Generation, characterization, and potential food applications of a novel red colorant formed by oxidative coupling of chlorogenic acid and tryptophan

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Abstract

Due to growing consumer skepticism regarding synthetic food colorants and their potential health effects and environmental impact, there is a rising interest in novel natural compounds derived from plants. A red-colored pigment was produced via the oxidative coupling of chlorogenic acid (CQA), using sodium periodate ($NaIO_4$) as the oxidizing agent, with tryptophan (Trp).

The first part of the thesis deals with the preparation and characterization of the new red dye. The colorant generated was precipitated, freeze-dried, and purified by size exclusion chromatography before undergoing characterization with UHPLC coupled with mass spectrometry and NMR spectroscopy. Additional mass spectrometric studies were conducted on the reaction product generated with ^{15}N and ^{13}C labeled Trp educts. Based on the data obtained, a complex compound with two Trp moieties and one caffeic acid moiety was identified and a possible formation pathway was proposed.

In the second part, the investigation focused on the optimization of the process by examining the effect of temperature and concentration of Trp and $NaIO_4$ on the reaction progress. Once the optimal variables were determined, three different pH values were tested. This way, the reaction time decreased from 72 to 24 hours yielding 46 ± 2% *w/w* based on the quantity of CQA. The solubility of the pigment was evaluated, and the stability of its hydrolyzed form was analyzed under various pH values, storage conditions (in light and darkness for over 28 days), presence of reducing agents, and heat resistance. Ultimately, it was found that the pigment effectively colored various food matrices in quantities ranging from 0.005-0.01% *(w/w)*.

In conclusion, this thesis improves the understanding of the production of a new red pigment resulting from the oxidative coupling of CQA and Trp. It also provides valuable insights into the production and characterization of this red dye. Additionally, the potential application of this pigment in different food matrices was tested.

Kurzfassung

Aufgrund der zunehmenden Skepsis von Verbrauchern gegenüber synthetischen Lebensmittelfarbstoffen und deren möglichen Auswirkungen auf Gesundheit und Umwelt wächst das Interesse an neuartigen Farbstoffen aus natürlichen Stoffen. Ein rotes Farbpigment wurde durch die oxidative Kopplung von Chlorogensäure (CQA) und Tryptophan (Trp) unter Verwendung von Natriumperiodat ($NaIO_4$) als Oxidationsmittel hergestellt.

Der erste Teil der Studie befasst sich mit der Herstellung und Charakterisierung des neuen roten Farbstoffs. Der Farbstoff wurde ausgefällt, gefriergetrocknet und durch Größenausschlusschromatographie gereinigt, bevor er durch UHPLC mit Massenspektrometrie und NMR-Spektroskopie charakterisiert wurde. Zusätzliche massenspektrometrische Untersuchungen wurden an Reaktionsprodukten durchgeführt, die mit ^{15}N- und ^{13}C-markierten Trp-Reaktanten hergestellt wurden. Basierend auf den erhaltenen Daten wurde eine komplexe Verbindung mit zwei Trp-Anteilen und einem Kaffeesäure-Anteil identifiziert und ein möglicher Bildungsweg vorgeschlagen.

Der zweite Teil der vorliegenden Arbeit konzentriert sich auf die Optimierung des Prozesses, indem der Einfluss der Temperatur sowie der Trp- und $NaIO_4$-Konzentrationen auf den Reaktionsprozess untersucht wurden. Nach der Bestimmung der optimalen Parameter wurden drei verschiedene pH-Werte getestet. Auf diese Weise konnte die Reaktionszeit von 72 auf 24 Stunden verkürzt werden. Die Löslichkeit des hergestellten Pigments wurde bewertet und die Stabilität seiner hydrolysierten Form unter verschiedenen pH-Werten, Lagerungsbedingungen (Licht und Dunkelheit für mehr als 28 Tage), Anwesenheit von Reduktionsmitteln und Hitzebeständigkeit analysiert. Es konnte schließlich festgestellt werden, dass das Pigment verschiedene Lebensmittelmatrizes wirksam färbt.

Zusammenfassend kann gesagt werden, dass diese Arbeit das Verständnis für die Herstellung eines neuen roten Pigments durch oxidative Kopplung von CQA und Trp erhöht. Die vorliegende

Arbeit liefert wertvolle Einblicke in die Herstellung und Charakterisierung dieses roten Pigments. Darüber hinaus wurde die potenzielle Anwendung dieses Pigments in verschiedenen Lebensmittelmatrizes getestet.

Table of Content

Preliminary remarks

List of abbreviations

ADHD	Attention deficit hyperactivity disorder
ADI	Acceptable daily intake
COSY	Correlated spectroscopy
CQA	Chlorogenic acid
3-CQA	3-Caffeoylquinic acid
4-CQA	4-Caffeoylquinic acid
5-CQA	5-Caffeoylquinic acid
CQAQ	Chlorogenic acid quinone
DEPT	Distortionless enhancement by polarization transfer
DOE	Design of experiments
eV	Electronvolts
HPLC	High-performance liquid chromatography
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
JECFA	Joint FAO/WHO Expert Committee on Food Additives
m/z	Mass-to-charge ratio
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PPO	Polyphenol oxidase
(U)HPLC	(Ultra) high-performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet

Vis	Visible
w/w	weight by weight

List of publications

1) Santarcangelo, A., Weber, F., Kehraus, S., Dickschat, J. S., & Schieber, A. (2023). Generation and structure elucidation of a red colorant formed by oxidative coupling of chlorogenic acid and tryptophan. *Food Chemistry*, 425, 136473. https://doi.org/10.1016/j.foodchem.2023.136473

2) Santarcangelo, A., Schulze-Kaysers, N., Schieber A. (2024). Improved generation, physicochemical characteristics, and food application studies of a red colorant obtained from oxidative coupling of chlorogenic acid and tryptophan. *Foods,* 13, 686. https://doi.org/10.3390/foods13050686

Reprints of all mentioned publications and related supporting materials are given in the Appendix A-D of this thesis.

Conferences

Santarcangelo, A., Weber, F. Schieber, A. (2022). Formation of pigments by reaction of chlorogenic acid quinone with tryptophan. 15th World Congress on Polyphenols Applications, Valencia, Spain, September 28–30, *Book of Abstracts*, 142. [Poster]

Santarcangelo, A., Schulze-Kaysers, N., Schieber, A. (2023). Production and characterization of a red pigment by reaction of chlorogenic acid quinone with tryptophan. 16th World Congress on Polyphenols Applications, Valletta, Malta, September 28–29, *Book of Abstracts,* 52. [Oral Presentation]

Chapter 1

Introduction

1. Color

Color perception in individuals with normal vision is mediated by the human visual system and plays a fundamental role in understanding the world around us. This perception results from neurological and physiological processes that enable the brain to interpret the wavelengths of light emitted or reflected from surrounding objects (Birren, 1976).

In the past, color primarily served survival and communication functions within species. However, over time, it has acquired more complex meanings and has influenced aesthetic and behavioral aspects of human life, including decision-making and purchasing, particularly in the context of food (Viera et al., 2019).

Before discussing the use and function of food colorings, it is important to define the concept of color and its origins first.

One of the most significant observations regarding color was made by Isaac Newton when he passed a ray of sunlight through a glass prism in a room without artificial light. The light that passes through the prism is the same that enters it. However, upon exiting the prism, the beam is transformed into a band of light that, when reflected from a white surface, creates a variety of color experiences for the observer's visual system, which are known as the colors of the electromagnetic spectrum (Westfall, 1962). Everyone who has already observed a rainbow after a rainfall on a sunny day witnessed the same effect.

