubility issues. The same solvent served as a blank.

Samples containing a combination of 6 mM HQ and 12 mM glycine in 5 mL of 3:2 H_2O/methanol were placed in a sealed 25 mL glass vial in a sand bath at 70 °C for 24 hours. They were then uncapped to allow complete evaporation and immediately dissolved in 5 mL of acetic acid and analyzed as described above. All samples were prepared in duplicate and analyzed using an Evolution 300 UV-visible spectrophotometer (Thermo Scientific, USA) with data processed using the VISION Pro software (Thermo Scientific, USA).

3.2.7 Color evolution of (+)-catechin/glycine models under alkaline conditions

Samples were prepared using 30 mM of (+)-catechin hydrate in 2 mL of 4:1 methanol/H_2O in 12 mL capped glass vials and left at room temperature (~23 °C) for 3 days. 0.2 mL of 45 mM KOH in methanol was then added to each sample and left to further react at room temperature. Additional samples were prepared with 60 mM of added glycine or 30 mM of added ascorbic acid. Controls were also prepared by subjecting 30 mM ascorbic acid without (+)-catechin to the same treatment. (+)-Catechin samples were also analyzed prior to any time or alkalizing treatment to study its original properties.

Absorbance spectra of all samples were acquired 24 and 28 hours after the KOH addition using an Evolution 300 UV-visible spectrophotometer (Thermo Scientific, USA) between emission wavelengths of 350 and 850 nm. Measurements were corrected using blank solutions of

the solvent. Measurements were taken from samples in a quartz cuvette with a 1 cm pathlength. Data was processed and manipulated using the VISION Pro software (Thermo Scientific, USA). Absorbance curves were smoothed, and peak maxima were identified using a 2nd order Savitsky-Golay 2nd derivative at 31 points. Peak areas were determined using the software's function by manually picking the peaks' start and end points. All samples were prepared in duplicate.

3.3 Statistical treatment of data

Sample sets of duplicates were compared using a two-tailed T-test for unpaired samples with a confidence interval of 95%.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Identification of (+)-catechin model reaction products with glycine

The hypothesis based on the regeneration of flavonoids' antioxidant activity via reactions with amino acids relies on the formation of products derived from Schiff base formation, Strecker degradation and Michael addition reactions. Previous studies have demonstrated that simple flavonoids' B-ring and gallate moiety (in galloylated catechins) undergo oxidation which creates an unstable and reactive electrophile[306]. Flavan-3-ol units, which do not contain a gallate moiety, undergo reactions involving their B-ring with thiols and amines in nucleophilic substitution reactions[176, 226, 307]. Although the B-ring has an *ortho*-dihydroxybenzyl (catechol-type) moiety, HQ (*para*-dihydroxybenzene) was used as a model reactant. Catechol and HQ are isomers and thus have slightly different reactivities but participate in similar reactions with the same nucleophiles[308]. Therefore, the reaction between HQ and glycine was studied as a model for flavonoid/amino acid interactions. The reaction intermediates and products formed will be discussed in this section.

4.1.1 Chemical composition of reaction products detected by HRMS

The HQ/glycine (2:3) model systems were exposed to a temperature of either 22 °C (room temperature) for 24 hours or 120 °C for 2 hours. These conditions were selected based on preliminary analyses involving a range of time and temperature conditions as well as their similarity to storage and cooking conditions, respectively. Analysis of the samples indicated the formation of compounds with greater molecular masses than either of the reactants', demonstrating the occurrence of reactions such as condensation, addition, and/or polymerization. The study of these reactions using isotope-labelled glycine confirmed the incorporation of nitrogen atom(s) in some of the ions detected. The analysis of HQ/glycine and HQ/[^{15}N]-glycine reaction mixtures confirmed the molecular formula of glycine-quinone adducts of interest (Table 4.1). Compounds were considered potential adducts of interest based on their number of carbon, oxygen, and nitrogen atoms. The peak shift associated with glycine's nitrogen isotopes confirmed the interaction between the two reactants as well as the number of nitrogen atoms integrated in the adduct, and therefore the stoichiometric proportion of glycine that reacted with HQ.

Table 4.1 Ions of interest detected by HRMS in the negative ionization mode.

Structure	Elemental composition	Error (ppm)*	Hydroquinone/glycine			Hydroquinone/[^{15}N]-glycine		
			[M-H]-	Relative Intensity (%)		[M-H]-	Relative Intensity (%)	
				22 °C, 24 h	120 °C, 2 h		22 °C, 24 h	120 °C, 2 h
Adduct 1	$C_7H_9NO_2$	1.42	138.0557	3.4	3.8	139.0559	2.1	3.2
Adduct 2	$C_8H_{10}N_2O_2$	1.80	165.0667	100	100	167.0632	100	100
Adduct 3	$C_9H_{10}N_2O_4$	0.80	209.0564	48.3	55.8	211.0506	32.4	44.1

*Error in the calculation of the adduct's elemental composition based on the unlabeled model.

Both adducts 2 and 3 comprised two nitrogen atoms, whereas adduct 1 had a single nitrogen atom. Adducts 2 and 3 were detected at high relative intensities in samples that were heated at 120 °C and left at room temperature. This suggests that the reaction can proceed under a variety of time and temperature conditions representative of the conditions to which foods may be exposed in aqueous media.

4.1.2 Tandem mass spectrometric analysis

The ions observed at m/z 165 and 209 in the HQ/glycine sample heated at 120 °C for 2 hours were subjected to MS/MS analysis under the negative ionization mode to elucidate their structures. The fragmentation peaks produced are detailed in Table 4.2.

Table 4.2 MS/MS fragments produced by collision-induced dissociation of Adduct 3.

Fragment [M-H]-	Elemental composition	Error (ppm)	Relative intensity (%)	
			8.0 eV	15.0 eV
HQ/glycine (m/z 209)				
123.0317	$C_6H_5NO_2$	-2.67	ND	13.3
135.0308	$C_7H_5NO_2$	-9.10	ND	7.2
138.0548	$C_7H_8NO_2$	-5.10	<5	30.3
165.0654	$C_8H_9N_2O_2$	-6.07	100	100
HQ/[^{15}N]-glycine (m/z 211)				
166.0665	$C_8H_9{}^{15}NNO_2$	18.44	7.2	NS
167.0630	$C_8H_9{}^{15}N_2O_2$	15.13	100	NS

*Error in the calculation of the ion's elemental composition. ND, not detected; NS, sample not submitted for MS/MS analysis at the given collision energy and data was therefore not collected.

The adduct detected at m/z 165 was not fragmented by CID at a low collision energy (5.0 eV) and must therefore have a relatively stable structure that prevents its breakdown under the applied conditions. The adduct observed at m/z 209 produced fragments with m/z 165 and 138 at a collision energy of 8.0 eV. At a higher collision energy (15.0 eV), additional fragments were found at m/z 135 and 123 and the parent ion at m/z 209 was completely fragmented. These smaller fragments are likely radical fragment ions, which are commonly produced by organic 1,3-dipolar and aromatic compounds[309]. The elemental composition of the fragment at m/z 165 produced from Adduct 3 corresponds to Adduct 2. Similarly, the elemental composition of the fragment at m/z 138 from Adduct 3 corresponds to Adduct 1. The presence of these fragments indicates that adducts 1 and 2 have structures similar to Adduct 3, which may either be a precursor to their formation, or be produced via a similar reaction. Based on the structures of the reactants and the molecular formulae of the ions, the fragmentation pattern of the 209 m/z ion is proposed below (Figure 4.1).