Chapter 1

As we know today, light is a type of electromagnetic radiation that carries energy through space (Purcell & Morin, 2019). Although it was first observed that the behavior of light is similar to that of waves, we know since the early 1900s that light consists, in fact, of individual packets of energy, known as photons or quanta (Ekspong, 1999). This discovery was the starting point of the theory of "quantum mechanics", whose nature and phenomena are still being studied nowadays.

The photons that form the electromagnetic radiation have a specific energy E, which is given by the equation $E = hv$, where v is the frequency and h is Planck's constant. The energy is directly proportional to the frequency v and inversely proportional to the wavelength λ of the radiation, which can be calculated using $\lambda = c/v$, with c being the speed of light in a vacuum. As frequency increases, the energy increases, while as wavelength increases, the energy decreases. Typically, the energy of photons is given in units of electron volts (eV).

Electromagnetic radiation can be monochromatic, i.e., composed of only one wavelength, or polychromatic, meaning that it contains a mixture of multiple wavelengths (Kuehni, 2012). Within the "wave-picture" of light, wavelength (usually measured in nanometers) represents the distance between two peaks of the electromagnetic wave (Hecht, 1987). As an example, the human eye is only sensitive to a narrow wavelength interval of electromagnetic radiation, known as the visible spectrum. It is found between wavelengths of approximately 400 and 700 nanometers (nm), which enable the perception of colors and objects for humans. The broad electromagnetic spectrum extends all the way from large wavelength radio, infrared and micro waves to small wavelength UV, X and gamma rays, each with specific properties and applications. X-rays (10-0.01 nm), for example, can penetrate biological tissues, while UV rays (400-10 nm) can cause photochemical reactions in the skin (Kuehni, 2012).

The interaction of electromagnetic radiation with matter can occur in a number of ways:

- Absorption: Quanta are absorbed by matter, interact with it in certain ways, and after loss of some energy are reemitted
- Transmission: Quanta pass through matter unchanged; certain forms of matter impede the speed of the quanta which, at interfaces of two different kinds of transmitting matter, can result in a change of direction (refraction)

- Scattering: Certain matter is impenetrable to quanta and they are scattered or reflected by it, in the process changing direction.
- Interference: Quanta can interact with neighboring quanta in certain conditions.

For the studies performed in this thesis, it is the visible part of the electromagnetic spectrum that we need to consider. While the above explanation of wavelength might hint that every individual associates a specific wavelength to a specific color, it was actually discovered that color perception is subjective and can vary from person to person (Harman, 1996). However, all chromatic phenomena share a common element: the presence of light.

2. Food colorants

Color is a crucial sensory attribute for determining consumer acceptance or rejection of food products, and is accounting for 62-90% of the evaluation process (Singh, 2006). As we know, food colorants are nowadays frequently used in a variety of applications, such as carbonated beverages, salads, juices, ice cream, and desserts. The total global sales of food coloring correspond to approximately 8,000 metric tons per year (Khan et al., 2020). The use of colorants as additives is important because it helps to guarantee uniformity in color among batches, enhances color intensity, and allows for the generation of colored products that were previously colorless, among other benefits (Ranaweera et al., 2020).

Food colorants can be classified according to their source (plant, animal, bacterial, fungal, etc.), color (red, yellow, purple, blue, green, etc.), or chemical structure: flavonoid derivatives (anthocyanins), isoprenoid derivatives (carotenoids), nitrogen-heterocyclic derivatives (betalains), and pyrrole derivatives or chlorophylls responsible for blue and green hues (Viera et al., 2019). Another common method for distinguishing colorants is to categorize them as natural or synthetic (Ranaweera et al., 2020). Although the meanings of the terms "natural" and "artificial" are widely understood, there is no official or universally accepted definition of what constitutes a natural or an artificial colorant. Indeed, neither the United States nor the European

Union have established a legally recognized definition for the term "natural colorant", which remains ambiguous in the current regulatory framework (Viera et al., 2019).

This thesis considers natural dyes to be present or derived from sources present in nature such as plants, fruits, vegetables, spices, algae, or minerals. In contrast, synthetic dyes are produced (in laboratories) through chemical processes and are derived from petroleum-based raw materials or non-natural sources.

This distinction is important since the demand of synthetic colorants in the late 19th century has increased compared to the use of natural colorants in industrial applications due to several advantages such as low cost, simple and fast synthetic processes, vibrant hues, long-lasting durability, a wide range of colors, superior color fastness, and cost-effectiveness (Safapour et al., 2022). However, in recent years, consumer acceptance of synthetic dyes has declined dramatically, due to studies such as the Southampton Six, which suggested a possible link between artificial dyes and hyperactivity in children (McCann et al., 2007).

For this reason, there is a growing trend globally to favor the natural side of foods. In particular, the focus has been on plant-based dyes rather than their animal-based counterparts. This trend can be attributed to the growing number of vegans and vegetarians who avoid animal products in their diets. Moreover, the study of food dyes can be considered a very profitable and industrially interesting research topic (Viera et al., 2019).

3. Red colorants in food stuff

Synthetic red dyes, particularly monoazo dyes like amaranth and allura red, are commonly utilized in the food industry as color additives due to their desirable properties, including high stability to oxygen, light, pH, low levels of microbiological contamination, and relatively low costs. Despite their advantages, there is increasing concern about their potential negative health effects (Monisha et al., 2023). They can be considered safe when consumed within the acceptable daily intake (ADI) limits. However, higher doses can have adverse health effects, particularly in children (Aquino & Conte-Junior, 2020). Potential toxicities associated with azo dyes include allergies,

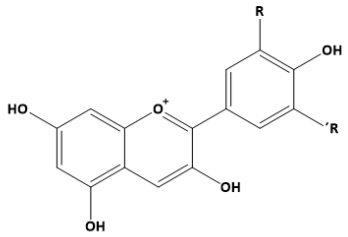
attention deficit hyperactivity disorder (ADHD), asthma, anxiety, cytotoxicity, and genotoxicity/cancerogenicity (Kaya et al., 2021).

Potential toxicity appears to be associated with the formation of aromatic amines, which are produced as a result of the catabolic reductive cleavage of azo dyes in various organs, including the liver and kidney, and are known to be mutagenic and carcinogenic (Amin et al., 2010; Elbanna et al., 2017). Hence, replacing food dyes with more natural colorants represents a crucial topic for future research. From a natural point of view, the most common red food colorants from plants are anthocyanins and betalains, which are not found in the same plant species at the same time (Esquivel, 2024; Moccia et al., 2021). Another dye, but this time of animal origin, commonly used in food applications is carmine, a colored extract obtained from the aphid *Dactylopius coccus* COSTA (Müller-Maatsch & Gras, 2016).

3.1. Anthocyanins

Anthocyanins are regarded as a crucial class of plant pigments and are frequently employed in the production of natural dyes for use in food products, such as in beverages, baked goods, and dairy products (Karakaya et al., 2016; Morata et al., 2019; Pires et al., 2019). They occur in high concentrations in various berries, currants, grapes, and same tropical fruit (Khoo et al., 2017) and are responsible for the vibrant red, purple, and blue colors found in various fruits and vegetables (Wijesekara & Xu, 2024). Anthocyanins are a specific type of flavonoids, which are secondary plant metabolites that have a typical C6-C3-C6 backbone. These demonstrate a high structural diversity and are widely present in higher plants, excluding the Caryophyllales family. Figure 1 illustrates six of the most common anthocyanins.

Anthocyanins are glycosylated anthocyanidins and are widely distributed throughout the plant kingdom. In addition, the sugar molecules bound to anthocyanins tend to increase their water solubility and stability in comparison to aglycons (Kammerer, 2024).



Compound	R	´R
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

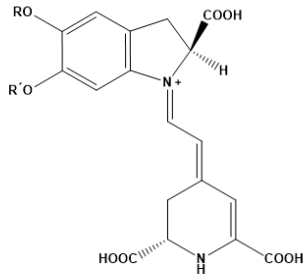
Figure 1. Structures of the six most frequently occurring anthocyanidins (Kammerer, 2024).

Anthocyanins show considerable sensitivity to pH, which directly influences their chemical structure and, consequently, the color they manifest. In very acidic environments (pH < 3), anthocyanins take the form of flavylium cations, characterized by a positive charge on the oxygen atom of the C ring, giving intense red or orange coloration. As the pH increases slightly (pH 4-5), they are present in the hemiketal form, in which the conjugation and electronic delocalization between the three anthocyanin rings are disturbed, preventing color formation. Under neutral pH conditions (pH 6-7), anthocyanins acquire a violet coloration, this color change is due to their conversion to uncharged quinones. When the environment becomes slightly alkaline (pH 7-8), these compounds take on a blue color due to the formation of their anionic form. Finally, under strongly basic conditions (pH >11), anthocyanins turn into an unstable dianionic form with a blue-green coloration, subject to degradation processes that lead to loss of pigmentation (Mattioli et al., 2020). Consequently, the dependence of anthocyanins on pH is considered a limitation for their use. Therefore, their use is restricted in most cases to coloring foods with stable acid characteristics, such as jams, sweets, purple-colored beverages, or yogurt-based beverages. In addition to pH limitations, these pigments are also highly unstable at elevated temperatures, which significantly restricts their applicability in the baking process (Oancea, 2021). Anthocyanins are also susceptible to degradation and have poor intrinsic stability. To improve their overall stability, often macromolecular components such as proteins and polysaccharides are present in the food matrix (Zang et al., 2022).

3.2. Betalains

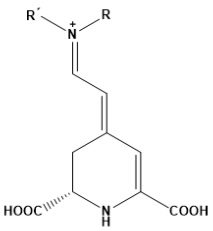
Betalains are considered to be one of the most important plant pigments and are frequently utilized as natural food colorants. They are primarily found in the roots of red beets or in certain fruits, where they provide unique red and yellow pigments that add diversity to the color palette of natural foods. These pigments are water-soluble, and the nitrogen-containing betalains can usually be divided into two categories: red betacyanins and yellow betaxanthins, as shown in Figure 2 (Esquivel, 2024).

A)



Compound	R	´R
Amaranthin	2-O-(β-glucuronic acid)-β-glucosyl	H
Betanin	β-glucosyl	H
Hylocerenin	5-O-methyl-glutaryl-β-glucosyl	H
Phyllocactin	5-O-malonyl-β-glucosyl	H

B)



Compound	R	´R
Dopaxanthin	H	L-Dopa
Vulgaxanthin I	H	Glutamine
Indicaxanthin	H	Proline

Figure 2. (A) Main betacyanins and (B) main betaxanthins in betalain-rich sources (Esquivel, 2024).

Temperature is a critical factor affecting the structure of betalains. An increase in temperature leads to a higher rate of degradation. The stability is compromised not only by temperature but also by various factors, such as pH, oxygen presence, light exposure, and the presence of metals. These influences significantly reduce their potential for use in food products (Calva-Estrada et al., 2022).

3.3. Carmine

Carmine is a natural dye extracted from an insect called cochineal (genus *Dactylopius*). This insect produces a bright red pigment called carminic acid, which is extracted from its dried carcasses. Carminic acid (compound A, Figure 3) is the major pigment and coloring principle of cochineal and cochineal extract, while carmine (compound B, Figure 3) is the hydrated chelate complex obtained from cochineal extract by adding aluminum or calcium.

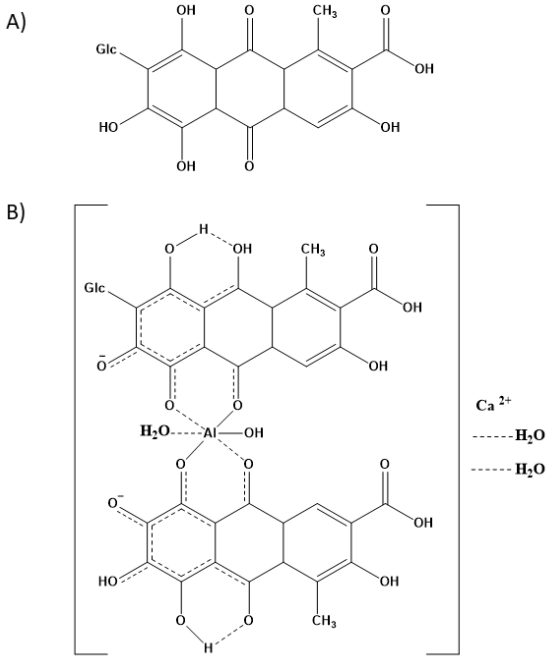


Figure 3. Chemical structures of carminic acid (A) and carmine (B) (Müller-Maatsch & Gras, 2016).

In the European Union, this colorant is permitted under certain conditions in several food categories. These include dairy products, edible ice, fruit and vegetables, confectionery, cereals and cereal products, meat and fish products, and beverages (Müller-Maatsch & Gras, 2016). Cochineal and its processed products are recognized as food additives with an ADI of 0-5 mg/kg body weight set by the JECFA in 1982 (JECFA, 2000 a,b).

Advantages of carmine include its remarkable resistance to heat and light. However, skepticism surrounds its use due to several factors: it is an animal by-product, it contains aluminum, it raises

microbiological concerns, and it can cause severe allergic reactions (Müller-Maatsch & Gras, 2016).

4. Exploring alternative sources: searching for new potential dyes

In the field of food research, there is a growing interest in identifying new natural compounds to meet the needs of the food industry in a healthy and sustainable way. In specific, researchers (like the author of this thesis) are focusing on plant-derived substances due to the limitations and challenges associated with the use of current food dyes, such as anthocyanins and betalains (Fernández-López et al., 2013). Chlorogenic Acid (CQA), present in many plant foods, has earned attention for its antioxidant properties and potential to produce coloring compounds. Research into the potential of CQA and its derivatives as a natural alternative for food dyes is a promising area that could revolutionize the food industry.

5. Chlorogenic Acid

The term "chlorogenic acids" (CQAs) covers a large group of compounds, most of which occur naturally and are synthesized in plants by esterification of a C6-C3 *trans*-hydroxycinnamic acid with a 1_L-(—)-quinic acid. The main hydroxycinnamates are *p*-coumaric, ferulic, sinapic and caffeic acids (Clifford et al., 2017).

The first reported CQA can probably be attributed to Robiquet & Boutron (1837). The term CQA was introduced years later to describe the phenolic material in green coffee beans (Payen, 1846). The term 'chloro' is considered misleading as it suggests the presence of chlorine in the structure. This term can be traced back to Payen's publication in 1846, where it was observed that CQA produces a green pigment when reacted with ammonia under aeration. Furthermore, this name was based on the Greek origin of the word "khloros", which means light green, rather than chlorine. The first time that the 3-caffeoylquinic acid (3-CQA) got isolated was in 1932 (Fischer & Dangschat, 1932), while the presence of 5-caffeoylquinic acid (5-CQA) in coffee and other isomers was reported by Barnes et al. (1950).

CQA consists of caffeic acid esterified with quinic acid, which can isomerize given the presence of the stereocenter connecting quinic acid (Dawidowicz & Typek, 2010).

Quinic acid, also known as 1_L-1(OH),3,4/5-tetrahydroxycyclohexanecarboxylic acid according to the IUPAC nomenclature system, can be bound to the caffeic acid group in positions 3, 4, or 5, but not commonly in position 1 (Clifford et al., 2003).