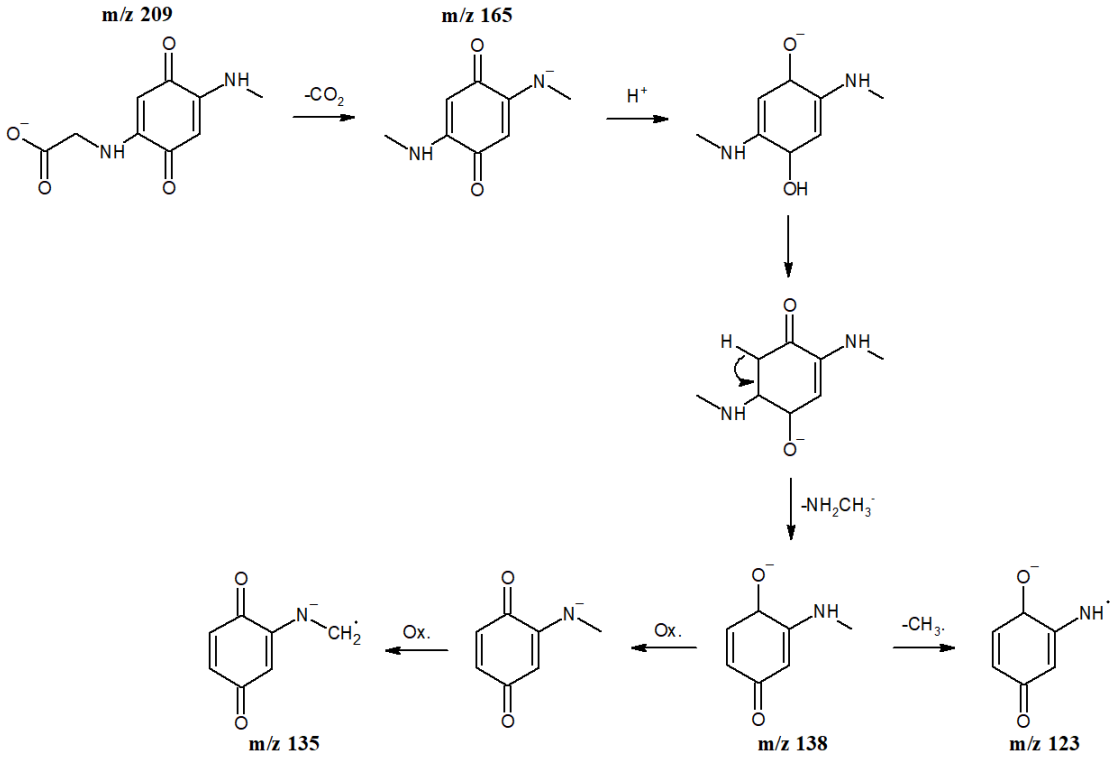


Figure 4.1 Proposed MS/MS fragmentation patterns of m/z 209 at 15.0 eV.

The proposed structures of adducts 1-3 all correspond to Michael addition products, which is the main identified reaction of amino and thiol compounds with other dicarbonyls and quinones

found in the literature, such as (+)-catechin[176] and *p*-BQ[310], or between HQ and alkylamines[311]. The targeted adducts are the result of reactions occurring in a 2:1 and 1:1 glycine/HQ molecular ratio. Adduct 1 was detected at much lower relative intensities than Adducts 2 and 3. It is possible that the oxidized glycino-hydroquinone adduct is highly electrophilic, thus favoring a further addition reaction. This would render the initial 1:1 glycino-hydroquinone adduct's detection at a lower intensity with the chosen detection method. This initial adduct may also be derived from the breakdown of diglycino-hydroquinone adducts, although such mechanism was not deemed favorable. Kinetic studies would be necessary to further elucidate the mechanism involved in the generation of adduct 1. Although the addition of glycine to adjacent carbon positions is possible, it is unlikely due to steric hindrance caused by the initial glycyl group, which would also explain why triple and quadruple additions of glycine were not observed. The proposed structures are in accordance with recent literature[312].

None of the ions detected in the negative ionization mode at high relative intensities indicated the formation of Schiff bases or their rearrangement and degradation products. The condensation products are influenced by environmental pH[313] and favored by low water activity[314], which may not have been optimal for this type of reaction in the present study. The formation of Schiff bases between quinones and glycine has been reported in cases where the aqueous solvent was left to evaporate, thus decreasing the water activity[176]. Although Maillard-type reactions and Michael additions have been extensively studied, they are complex and rarely undergo comparative studies to determine which conditions favor one reaction pathway over the other although they involve similar reactants.

Finally, the presented model and its analysis method has demonstrated that the Michael addition of glycine to HQ occurs at storage temperature and during heating over a relatively short period. The combination of high-resolution mass spectrometry, isotope-labelling and tandem mass spectrometry proved suitable for the structural elucidation of the products generated. Future studies should focus on the extent of the reaction and altering parameters such as the pH, solvent, duration of incubation, and the type of amino acid and phenolic compound to assess how these affect results.

4.1.3 Proposed reaction mechanism and impact on redox activity

The oxidation of HQ is catalyzed by oxygen as well as its own oxidation product, following an autoxidation chain reaction[315]. This allows the compound to transfer hydrogen atoms and

contributes to its antioxidant activity[271]. The oxidation of HQ converts the compound to its p-BQ form and reduces the oxidizer. In the presence of glycine, adducts are readily formed with p-BQ under both mild heating and ambient temperatures. The addition of each glycine moiety to the aromatic ring restores a hydroxyl group, allowing the structure to tautomerize and restore the second hydroxyl group. The resulting *p*-dihydroxy structure is subject to an additional oxidation step. Once oxidized, the compound could react once again with glycine in a similar step-wise process to the first glycine addition. The diglycino-hydroquinone adduct subsequently formed and the glycino-hydroquinone adduct initially formed can then be decarboxylated before being further oxidized and donating two additional hydrogen atoms, conferring additional antioxidant activity to the intermediate adduct compared to unreacted HQ. The decarboxylation of glycino-hydroquinone results in the structure previously identified as Adduct 1 whereas the decarboxylation and oxidation of the diglycino-hydroquinone adduct results in the production of Adducts 2 and 3. The proposed reaction sequence is illustrated in Figure 4.2.

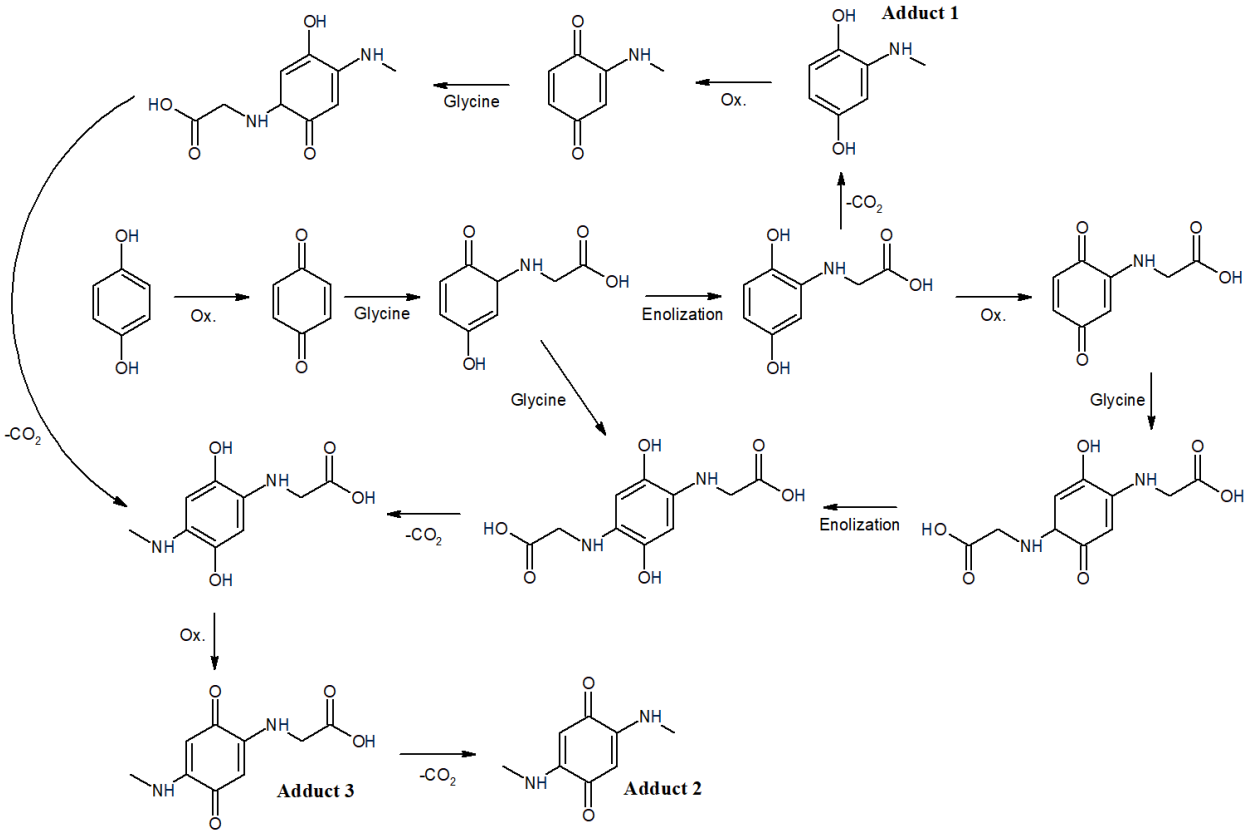


Figure 4.2 Proposed mechanism of the reaction of glycine and hydroquinone.

The presented mechanism implies that in the presence of glycine, each HQ molecule can undergo one to two additional oxidation steps compared to unreacted HQ. These transferred hydrogen atoms could terminate autoxidation reactions by quenching free radical species, such as those involved in polyphenol and lipid autoxidation. By limiting the effects of oxidation in food systems, this type of reaction could help extend the length of food products' high-quality shelf-life.

4.1.4 Toxicity prediction of the detected adducts

The toxicity approximation of the three adducts detected was attempted using an *in silico* toxicology prediction software (Lazar)[316]. The database used to evaluate toxicity parameters did not contain enough similar compounds to draw conclusions on the adducts' acute toxicity, carcinogenicity, mutagenicity, maximum recommended daily dose, and lowest observed adverse effect level in either animal or bacterial models or humans. However, the formation of related hydroquinone-thiol adducts has piqued interest in relation to their potential in benzene toxicity mediation[317].

4.1.5 Extrapolation of results to (+)-catechin and other polyphenols

Although the HQ/glycine model serves to understand the reactions involving quinones and simple amino acids, it may not directly translate to more complex flavonoids and polyphenols, which have a bulky group attached to the *o*-quinone ring of their oxidized counterparts, and in some cases (e.g., epigallocatechin), an additional hydroxyl group on their redox active benzene ring. This may decrease the likeliness of double Michael additions due steric hindrance or the substitution of the targeted carbon position(s). Phenolic structures of flavonoids are most often in an *ortho* or a *meta* rather than a *para* conformation, which can affect reaction kinetics, although *ortho* and *para* quinones exhibit very similar behaviours[274]. The type of amino acid can also impact the reaction due to the nature and size of their sidechains. Therefore, the previous results should be taken into consideration in the study of larger polyphenols but do not directly translate to more complex compounds. Further studies are necessary to confirm the formation and identify the structure of potential adducts formed between amino acids and polyphenols.

In the case of (+)-catechin, the Michael addition of glycine to the antioxidant could potentially yield an adduct in a 1:1 or 1:2 glycine/(+)-catechin molar ratio, depending on the carbon position(s) at which addition takes place. Michael additions of amines generally proceed as a 1,4-

addition: the conjugate addition product is much more stable than the direct addition product, of which the formation is reversible due to the nucleophile being a weak base[204]. In the case of dehydrocatechin (oxidized (+)-catechin) the 1' and 6' carbon positions would be considered as β-carbons. However, one of these β-carbons is covalently linked to (+)-catechin's C-ring and is unavailable. Studies have shown that epicatechin, an epimer of (+)-catechin, can react with CML to form adducts that incorporate one or two molecular equivalents of CML. Although their collected data does not elucidate the exact structure of the compounds, such as the carbon ring positions at which addition takes place, the authors suggest that addition takes place at the 2' and 5' positions of the C ring[212, 226]. This would rather suggest a 1,6-conjugate addition relative to each carbonyl, possible due to this system's α,β,γ,δ-unsaturated carbonyl structure[318]. Based on the previous results and existing literature, the reaction mechanism for the reaction between (+)-catechin and glycine is proposed for both 1,4- and 1,6-conjugate addition mechanisms (Figure 4.3).This mechanism involves three oxidation steps and liberates six hydrogen atoms, which could triple (+)-catechin's ability to terminate oxidation reactions. As the hydroxyl groups are regenerated, the adducts may even retain their metal chelation properties.

Similarly to HQ, the Michael addition of glycine and other amino acids has the potential to confer increased antioxidant activity to the compounds through the regeneration of the redox active B-ring. Again, up to four additional hydrogen atoms as compared to free (+)-catechin, for a total of six hydrogen atoms overall, could be transferred to free radicals, hereby quenching them. Therefore, (+)-catechin in the presence of excess glycine and under favorable conditions could have up to triple the antioxidant efficiency compared to an equal amount of unreacted (+)-catechin.

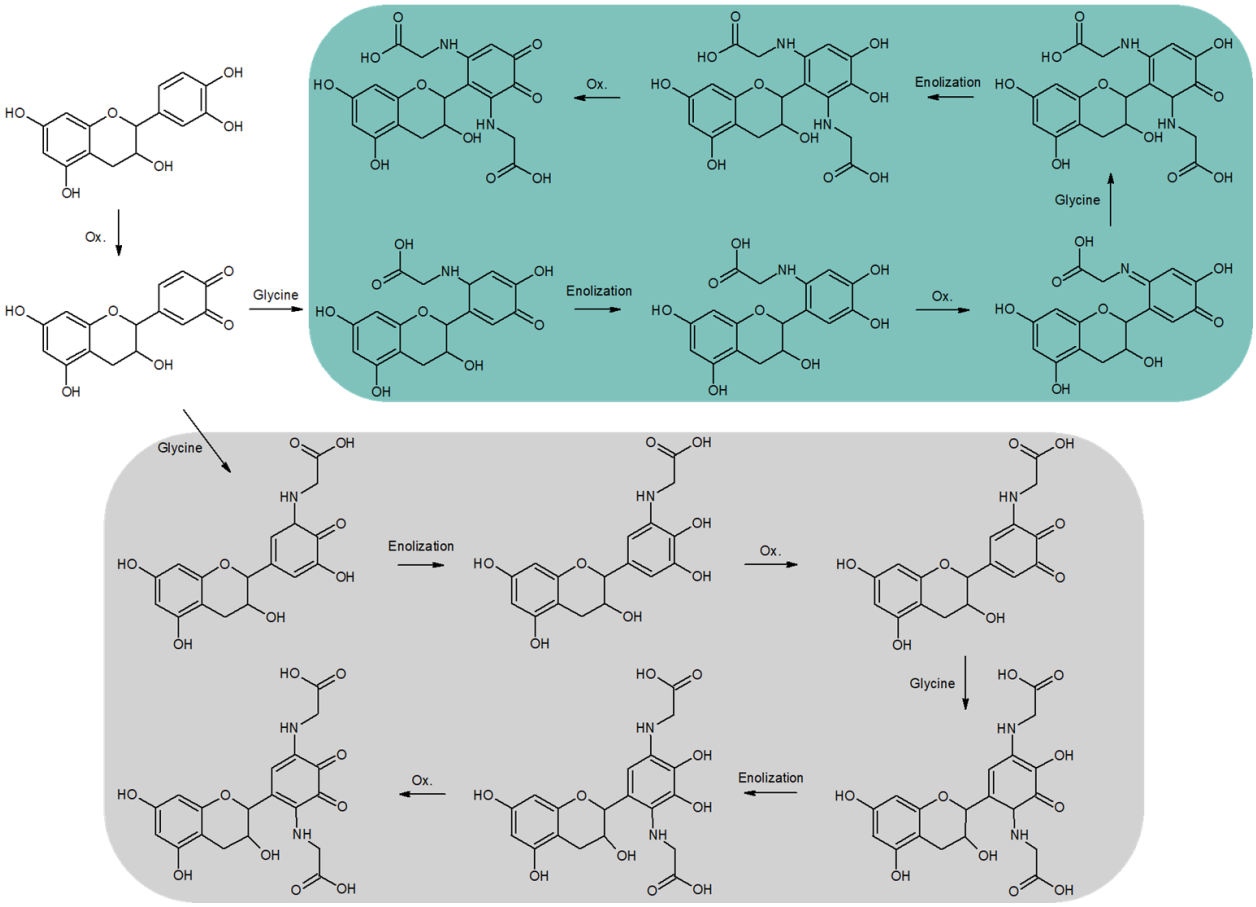


Figure 4.3 Proposed mechanism of the reaction of glycine and (+)-catechin.