A stereocenter is defined as an atom, typically carbon, that exhibits four different substituents. Stereocenters are significant because they can lead to molecules with chiral isomers, which are molecules that have the same atomic structure but differ in the spatial arrangement of their atoms. This spatial arrangement can impact the chemical and biological properties of the molecule (Mislow & Siegel, 1984).

In the IUPAC system, 3-CQA of Fischer and Dangschat became 5-CQA (Fischer & Dangschat, 1932). Therefore, neochlorogenic acid, cryptochlorogenic acid, and chlorogenic acid (Figures 3 A-C) can be identified as 3-, 4-, and 5-caffeoylquinic acid, respectively. These are also considered the most common isomers of CQA (Meinhart et al., 2018). Due to its prevalence in nature, the *trans*-isomer

of 5-CQA is usually preferred compared to other isomers, as in this thesis. Unfortunately, in some studies, non-IUPAC notation is used, which has a different numbering system, often causing much confusion (Clifford et al., 2017; Kremr et al., 2016).

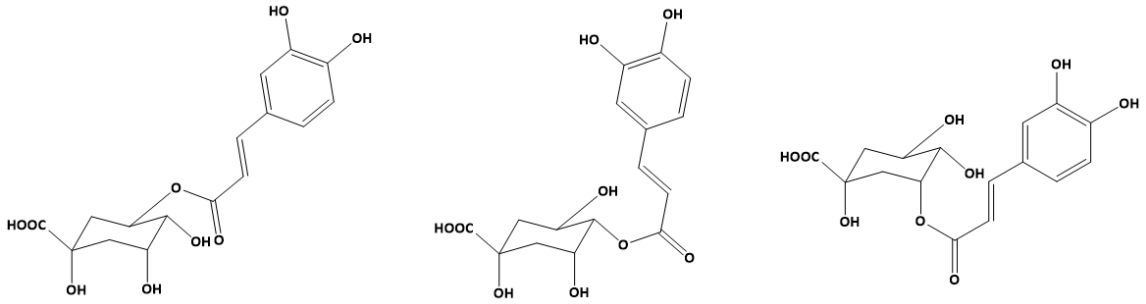


Figure 4. Representation of the most common isomers of chlorogenic acid.

5.1. Behavior of 5-CQA under different conditions

trans-5-CQA acid is known for its instability and tendency to degrade through various mechanisms, including isomerization. Two isomerization behaviors can be observed for *trans*-5-CQA: positional isomerization and *cis*/*trans* isomerization. The former depends usually on pH and/or the temperature of the solution, while the latter is induced by UV light (Xie et al., 2011). The formation of the two isomers 3-CQA and 4-CQA from 5-CQA is usually observed by subjecting CQA to heat sources or alkaline conditions (Clifford et al., 2017; Dawidowicz & Typek, 2010). While CQA can be oxidized at alkaline pH, it remains stable at acidic pH and moderate temperatures (Friedman & Jürgens, 2000).

6. Formation of quinones

The generation of quinones is a crucial step in the interaction of CQA due to their high reactivity. They often serve as the initial step in the reaction. *o*-Quinone can be generated by oxidation of *o*-diphenol, which can happen through enzymatic oxidation, autoxidation process in presence of oxygen, heat, metal, alkaline pH or $NaIO_4$ (Fulcrand et al., 1994; Geng et al., 2023; Schieber, 2018).

6.1. Enzymatic oxidation

Oxidation to quinones can occur through the action of two types of enzymes: polyphenol oxidases (PPOs) and peroxidases. Peroxidases produce quinones through the formation of radicals, which typically require the presence of H_2O_2 (Matheis & Whitaker, 1984). Therefore, their role in foods is limited compared to the action of PPOs. Phenolics are usually oxidized by PPOs after disintegration of the plant tissue and exposure to molecular oxygen (Schieber, 2018). The essential characteristics for enzymatic oxidation are in fact the presence of a substrate, the oxygen and the enzymes. Catechol oxidase and laccase are enzymes classified as PPOs (McLarin & Leung, 2020). PPOs can either oxidize *ortho*-dipenols (*o*-diphenols) to *ortho*-quinones (*o*-quinones) through catechol oxidase, which is a bisphenol oxidase (Kampatsikas & Rompel, 2021), or catalyze the hydroxylation of monohydroxy phenols into *ortho*-dihydroxy (*o*-dihydroxy)-phenols through monophenolase or cresolase activities (García-Molina et al., 2022).

Although laccase can catalyze the oxidation of *o*-phenol and *para*-phenol (*p*-phenol), it has been shown to be ineffective in oxidizing phenol and *meta*-phenol (Mohit et al., 2020).

PPO activity can be inhibited by removing oxygen or the phenolic substrate. The enzyme can also be inactivated by direct interaction with an inhibitor or by adding reducing compounds such as vitamin C or certain thiols (Prigent, 2005). These compounds revert the oxidized phenolic back to its original form (Negishi & Ozawa, 2000; Prigent, 2005; Richard-Forget et al., 1992).

6.2. Non-enzymatic oxidation

Quinones can be formed even without the presence of an enzyme. Non-enzymatic oxidations can be classified into two main categories: auto-oxidation of polyphenols catalyzed by metal ions or oxidation by other *o*-quinones through electron transfer reactions.

The first group is based on the reaction of polyphenols with oxygen through catalysis of metals, especially iron or copper, to form *o*-quinones (Geng et al., 2023). Auto-oxidation can also occur at alkaline pH or in the presence of $NaIO_4$, the latter of which has shown to be able to produce a profile of oxidation products similar to that obtained from PPOs (Fulcrand et al., 1994; Schieber, 2018). Increasing the pH causes the deprotonation of the phenolic hydroxyl group, which eventually may result in the formation of quinones (Prigent, 2005).

Figure 5 shows a scheme of quinone formation in case of CQA.

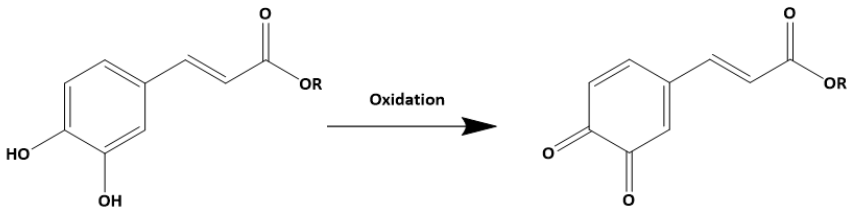


Figure 5. Quinone formation exemplified for CQA (R, quinic acid).

6.3. Reaction of quinones

o-Quinones that are generated from enzymatic or non-enzymatic oxidation tend to be very reactive and electron deficient intermediates that can be involved in different types of reactions. They do not exist in large quantities as they tend to react to form more stable products.

One of the possible reactions is to react with nucleophiles in a so-called Michael addition (Schieber, 2018). Nucleophilic additions of *o*-quinones are to be considered irreversible following the generation of stable aromatic systems (Uchimiya & Stone, 2009).

During the oxidation of polyphenolic compounds, *o*-quinones can undergo a condensation reaction in the absence of other suitable reagents. This reaction can result in the formation of oligomers, which are larger molecules consisting of a series of basic units bound together (Cheynier et al., 1988; Fulcrand et al., 1994; Schieber, 2018).

It is important to underline that quinones, in addition to being Michael acceptors, can also generate reactive oxygen species through redox cycling with their semiquinone radical anions. This redox cycling can be induced by both enzymatic and non-enzymatic reactions that give as result the formation of superoxide radical anions, hydrogen peroxide, and hydroxyl radicals (Schieber, 2018). These reactions are a cause of concern, particularly when they occur in biological systems, due to their potential harmful effects, such as acute cytotoxicity, immunotoxicity, and carcinogenesis (Bolton et al., 2000).