In teal, 1,4-conjugate addition mechanism; in grey, 1,6-conjugate addition mechanism. Decarbolyxation reactions may also take place.

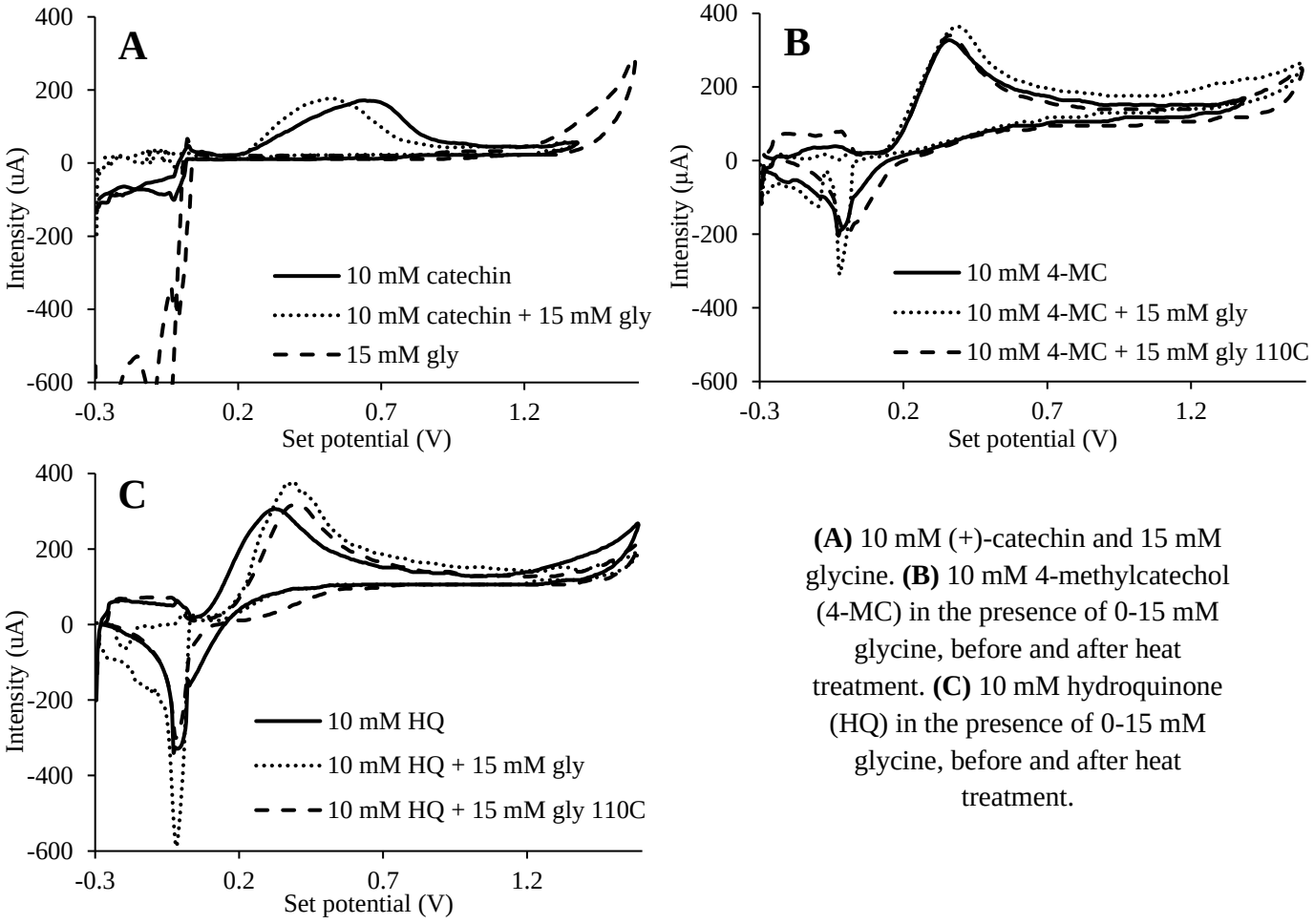
4.2 Antioxidant activity experiments

4.2.1 Cyclic voltammetry

The electrochemistry involved in the redox reactions of simple phenols, as models for more complex polyphenols, as well as reactions between these phenols and amino acids was studied using CV. The resulting cyclic voltammograms are shown in Figure 4.4.

All samples analyzed, including blank (not shown) and glycine solutions which should not be electrochemically active, displayed irregularities below approximately 0 V. These were not reproducible between replicates. Extensive cleaning of the GCE working electrode and surface conditioning by anodic polishing temporarily decreased noise and interference but these remained issues. Peak intensity, related to the sample concentration, had a low reproducibility, likely due to adsorbed analytes on the GCE surface. In some sample replicates, anodic peak potentials shifted

unexpectedly. The manufacturer deems an error of ±0.2V acceptable; however, this margin can have a significant impact on the interpretation of results.



(A) 10 mM (+)-catechin and 15 mM glycine. **(B)** 10 mM 4-methylcatechol (4-MC) in the presence of 0-15 mM glycine, before and after heat treatment. **(C)** 10 mM hydroquinone (HQ) in the presence of 0-15 mM glycine, before and after heat treatment.

Figure 4.4 Impact of glycine on the cyclic voltammograms of phenolic compounds.

(+)-Catechin produced a wide anodic (oxidation) peak with a lower peak intensity at a higher potential than 4-MC and HQ (Table 4.3). It had no clear reduction peak, indicating its irreversibility. Upon a second cycle of applied potential on a same sample, no oxidation peak formed; adsorbed (+)-catechin blocking the GCE surface[177] could explain the lack of current as well as the perceived electrochemical irreversibility. In the presence of glycine, there was still no anodic peak upon a 2nd cycle. The asymmetry of the anodic peak is unusual and was confirmed by a replicate (not shown). In the presence of glycine, the anodic peak potential decreased, indicating enhanced antioxidant activity. It is unclear whether this effect can be attributed to the spontaneous

oxidation of (+)-catechin followed by its condensation with the amino acid to reduce the compound. This scenario would imply very rapid and efficient reactions, as the sample was analyzed quickly after its preparation and the oxidation peak associated with unreacted (+)-catechin completely disappeared. This is in accordance with results obtained by Wang et al., who observed the gradual appearance of a new anodic peak accompanied with the disappearance of HQ's anodic peak in the presence of increasing proline concentrations[276]. Other factors such as instrument and sample variability could explain the deviation. This shift was not observed in samples prepared using 4-MC or HQ.

As for HQ and 4-MC, the diphenols produce anodic peaks at similar potential and intensities, regardless of the phenol involved and the presence of glycine, heated or not. The 4-MC and HQ samples turned blueish-grey and peach, respectively, after the heat treatment, indicating that a reaction had taken place. Their E_{pa} remained close to those of the phenols in the absence of glycine, indicating that their electron-donating ability was relatively unchanged by the process. Studies have demonstrated that the E_{pa} is correlated to a compound's radical-scavenging activity[280]. Taking into consideration the proposed reaction mechanism between glycine and diphenols, this would imply that the glycine/phenol adduct has an antioxidant activity that is comparable to the initial phenol. Essentially, the activity of the phenol can be doubled in the presence of glycine.

Table 4.3 Anodic peak data of phenolic compounds in the presence of glycine.

Sample	I_{pa} (µA)	E_{pa} (V)
Gly	-	-
Catechin	172	0.64
Catechin + gly	122	0.52
4-MC	327	0.36
4-MC + gly	365	0.39
4-MC + gly (110 °C)	340	0.37
HQ	306	0.32
HQ + gly	352	0.42
HQ + gly (110 °C)	340	0.40

Where I_{pa}, anodic peak intensity; and E_{pa}, anodic peak potential.

Although cyclic voltammetry has successfully been implemented as a suitable analysis method for the reaction of quinones and amines, multiple factors hinder its practicality. Notably,

a vast array of possible instruments and test parameters, such as reference and working electrodes, solvents, and working electrode pre-treatments, make it impossible to compare data from different laboratories and come to a consensus on any topic based on the method. In fact, although it is generally accepted that CV can be used to assess antioxidant activity, researchers do not agree on the best CV parameter to assess it: every parameter, whether it involves the anodic or cathodic peak, the intensity or potential, peak integration, or even a combination of these, has been used. Additionally, the method is highly sensitive to disturbance caused by dissolved oxygen, adsorbed analytes on the WE, contaminants, and more. Pristine conditions are fundamental and require laborious electrode cleaning before each analysis as well as analytes, solvents, and electrolytes with high purity to be achieved. Finally, the system is fragile and must be manipulated delicately. Electrode connectivity is a frequent issue due to the failure of connectors and the loosening screw attachment of the RE. The GCE is prone to bending and is poorly protected by a thin, soft plastic tube. Both the exaggerated impact of slight analysis condition fluctuations and the constant impairment of the device make results difficult to reproduce. Although the manufacturer aims to offer a more standardized analysis equipment that is accessible to most labs, the inconvenience of the laborious preparation for a low sample throughput and the lack of reference material and methods prevent the Electrasyn 2.0 CV from being worthy of its place in a lab setting.

4.2.2 Evaluation of the effect of added glycine on red wine's oxidative stability

Glycine was added to 50 % red wine diluted in water to investigate its effect on wine's oxidation process by measuring oxygen consumption. After 17.5 hours, the wine samples had consumed on average 1.9 % of the applied O_2 pressure whereas the samples to which glycine was added had only decreased the O_2 pressure by 1.2 % (Table 4.4). This translates to a longer induction time for wine samples containing glycine and indicates that the amino acid provides an additional oxygen uptake-limiting effect, possibly through antioxidant activity.

The wine samples were composed, in part, of water with a small alcohol content, mainly ethanol (~7 %), and were heated at 80 °C while exposed to oxygen: conditions comparable to those under which the amine/quinone Michael additions have been shown to take place in the present study as well as in previous research[226]. Out of the 20 common amino acids, glycine has the lowest antioxidant activity, as measured by the oxygen radical absorbance capacity assay[319]. Thus, glycine alone does not confer antioxidant protection and its stabilizing quality must come from interactions

with other wine components. By extension, the Michael addition of glycine to certain polyphenols naturally present in red wine is suspected to enhance its oxidative stability. Although the Beaujolais wine in question is considered a dry wine with a reported sugar content of 3.9 g/L, some of these sugars can react with the added glycine through a typical Maillard reaction mechanism. Maillard reaction products have been reported to have antioxidant effects[320], including those formed between glycine and glucose[321]. These could offer an alternate explanation for the decrease in oxygen consumption observed when glycine was added to wine.

Table 4.4 Oxygen pressure drop associated with the oxidation of Beaujolais red wine, in the presence of glycine.

Sample	Pressure drop (%)	Error (%)
Control	1.9[a]	0.05
1% glycine	1.2[b]	0.05

Letters in superscript indicate a statistically significant difference at p=0.05. The experiment was conducted using a 50 % wine/water solution at 80 °C for 17.5 hours under an initial pressure of 700 kPa.

Overall, the addition of glycine to red wine could help limit the undesirable effects caused by the presence of excess oxygen. Its effect on color stability is discussed in section 4.4. This method based on the measurement of oxygen consumption over time proved to be a simple, straightforward analysis method for the oxidation of wine. Although the process may be lengthy depending on the sample's oxygen consumption, very minimal sample preparation, material and clean-up are necessary, and there is no need for intervention during the analysis, which could be done overnight for the present study.

4.2.3 Evaluation of the effect of added 4-methylcatechol and glycine on the oxidative stability of flaxseed oil

Although glycine and 4-MC show a poor solubility in FSO at room temperature, their solubility slightly increases with temperature, so it was assumed that at low concentrations and at 120 °C, most of the additives would be available to interact with the oil's oxidation, if applicable. Although stirring should facilitate the dissolution of the analytes, it also increases the oil's contact with oxygen and accelerates its dissolution as well, leading to faster oxygen consumption in all samples (Table 4.5). The addition of 4-MC was found to slow oxygen consumption, regardless of stirring, confirming its antioxidant effect. In the presence of glycine, the time to reach a 60 % oxygen pressure drop (t_{60}) slightly decreases, although the significance of the difference is unclear

due to the lack of replicates. It is possible that the analytes' limited solubility alongside other solvent effects prevent glycine from reacting with 4-methyl-1,2-benzoquinone, the oxidized form of 4-MC. It is unclear why glycine would lower the t_{60} of 4-MC in FSO compared to samples where the amino acid is absent.

Table 4.5 Effect of 4-methylcatechol and glycine on the time to reach a 60 % drop in oxygen pressure in flaxseed oil solutions.

	t_{60} **(minutes)**	
Sample	**Not stirred**	**Stirred at 100 RPM**
Control	34.1	27.3
0.2 % glycine	49.4	26.2
0.1 % 4-MC	50.2	41.9
0.1 % 4-MC + 0.2 % glycine	48.9	39.4

t_{60}, time, in minutes, to achieve a 60 % drop in oxygen pressure; RPM, rotations per minute; 4-MC, 4-methylcatechol. The experiment was conducted using 4 g of flaxseed oil heated to 120 °C with an initial pressure of 700 kPa.

Surprisingly, in unstirred samples, glycine exhibited slowed oxygen consumption to a similar extent to 4-MC, at double the latter's concentration. As previously stated, glycine is not known to have any antioxidant activity[319]. When stirred, the protective effect was completely lost and the t_{60} of FSO was low, regardless of the presence of glycine. The trend was confirmed by the analysis of replicates. We hypothesized that glycine, when undisturbed, may form a physical layer atop the oil sample which would act as a barrier to the dissolution of oxygen by limiting its contact with the oil. This physical barrier would be disrupted by stirring.

The measurement of oxygen consumption was a simple, reliable tool for the evaluation of antioxidants in FSO. Although the technique does not provide information on the type of oxidation products formed, the induction time can be measured, which directly relates to shelf-life and quality. Therefore, this method is suitable for straightforward quality analyses. However, it may be inappropriate to study reactions between most phenols and amino acids in most oils using any method due to solubility limitations which impede on the extent of their reaction.

4.3 Reactions of hydroquinone and glycine reactions and the *p*-anisidine value of flaxseed oil

Attempts were made to study the impact of HQ, glycine, and combined HQ/glycine on the oxidation state of FSO. Fresh FSO and FSO subjected to heating were compared to ensure the efficacy of the accelerated oxidation process. The pAV, initially at 3.4 for fresh FSO, increased to 4.4 in FSO left at room temperature for 24 hours and even up to 32.8 when heated at 70 °C, uncovered, for 24 hours.