At low pH values quinones are found in the form of hydroquinone, which are generally more stable and less reactive, whereas at higher pH values quinones become the predominant species and show greater reactivity, becoming more likely to form dimers through oxidative coupling reactions, both with other quinone molecules and with other phenolic compounds in the environment (Prigent, 2005).

Several phenols, including gallic acid, caffeic acid, and catechin have been observed to undergo oxidation induced dimer formation. Among these, caffeic acid emerges as a particularly interesting case. When exposed to oxidizing conditions, this compound is not limited to the formation of dimers, but can also generate more elaborate structures such as trimers or oligomers of higher complexity. Remarkably, one of these caffeic acid-derived trimers showed a

characteristic red coloration consisting of four carbon rings and two unsaturated acyl side chains (Figure 6) (Weber et al., 2019).

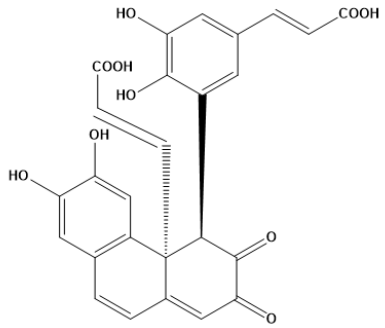


Figure 6. Proposed structure of the isolated compound reported by Weber et al., 2019.

Antolovich et al. (2004) demonstrated that also CQA dimerizes, resulting in the formation of various dimers, as observed by reverse-phase chromatography. The dimerization reaction of CQA can be compared to that of ethyl caffeate and can be applied to the entire class of caffeic acid esters. These compounds are susceptible to oxidative dimerization reactions due to the presence of the caffeic moiety. Figure 7 illustrates one of the proposed pathways for the formation of an ethyl caffeate dimer. This process can be considered critical because it is a key step in the formation of trihydroxy benzacridine (TBA), which will be discussed in detail in the next section.

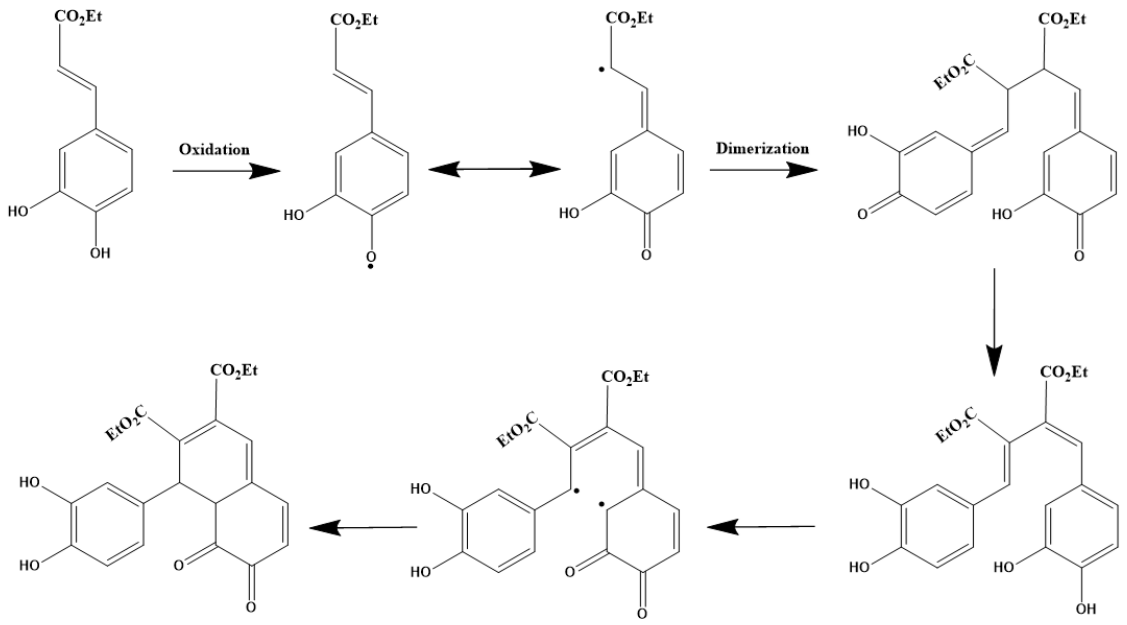


Figure 7. Proposed mechanism of dimerization of ethyl caffeate at alkaline pH (Namiki et al., 2001; Prigent, 2005; Yabuta et al., 2001).

7. Reactions of quinones with amino acids or proteins

Amino acids and proteins are important nucleophiles, especially under alkaline conditions, and can react with quinones by nucleophilic addition. In plants, CQA and proteins typically do not interact as they are located in separate compartments. However, mechanical processing, which can occur during activities such as harvesting, can cause these compartments to rupture and allow for interaction (Bittner, 2006).

Phenolic compounds can interact with proteins in two ways: noncovalent (reversible) and covalent (usually irreversible). Phenol-protein interactions, both covalent and noncovalent, depend not only on phenol/protein ratios, but also on factors such as steric hindrance and polarity of both protein and phenol. Therefore, the nature and sequence of amino acid residues in the protein chain are particularly important (Prigent, 2005).

The interactions between protein and phenol may result in changes of properties and the creation of new features, such as the development of colors. An example of this interaction is the appearance of green color in some foods and cooking methods, such as baking and frying, and in some vegetables, such as sweet potatoes, burdock, and sunflower seed meal (Yabuta et al., 1996). This phenomenon is considered of particular interest because of the infrequency of green color in natural food systems. Chlorophylls and their derivatives are currently commonly used as green dyes, but their instability during the coloring process is a major concern (Namiki et al., 2001).

Yabuta et al. (1996) explain that this green shade can be attributed to the interaction between caffeates and primary amines, such as amino acids (excluding serine, proline, cysteine, and threonine), but not with secondary and tertiary amines.

In a subsequent study, various caffeic acid esters with different amine groups were tested to gain a better understanding of the role of the different functional groups involved in the reaction. The study concluded that an *o*-diphenolic structure with a carbonyl side chain, similar to that found in CQA, and the presence of a primary amine group (R-NH_2) were necessary for the greening phenomenon. It was also observed that the intensity of the green color changed in relation to the amino compound. The green color intensified in the following order: Glycine, Alanine > Histidine > Phenylalanine >> Glutamic acid. Additionally, the λ_{max} observed was slightly different related with the amino group used (Yabuta et al., 2001).

In the case of CQA alone, browning occurred probably due to melanin formation, as hypothesized by Bongartz et al. (2016) and supported by other findings by Kammerer (2024). This behavior was previously observed by Namiki et al. (2001) with caffeine derivatives that developed a brown coloration of the solution after oxidation.

Namiki et al. (2001) and Yabuta et al. (2001) found, as previously described, that quinones tend to form a dimer upon oxidation. This dimer is able to react with an amine compound and lead to the formation of a new benzacridine ring structure through a Michael addition and subsequent nucleophilic cyclization (Namiki et al., 2001).

It appears that pH plays a crucial role in this type of reaction. At acidic pH, the rate constant of the reaction between quinones and amino acids is minimal due to the lack of reactivity. This can be explained by the fact that under acidic conditions, amine groups are protonated, which reduces their nucleophilicity since they are below the isoelectric point (Oliveira et al., 2017).

The presence or absence of an amino residue in the resulting structure was shown to depend on which amino group was involved. When CQA dimers react with α-amino groups, it results in the absence of an amino residue in the resulting TBA. On the other hand, when they react with β-alanine and ε-lysine, it leads to the formation of TBAs with an amino acid residue (Bongartz et al., 2016; Schieber, 2018).