The experiment was repeated with purified p-anisidine and with HQ or HQ/glycine added to the FSO before the heating process. The pAV of FSO with 6 mM HQ was 7.4 and was almost double (pAV=14.3) in an equivalent sample with 12 mM glycine. The basis of the *p*-anisidine assay is the reaction of the indicator with aldehydes and ketones present as a result of an oil or fat's oxidation[292, 322]. Based on HQ and glycine's structures and intermediates involving ketones and carbonyl structures, we hypothesized that these additives or the products formed during their reaction may interference with this assay. It is also possible that p-anisidine, which has an amine group, reacts with HQ via Michael addition, just like glycine, and exaggerates the pAV by absorbing light at 350 nm. The reactions of glycine and phenols with *p*-anisidine in the absence of FSO were then studied (Table 4.6).

Table 4.6 Absorbance at 350 nm of the potentially interfering additives studied.

Sample	A (Reacted)	A (Unreacted)	ΔA
6 mM HQ	0.066	0.040	0.026
6 mM p-BQ	0.080	0.047	0.033
12 mM glycine	0.056	0.046	0.010
6 mM HQ/12 mM glycine, heated	0.115	0.097	0.018

HQ, *p*-BQ, glycine and heated HQ/glycine mixtures were analyzed using a method based on ISO 6885:2016, replacing the oil with isooctane. The *p*-BQ sample had the most pronounced effect on the absorbance measurements taken at 350 nm, followed by HQ, meaning these react with *p*-anisidine and increase the apparent pAV of FSO, causing interference. Even unreacted samples had some absorbance at this wavelength and could complicate the comparison of oil

samples with different additives. However, their contribution to the absorbance at 350 nm may be insignificant at low concentrations when compared to fats and oils. It is possible that oxidation intermediates in the FSO have a pro-oxidant effect on HQ and increase its reactivity towards *p*-anisidine.

In addition, FSO oxidation causes the formation of a polymeric film at the surface exposed to air[323], resulting in heterogenous samples. Some FSO samples also became turbid when dissolved in isooctane. The pAV assay requires clear material to measure absorbance, as reflectance would be an issue.

Overall, the pAV test may be inappropriate for the evaluation of the antioxidant activity of compounds with ketone and aldehyde structure added to fats and oils. The accelerated oxidation of FSO exposed to air is also unsuitable, particularly for this method, due to complications involving its drying and heterogeneity. As pointed out previously, using oils as a reaction medium may also inhibit the reaction between phenols and amino acids due to their incompatibility, thus invalidating the potential benefits of their combination. *p*-Anisidine's instability made it necessary to frequently purify it, possibly causing deviations between batches and delaying experiments. Other inconveniences include a low sample throughput and the use of large organic solvent volumes, which bears a high environmental cost. However, the assay does not require any special equipment, making it accessible in most laboratory settings. It would be worthwhile to study the volatile oxidation products formed in the presence of phenols and amino acids by headspace gas chromatography coupled to mass spectrometry to evaluate the extent of oxidation of oils[324]. This could help determine the antioxidant efficiency of the phenol and amino acid combination as a strategy to reduce quality loss in foodstuff.

4.4 Impact of glycine on the color evolution of (+)-catechin caused by oxidation

One of the most common indicators of oxidation of catechins and to a larger extent, most polyphenols, is a change in color associated with cross-linking[325]. (+)-Catechin solutions subjected to oxidative conditions evolve from colorless in their fresh state to yellow and brown, depending on the extent of oxidation[326]. Whereas acidic conditions tend to stabilize (+)-catechin, basic solutions of the compound exposed to oxygen oxidize quite rapidly[47]. In fact, a barely visible yellow colour began to appear in the (+)-catechin hydrate in aqueous basic samples in contact with air.

The absorbance spectra of fresh and oxidized (+)-catechin as well as oxidized (+)-catechin/glycine mixtures in basic aqueous solution in contact with air are reported in Figure 4.5. As reported by Bark *et al.*[326], fresh (+)-catechin absorbed a low level of emission in the shorter wavelengths, producing a small shoulder. This could be explained by a small amount of the (+)-catechin being oxidized during storage of the solid compound or immediately when exposed to the solution conditions.

To confirm that both observed peaks were related to the oxidation of (+)-catechin, the experiment was repeated with added ascorbic acid, a potent antioxidant known to regenerate catechins[327]. Over the 28 hours following the addition of KOH, the absorbance spectra obtained from the (+)-catechin/ascorbic acid samples remained identical to the spectra obtained from fresh (+)-catechin. Glycine and ascorbic acid do not absorb in the visible range. Therefore, Peaks 1 and 2 are deemed to be produced due oxidative processes, in accordance with the current literature[326, 328].

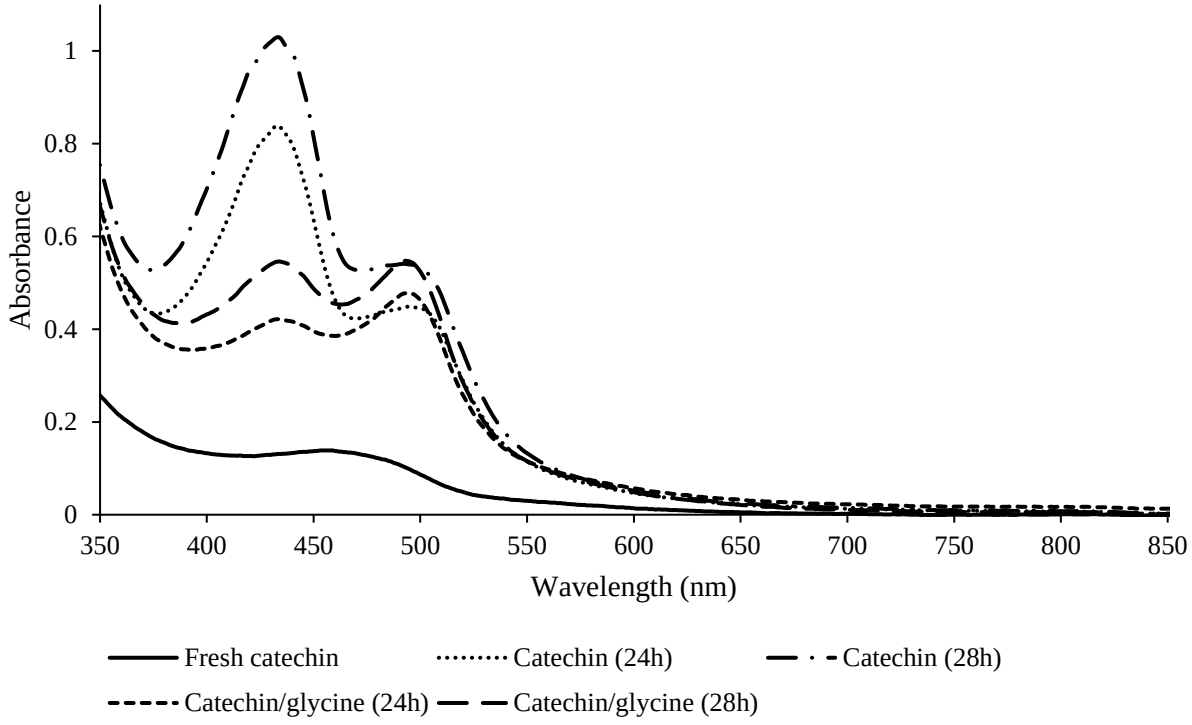


Figure 4.5 Impact of added glycine on the absorbance spectra of (+)-catechin under oxidative conditions.

After 24 hours, two peaks formed in the (+)-catechin samples: Peak 1 around 435 nm and Peak 2 around 495 nm. These peaks correspond to those found in previous studies[329-330]. Because peak separation was not achieved, peak areas were calculated (Figure 4.6). The addition of glycine drastically lowered Peak 1, cutting its absorbance by half. Glycine did not have a significant impact on Peak 2. Over time, (+)-catechin's peak areas increased whereas in the presence of glycine, they remained more stable. The increase in Peak 1 in the absence of glycine was accompanied by a visually noticeable increase in yellow tint.

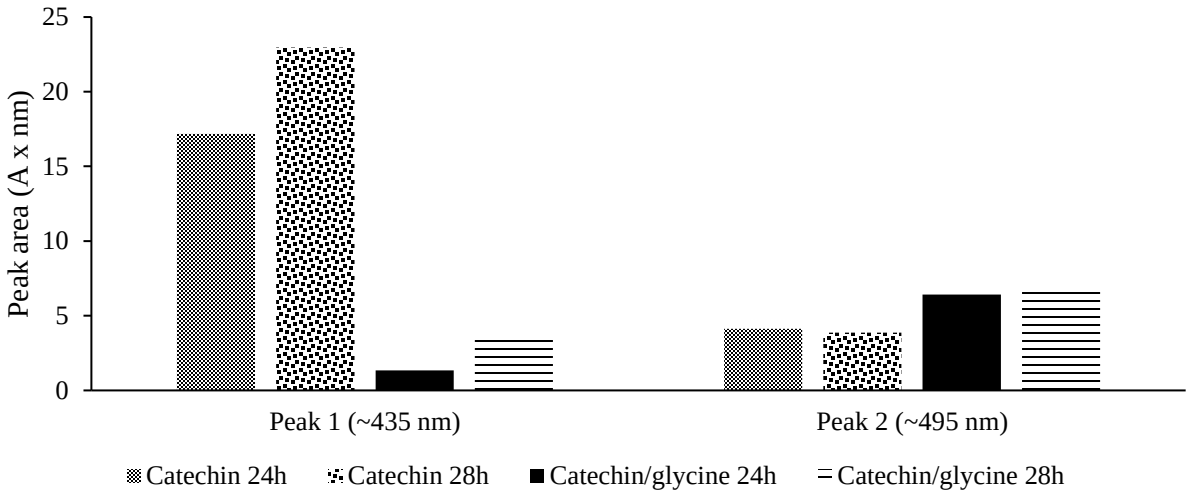


Figure 4.6 Areas of visible light absorbance peaks for oxidized (+)-catechin samples.

Because both peaks evolve differently with time and with added reactants, it can be assumed that they arise from different compounds. As mentioned previously (Section 2.4.2), (+)-catechin can epimerize under the applied conditions, forming epicatechin. Dehydrocatechins dimerize via interflavanic bonds, forming DhC_2 or procyanidins. Peak 1 must correspond to yellow-colored A-type catechin dimers and trimers[197] or procyanidins[331]. Very little visible absorbance data is available; however, at pH=6, γ-DhC_2 shows a peak at 385 nm and δ-DhC_2, at 412 nm[194]. The different analysis method used may be the cause of deviation. The Michael addition of glycine to (+)-catechin could inhibit catechin dimers involving two adjacent B-ring carbons, as double Michael additions would occupy one of these carbons. All DhC_2 A (γ, δ, and the likely intermediate α) involve adjacent ether and C-C bonds between one unit's A-ring and the other's B-ring. This would explain the decrease in Peak 1: because a proportion of the oxidized (+)-catechin reacts with glycine, it cannot form yellow-colored compounds. The reaction is likely

incomplete, allowing the remaining oxidized (+)-catechin to form the colored oligomers. DhC_2 B (β and ε) may still be formed, as they involve a single interflavanic linkage, although steric hindrance could play a role. These compounds are colorless, and their production cannot be monitored by visible spectrometry.

As for Peak 2, although visible in published spectra for basic catechin solutions[329-330], few studies have identified (+)-catechin-related compounds that absorb around 500 nm, giving them a reddish color. In high pH aqueous (+)-catechin solutions heated to 60 °C for two hours in the presence of added H_2O_2, Germann, Stark & Hoffman found hydroxyxanthene-derived chromophores with absorbance maxima between 380 and 514 nm[332]. The compounds are reported to be reddish and absorb at 496 nm under basic conditions[332-333], which corresponds to Peak 2. The authors named the compounds formed in the presence of H_2O_2 "xanthenocatechins" and "xanthenoepicatechins"[332]. Although the solutions prepared in the present study did not contain added H_2O_2 and were left incubated at room temperature, the oxidation of (+)-catechin can generate H_2O_2 by reducing molecular oxygen. The extended incubation at room temperature could mimic results obtained from similar samples exposed to a higher temperature for a shorter period. The hydroxyxanthene-derived compound is similar in structure and color to xanthylium salt derivatives formed between two equivalents of (+)-catechin and glyoxylic or tartaric acid[334-336]. Similar structures have been reported following reactions between malvidin-3-glucoside and (+)-catechin, which first form a B-type dimer via a C-C link between their A- and C-rings, followed by dehydration to close the xanthelium structure[337]. The reaction mechanism involved in the formation of the xanthenocatechins and xanthenoepicatechins has not been described and would seem to involve several rearrangements[332]. All the xanthene-derived compounds found in the literature are the result of intermolecular reactions between A-rings, and the B-rings remain intact, suggesting that the Michael addition of glycine to (+)-catechin's B-ring would not interfere with their formation.

Samples containing glycine appeared more peach-hued than those without the amino acid, which were more yellow. This is explained by the slightly larger peak area for Peak 2 in the presence glycine, which could be an effect of the competition between the reactions involved in the formation of the compounds associated with the peaks. It is also possible that glycine plays a role in the formation of xanthylium salts identical to those described by Es-Safi *et al.*[333], if glycine

can replace glyoxylic acid in the proposed mechanism. The second derivative of the absorbance curves indicates a minor bathochromic shift for Peak 2 of samples containing glycine. This could be the result of the structural modification caused by the addition of the glycyl groups.

Visible spectrophotometry can be used as a simple tool to study the impact of oxidation on a food product's visual characteristics, which are crucial to its quality and consumer acceptance. Although absorbance peaks may represent different structurally similar compounds, their overall impact on color may be more relevant than the identification of the individual compounds causing color changes. The use of basic conditions to accelerate (+)-catechin oxidation and its reaction with glycine may not appropriately reflect processes involved in foods and should be modified if used as a model for specific purposes. However, using a higher pH effectively enhanced reaction rates compared to neutral conditions at room temperature.

This study highlights the need for further elucidation and identification of (+)-catechin's oxidation products in basic hydroalcoholic solutions. Namely, the characteristic absorbance wavelengths of oligomers and their rearrangement products should be collected to aid in analysis. Proper insight on the mechanism of production of the previously reported xanthenocatechins would help evaluate potential applications or control methods. An algorithm has been developed to predict the UV-visible spectra of compounds based on their structure using machine learning, which could help associate individual and/or combined peaks to the compounds identified in a mixture[338].

Overall, glycine can help control the development of yellow color in catechin-rich media, specifically under basic conditions, with a small increase in red color. In light of these results, along with glycine's ability to control oxygen uptake in red wine, it seems as though the addition of this amino acid could be suitable in red wine, where color stability and controlled oxidation are beneficial to the product's quality. During preliminary testing, glycine was not linked to particle deposition, contrarily to L-cysteine. Further research in this direction could provide more insightful conclusions as to this application.

This pigmentation could also prove useful in other foods where red colour is desirable. For example, oxidation-prone red meat suffers from a loss of its bright red hues over time[339]. Coating of the meat in a catechin solution or film could help in multiple ways: catechin itself would protect the meat's pigments and lipids against oxidation, before reacting with free amino acids present in

meat[340], thus regenerating the antioxidant activity. Finally, the red pigments formed by catechin/glycine oxidation products could help enhance the redness of the meat. Other foods in which a similar strategy may be promising include red fruit juices and smoothies, emulsions such as red pepper mayonnaises and aioli, and fried foods (beet chips, spice-coated fried snacks, etc.).