The reaction mechanism was reported in more detail by Bongartz et al. (2016) and illustrated in Figure 8.

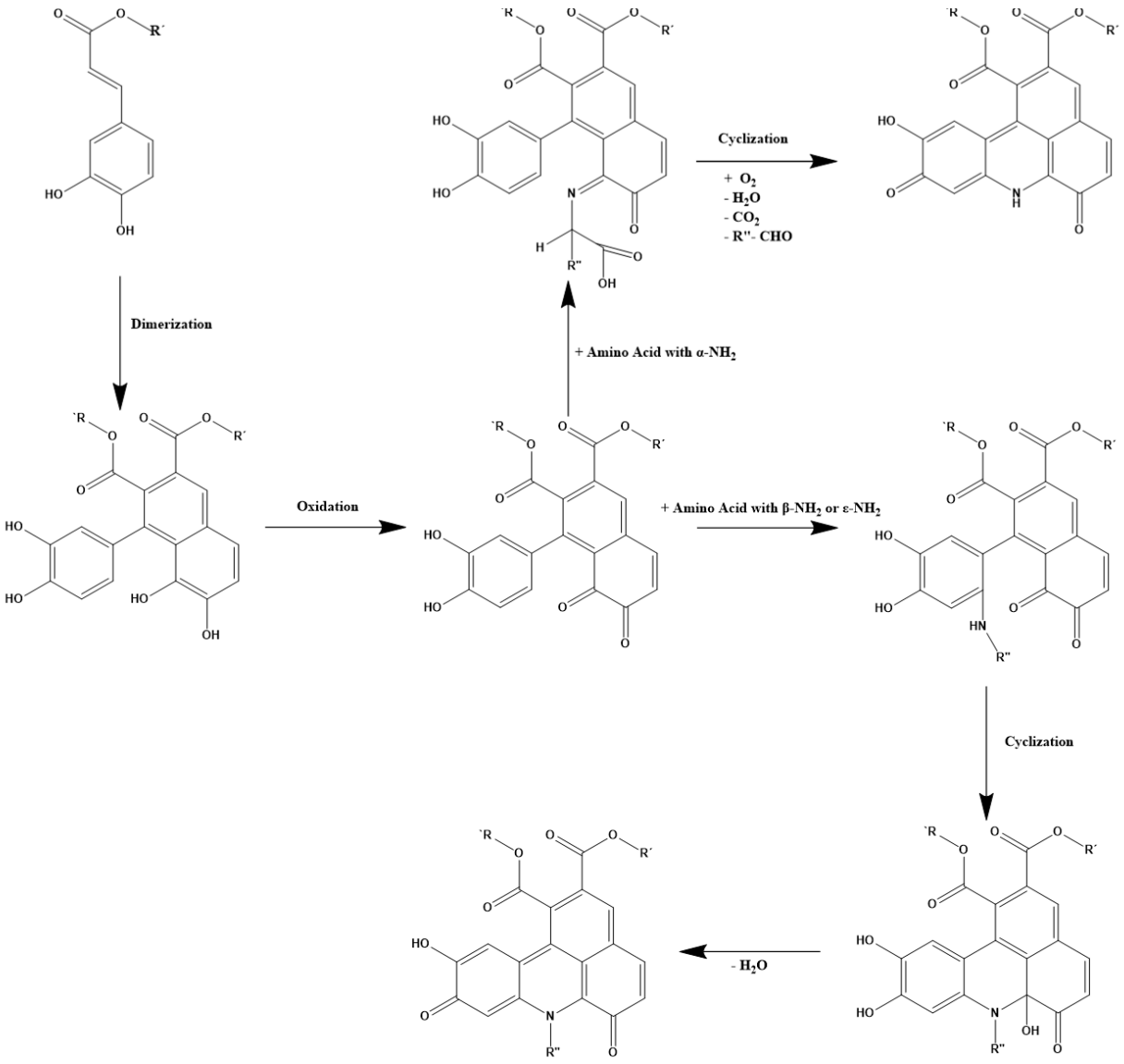


Figure 8. Proposed reaction mechanism by Bongartz et al. (2016) between CQA and individual amino acids, R´ = quinic acid, R" = amino acid side chain.

Despite extensive efforts to isolate the green pigment, Yabuta et al. (1996; 2001) were not able to complete this task, hinting towards the rather unstable and complex nature of the compound. Nevertheless, during the chromatographic process a bluish-green change of coloration was observed. Conversely, acidification to pH 2 with hydrochloric acid (HCl) resulted in a color change towards reddish-violet. Furthermore, treatment of the substance with ascorbic acid resulted in a color change to yellow. Namiki et al. (2021) attributed the yellow staining to the reduced form of TBA, which was found to be highly reactive with oxygen and produce quinone-type products with green and blue characteristics.

So far, the typical mechanism of interaction between CQA and amino acids seemed to involve first forming a dimer, which then reacts with the amino acid, but in the case of cysteine, something different happens. Cysteine is a strong nucleophile that reacts directly by addition with the CQA monomer (Figure 9), preventing the formation of the dimer (Poojary et al., 2023; Schilling et al., 2008).

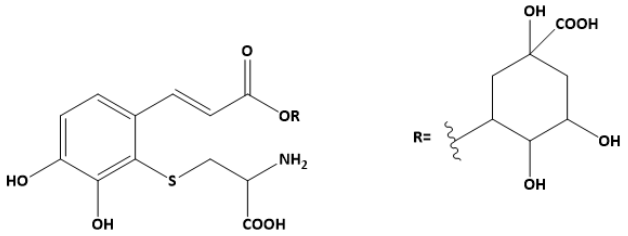


Figure 9. CQA-Cysteine adduct with R= quinic acid (Poojary et al., 2023).

Another noteworthy phenomenon is the reaction between chlorogenic acid quinone (CQAQ) and tryptophan (Trp), which exhibits a distinctive red coloration. This stands in contrast to the shades of green and brown observed in the reactions with other amino acids. Bongartz et al. (2016) proposed the first possible explanation, which suggests that the presence of the indole group may affect the chromophore. Later, Vercruysse (2019) observed for the first time that Trp generates a green or red pigment depending on the concentration of Trp. Exploring the reasons behind the variations in Trp behavior compared to other amino acids is a fascinating question that requires further scientific investigation.

Subsequent investigation by Moccia et al. (2021) formulated as major component of the red pigment an unusual benzochromeno (2,3-b) indole attached to a Trp moiety, featuring a cyanine type chromophore with a λ_{max} of 542 and 546 nm (Figure 10).

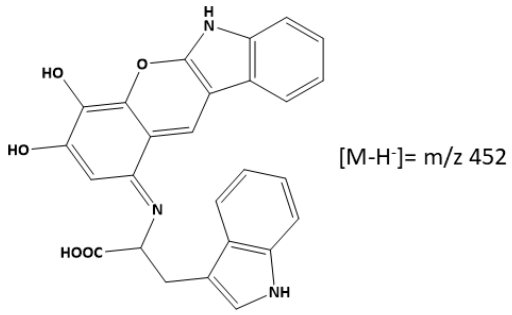


Figure 10. Representation of the structure proposed by Moccia et al. (2021).

As a result of further studies in our laboratory, different reaction products were identified, which prompted the author of this thesis to pursue further and more comprehensive investigations.

8. Analysis and characterization of a new compound

When a new compound is identified or produced, the analysis of its structure is critical. It provides pivotal information about the characteristics and properties of the compound, and guides its potential applications.

Finding the molecular structure can sometimes be really challenging and often requires multiple characterization techniques, as a single method rarely provides all necessary information. Therefore, scientists use combined tools and analytical approaches to gain a comprehensive view of the desired molecular structure.

8.1. UV/Vis spectroscopy

As explained in Section 7, the interaction between CQA and amino acids results in the creation of colored complexes. The visible UV spectrum can provide valuable information about the nature, color, and formation of these compounds (Bongartz et al., 2016; Iacomino et al., 2017).

UV/Vis spectroscopy is absorption or reflection spectroscopy in the ultraviolet and visible regions of the electromagnetic spectrum. It is a quantitative technique used in analytical chemistry to determine the concentration of various analytes, including transition metal ions, highly conjugated organic compounds, and biological macromolecules (Perkampus, 2013).

The basic principle of UV-Vis spectroscopy is based on the absorption of light by electrons in molecules. When light strikes a molecule, it interacts with the electrons within, causing them to become excited from a lower energy state (ground state) to a higher energy state (excited state), resulting in the absorption of energy. The concentration of the sample and the length of the optical path through which the light passes are directly proportional to the amount of light absorbed. This relationship is fundamental to the measurement of light absorption in spectrophotometry (Förster, 2004).

8.2. UHPLC coupled with mass spectrometry

Ultra-High-Performance Liquid Chromatography (UHPLC) is a form of liquid chromatography that separates different components of a mixture as they pass through a column with a stationary phase. This is achieved through targeted technological innovations that allow for higher flow pressure and smaller particle sizes, resulting in faster separation times and increased analytical efficiency (Dong, 2019).

UHPLC is frequently combined with mass spectrometry (MS), a potent analytical technique that offers comprehensive insights into the composition and molecular structure of the separated components. MS determines the mass-to-charge ratio (*m/z*) of the ions in the sample, enabling the identification and characterization of molecules and compounds in the mixture, as well as the determination of their elemental or isotopic composition (de Hoffmann & Stroobant, 2007).

This powerful combination of UHPLC and MS is widely utilized in various scientific fields and applications, including pharmacology, environmental chemistry, biology, and food chemistry (Frenich et al., 2014; Rathod et al., 2019). Due to its capacity to offer comprehensive data on both simple and complex mixtures, UHPLC-MS has become an essential tool for scientists.

8.3. Nuclear magnetic resonance

Nuclear Magnetic Resonance (NMR) spectroscopy is an analytical technique that provides detailed information about the structure of molecules by observing the behavior of atomic nuclei in a magnetic field. The absorption of radio-frequency radiation (100 to 1000 MHz) is measured after immersing the molecule under investigation in a strong magnetic field, which causes nuclear spin transitions. Specifically, atoms such as ^{1}H or ^{13}C are utilized.

A key parameter in this technique is the chemical shift, or δ, which indicates the position of the signal and provides details about the chemical context of the compound.

The splitting of the peaks and the intensity of the signal, in the case of the proton spectrum, are also important because they can reveal the number and type of neighboring nuclei and allow the identification of the number of protons present per signal. The shape of the signals is also important because it can provide information about chemical exchange (Jacobsen, 2007).

The ^{1}H-NMR and ^{13}C-NMR experiments are useful for identifying the presence and chemical characteristics of protons and carbons in the molecule. In addition, advanced one-dimensional techniques such as DEPT (Distortionless enhancement by polarization transfer) allow selective assignment of carbon signals and determination of the proton multiplicity associated with each carbon atom, facilitating structural elucidation.

Two-dimensional spectroscopies, such as COSY, HSQC, and HMBC, display correlations between coupled protons, protons and directly bonded carbons, and protons and carbons separated by multiple bonds, respectively (Ernst et al., 2023). For a comprehensive analysis of the chemical structure, as in the case of **Chapter 2**, it is necessary to combine one-dimensional and two-dimensional spectroscopies.

8.4. Purification

To achieve accurate determination of the molecular structure by NMR spectroscopy, it is essential to work with a purified compound. Indeed, the presence of other substances may compromise the correct assignment of structure. Consequently, purification of the sample is a crucial step in the process.

The purification of a compound in a liquid state can be achieved through the use of liquid chromatography, a separation technique based on the differential distribution of components present in a mixture between a mobile phase (the solvent) and a stationary phase (the packing material). A gel filtration resin that is commonly utilized for this purpose is Sephadex, which is derived from the cross-linking of dextran with epichlorohydrin. The different types of Sephadex have varying degrees of cross-linking, which affects their swelling and molecular fractionation range. This method, which was used the studies described in **Chapters 2** and **3**, is a form of molecular separation chromatography. Small molecules are retained in small compartments, while larger molecules elute first, making it an optimal method for separating small molecules from larger ones (Janson, 1987).

9. Optimization of the reaction

The optimization process represents a pivotal stage in numerous areas, as it aims to maximize efficiency and enhance the performance of both reactions and their products. This intricate process necessitates meticulous consideration of a multitude of variables that can influence the ultimate outcome. In this context, the application of Design of Experiments (DOE) is emerging as a key tool to address this challenge in a structured and scientific manner (Weissman & Anderson, 2015). Based on sound statistical principles, DOE enables scientists and engineers to investigate and comprehend the interrelationship between input variables, called factors, and output variables, which are the desired responses. This methodological approach provides a clear framework for the systematic analysis of the effects of various variables on the process under consideration. The use of DOE enables the accurate identification of the factors that exert the greatest influence on the final outcome, as well as the manner in which they interact with one another. This comprehensive analysis enables the targeted optimization of processes, thereby reducing errors and improving overall efficiency (Durakovic, 2017).

In conclusion, the use of DOE represents a scientific and rigorous approach to the complex optimization process, allowing for an accurate assessment of the variables involved and a better understanding of the dynamics that govern industrial and scientific processes.

Objective of the thesis

Due to the limited popularity of synthetic food colorants among consumers, there is an increasing demand for new and more natural compounds, preferably derived from plants. This has led to an interest in dyes obtained from the oxidation of CQA in the presence of various amino acids. It has been observed that chlorogenic acid quinone, produced either chemically or enzymatically, reacts with certain amino acids to form chromophores characterized by a green color. This phenomenon may explain the development of staining observed in certain foods and cooking methods, including baking and frying of vegetables such as sweet potatoes, burdock, and sunflower seed meal (Yabuta et al., 1996). However, a model system showed that with Trp a red pigment is produced instead of a green one (Bongartz et al., 2016). Due to the paucity of information on the nature of the red dye formed and the discrepancies observed in the product described by Moccia et al. (2021), one of the aims of this thesis was to analyze and characterize this red pigment.
Various analytical techniques, including UV-Vis and UHPLC-MS analysis, were utilized to attribute the red coloration to specific substances. The investigation then focused on isolating and purifying the red pigment in order to elucidate its structure by NMR and propose a mechanism of formation **(Chapter 2).**
Once the structure was known, another aim was to identify the optimal conditions to promote the formation of the pigment by evaluating the influence of various parameters on the red pigment formation. To get an initial overview of the properties of red pigment, some of its chemical and physical properties were also studied. The potential applications of the red pigment were also addressed, with particular interest in its stability in different food matrices, as this is critical information in assessing the practical utility and potential impact of the pigment in the food context **(Chapter 3)**.

The overarching goal of this thesis is to enhance the knowledge about the red pigment produced by the oxidative coupling of CQA and Trp, and to provide new insights for its production and complete characterization. The thesis also intended to emphasize the potential applications of this pigment in food systems.

The specific objectives of this thesis can be described as follow **(Chapter 2-3)**:

- Study the formation of a red dye given by the reaction of CQA with Trp **(Chapter 2)**.
- Isolation and purification of the red dye to elucidate its structure and propose a reaction mechanism **(Chapter 2)**.
- Optimization the reaction time to make the process more feasible **(Chapter 3)**.
- Investigation of the chemical and physical properties of such pigment **(Chapter 3)**.
- Possible applications and an initial study of the red pigment stability in different food systems **(Chapter 3)**.