CHAPTER 5. CONCLUSION

5.1 General conclusions

The reaction of glycine with (+)-catechin and catechin-related structures was investigated in this study, providing mechanistic insight that builds on previous research. Three adducts were detected and characterized using an isotopic labelling technique, ESI-HRMS and MS/MS, and indicated the occurrence of Michael additions of one and two glycine moieties to HQ. The proposed reaction mechanism involves additional oxidation and hydroxyl group regeneration steps, potentially allowing extended antioxidant capacity to the adducts formed compared to the unreacted phenolic compound. This mechanism was extrapolated to fit that of (+)-catechin and glycine's reaction, in accordance with previous studies.

Although the Michael addition of amines to quinones has been proposed to regenerate antioxidant activity elsewhere, this seems to be the first study to attempt to relativize the protective effect of the formed adducts. To counter the scarcity of studies reporting the antioxidant activity of combined polyphenols and amino acids, methodologies were also developed and evaluated according to the quality of results and their ease of use. These tests included UV-visible spectrometric, colorimetric, electrochemical and oxygen consumption methods. The acquisition of UV-visible spectrometry data could easily provide an indication of (+)-catechin's oxidation and the impact of glycine on its pigmented products. Although the method provides little structural information on the compounds responsible for the formation of each peak, these may be used as markers of oxidation and provide a meaningful representation of the products' impact on colour, strongly correlated with quality perception in many foods. Cyclic voltammetry was also attempted on solutions of phenolic compounds and amino acids. Glycine was only suspected to have a favorable antioxidant effect with (+)-catechin, and not with 4-methylcatechol or HQ. The functioning of the voltammeter proved to be an obstacle, and clean, reproducible results were not obtained, preventing the collection of meaningful cathodic peak results. Commercial cyclic voltammetry instrumentation needs further improvements to make their use worthwhile. The *p*-anisidine value assay was attempted in FSO heated with added HQ and glycine. In the oil, the addition of combined HQ and glycine increased the *p*-anisidine value compared to HQ alone. The reasons for this were not defined, but interference could not be ruled out. Finally, the oxygen

consumption of FSO with added 4-methylcatechol and glycine and of red wine with added glycine were measured using a new automated instrument. In FSO, the combination did not exhibit beneficial effects as compared to HQ alone, possibly due to solubility issues. However, glycine had an astonishing limiting effect on oxygen consumption in oil that was completely lost when stirred, highlighting an unexpected phenomenon. In wine, the addition of glycine effectively limited oxygen uptake, likely due to its interaction with the abundant flavonoids naturally present.

5.2 Suggestions for future research areas

Future work should be performed to enhance the understanding of the reactions between quinones and amines, notably regarding the reaction parameters that favour Schiff base formation and Michael addition. To do so, conditions such as pH, temperature, and most importantly solvents (and even lack thereof) should be modified prior to characterization of the products formed. To better understand the effects of (+)-catechin and glycine interactions in foods, models that are representative of the specific media should be used. These models should also be studied using different combinations of polyphenols and amino acids to generalize the conclusions or underline differences. To confirm the antioxidant effect, the reactions should be further investigated in foodstuff using more promising analytical methods, such as headspace gas chromatography coupled to mass spectrometry for the analysis of volatile secondary oxidation products of oils. Other emerging effects could also be surveyed, such as antimicrobial or pharmaceutical properties, as the adducts are underrepresented in the literature and could exhibit unanticipated behaviours. Toxicity evaluation of the products and sensory analyses could be pursued to ensure the strategy's suitability in food production.

CHAPTER 6. REFERENCES

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CHAPTER 7. APPENDICES

Appendix 7.1. Secondary oxidation products of fatty acid methyl ester by autoxidation ([20] as compiled by [21]; [22]).

Class	Oleic acid	Linoleic acid	Linolenic acid
Aldehydes	Octanal	Pentanal	Propanal
	Nonanal	Hexanal	Butanal
	2-Decenal	2-Octenal	2-Butenal
	Decanal	2-Nonenal	2-Pentenal
		2,4-Decadienal	2-Hexenal
			3,6-Nonadienal
			Decatrienal
			Propanedial
Carboxylic acids	Methyl heptanoate	Methyl heptanoate	Methyl heptanoate
	Methyl octanoate	Methyl octanoate	Methyl octanoate
	Methyl 8-oxooctanoate	Methyl 8-oxooctanoate	Methyl nonanoate
	Methyl 9-oxononanoate	Methyl 9-oxononanoate	Methyl 9-oxononanoate
	Methyl 10-oxodecanoate	Methyl 10-oxodecanoate	Methyl 10-oxodecanoate
	Methyl 11-oxo-9-undecenoate		
Alcohols	1-Heptanol	1-Pentanol	
		1-Octene-3-ol	
Hydrocarbons	Heptane	Pentane	Ethane
	Octane		Pentane

Appendix 7.2 Fatty acid profile of edible oils[28, 341].

Type of oil	*8:0*	*10:0*	*12:0*	*14:0*	*16:0*	*16:1*	*18:0*	*18:1*	*18:2*	*18:3*	*20:0*	*20:1*	*22:0*	*22:1*	*24:0*
Seeds															
Babassu	5.4	6	44.3	15.8	8.6		2.9	15.2	1.7		0.1				
Coconut	8.1	6.5	48.6	17.7	8.5		2.5	6.5	1.5		0.1				
Palm kernel	4	4.1	49.7	16	8		2.4	13.7	2		0.1				
Sunflower			0.1	0.2	6.8	0.1	4.7	18.6	68.6	0.5	0.4				
Sunflower[a]			0.1	0.2	3	0.4	5.9	82.4	7.4		0.3	0.3			
Rapeseed[b]				0.1	4.7	0.3	1.7	59	21.4	9.9	0.6	1.4	0.4	0.3	0.2
Soybean					10	0.2	3.5	21	55.3	9.2	0.5		0.3		
Cottonseed				1	23.9	0.5	2.9	18.5	52.5	0.3	0.4				
Peanut					10.1	0.2	3.5	51.4	27.3	0.1	1.6	1.3	3.1		1.4
Cocoa butter				0.1	26.2	0.3	34.4	34.8	2.9	1.1	0.2				
Flaxseed					4.9		3.7	21.3	18.1	50.6	0.1	0.1	0.1		0.6
Nuts															
Almond				0.1	8.5	1.1	1	57	31.4	0.6	0.1	0.1	0.1		
Brazil			0.1	0.2	14.4	0.5	7.9	31.2	45.1	0.1	0.3	0.1	0.1		
Cereals															
Corn					10.7	0.2	1.5	30.5	55.9	0.8	0.4				
Rice bran					13.9	1.9	2.7	41.1	36.4	2.3	1.8	0.2			
Wheatgerm				0.2	18.5	0.6	0.5	18.1	55.9	5.3	0.1	0.8			
Fruit															
Palm				1	43.8	0.5	5	38.5	10.5	0.3	0.4				
Olive					10.8	0.5	3	75.5	8.5	0.9	0.4	0.4			

Proportion of fatty acid (%) in oil. [a]High oleic acid. [b]Low erucic acid.

Appendix 7.3 Mid-IR potential peak assignment for (+)-catechin[193].

Group	Region (cm^{-1})	Characteristics
O-H	3600-3200	H-bonded, broad and strong
C-H	3100-3000	aromatic, medium
C-H	3000-2850	alkane, medium, sharp, stretching
C=C	1600-1400	aromatic, medium-weak, series of sharp bands
C=O	1820-1670	ester and carbonyl, mostly strong, conjugated lower
R-C-H	1480-1350	alkane, variable, bending
C-O	1300-1000	alcohol and ether, strong, ester has 2+ bands
1,2-disubstituted	1200-900	benzene ring, 3 peaks, 2 medium, 1 strong
1,3-disubstituted	1100-700	benzene ring, 4 peaks, 2 medium, 2 strong