Chapter 2

Generation and structure elucidation of a red colorant formed by oxidative coupling of chlorogenic acid and tryptophan

The research summarized in this chapter has been published as

Santarcangelo, A., Weber, F., Kehraus, S., Dickschat, J. S., & Schieber, A. (2023). Generation and structure elucidation of a red colorant formed by oxidative coupling of chlorogenic acid and tryptophan. *Food Chemistry*, 425, 136473.
https://doi.org/10.1016/j.foodchem.2023.136473

The reprint of this publication can be found in **Appendix A** of this thesis. Additional supporting material related to this publication is provided in **Appendix B**.

Author's contributions: My contribution to this research was to set up and carry out the experiment, analyze the data and write the original draft.
The structure elucidation was achieved through the group work I conducted together with Prof. Dr. Fabian Weber, Dr. Stefan Kehraus and Prof. Dr. Jeroen S. Dickschat. The hypothesis regarding the mechanism was formulated by Prof. Dr. Jeroen S. Dickschat.
Prof. Dr. Andreas Schieber, the supervisor of my doctoral dissertation, played a key role in the conceptualization of the work as well as in the writing and editing of the manuscript.

Summary

Color is an important feature in foods, as it has a great impact on consumer´s choice when purchasing.

Synthetic food dyes are currently viewed with skepticism by consumers due to a study linking their use to hyperactivity in children (McCann et al., 2007), despite their ability to provide attractive hues and stability in products, typically limited in natural compounds (Calva-Estrada et al., 2022; Meléndez-Martínez et al., 2022; Zang et al., 2022). Because of these challenges, the food industry is looking for alternative colorants derived from natural sources. Plant sources are usually preferred because ingredients of animal origin, such as carmine, are not accepted by consumers who follow a vegetarian or vegan lifestyle (Meléndez-Martínez et al., 2022; Müller-Maatsch & Gras, 2016).

Oxidative conversion of plant phenols with *o*-dihydroxy structure can lead to the formation of colored compounds. A red reaction product obtained after the oxidation of caffeic acid by polyphenol oxidase can be considered as an example (Weber et al., 2019). Another case involves substituted benzacridines, molecules that are green at pH 9 but turn yellow at lower pH values or in the presence of ascorbic acid, generated by the reaction of CQA with some amino acids (Iacomino et al., 2017; Schieber, 2018; Yabuta et al., 2001).

The study by Bongartz et al. (2016) found out that only the reaction of CQAQ with Trp leads to a red color, while the reaction with other amino acids produces brown or faintly greenish compounds. Subsequent investigations reported in the study by Moccia et al. (2021) demonstrated the formation of cyanine-like structures. However, different reaction products were identified in our laboratory as a result of further studies. Consequently, attention has been given to the isolation and structural elucidation in order to broaden the information on such compounds that might be considered future food colorants.

The CQA oxidation was performed according to previously reported protocols (Iacomino et al., 2017) but using $NaIO_4$ as the oxidizing agent in a TRIS buffer (0.2 M; pH 7.5). The mixture was left for reaction under vigorous stirring and periodically analyzed by UV/Vis spectroscopy and UHPLC-MS. This reaction resulted in the formation of several products, three of which displayed a red color. The red compounds **V, VI**, and **VII** (Figure 1 appendix A) displayed a λ_{max} at 564 nm and had

a molecular ion with an *m/z* value of 735 $[M+H]^+$. Their closely related structures were confirmed by identical fragmentation patterns (Table A1 Appendix B). The reaction was terminated by the addition of hydrochloric acid after 72 hours, resulting in the precipitation of the red pigment, which was subsequently collected by centrifugation and lyophilized. The resulting pigment was found to be partially soluble in water but fully soluble in 0.2 M NaOH solution, ethanol, and DMSO. However, exposure of the pigments to alkaline solutions resulted in a decrease in the peak intensity of all three products and the simultaneous generation of a compound **VIII** (Figure 2 Appendix A), which exhibited a molecular ion at *m/z* 561 $[M+H]^+$, as ascertained by UHPLC-MS analysis. The mass difference of 174 Da indicates the absence of a portion of quinic acid.

For the preparation and isolation of the pigment, the reaction was performed on a larger scale. The pigment was precipitated after 72 hours by addition of HCl and then dissolved in alkaline solution and purified through size exclusion chromatography with a yield of 40% (*w/w*) based on 5-CQA. To obtain a highly pure compound suitable for NMR analysis, the purification procedure was repeated. This yielded a 15% (*w/w*) compound from the 5-CQA precursor.

The compound thus purified was subjected to UHPL-DAD-MS analysis, which showed a single peak with a λ_{max} of 568 and a molecular ion of *m/z* 561 $[M+H]^+$. Its fragmentations are shown in Table A2 Appendix B. The accurate mass value of *m/z* 561.1405 $[M+H]^+$ and molecular formula suggestion of $C_{31}H_{20}N_4O_7$ of the compound **VIII** was obtained through UHPLC-ESI-ToF-MS analyses.

The molecular structure of **VIII** was elucidated by combining NMR data (Figures A2-A8, Appendix B) with UHPLC-MS analyses (Table A2, Appendix B). It was identified as a complex compound composed of two Trp moieties and one caffeic acid moiety (Figure 3A, Appendix A).

Further experiments were conducted as control and labeled compounds were also used. Replacing caffeoylquinic acid with caffeic acid ethyl ester produced a subtle pink hue. Further analysis detected a compound with absorption at 550 nm and a molecular ion at *m/z* 589 $[M+H]^+$ (Table A3 Appendix B). Fragmentation pattern by UHPLC-MS-MS analysis showed this to have the same core structure as **VIII**. Further efforts to isolate and characterize the compound were not pursued due to the extremely low signal intensity. When caffeic acid was oxidized under the reported conditions, no red pigment was formed, and no peak was detected. However, performing the reaction in a diluted solution (1:10 *v/v*) resulted in a slight red hue and detection

of a small peak with *m/z* 561 $[M+H]^+$, which exhibited the same fragmentation as compound **VIII**. The cause of the differing reaction behavior of caffeic acid at lower concentrations requires clarification. When N_α-methyl-L-Trp was used instead of Trp, no red color was observed. However, when 1-methyl-L-Trp was used, a red colored compound was produced. These findings suggest that an unsubstituted α-amino group of Trp was necessary for the reaction to take place. Important structural information of the red colorant was obtained from labeled compounds ($^{15}N_\alpha$)-L-Trp and (1-^{13}C)-L-Trp. Our findings revealed that the red pigment has two α-amino groups, which remain attached to the core during MS/MS experiments, and that the two carboxylic acid groups of Trp are positioned in the red pigment such that they serve as good leaving groups released during MS/MS. The complete fragmentation data are reported in Tables A6 and A7 in appendix B.

A mechanism for generating red pigment **VII**I was also proposed (Figure 5, Appendix A).

The identification of the red pigment is noteworthy as it verifies the notion that there is still inadequate knowledge about the reactions of quinones.

Chapter 3

Improved generation, physicochemical characteristics, and food application studies of a red colorant obtained from oxidative coupling of chlorogenic acid and tryptophan

The research summarized in this chapter has been published as:

Santarcangelo, A., Schulze-Kaysers, N., Schieber A. (2024). Improved generation, physicochemical characteristics, and food application studies of a red colorant obtained from oxidative coupling of chlorogenic acid and tryptophan. *Foods*, 13, 686.
https://doi.org/10.3390/foods13050686

The reprint of this publication can be found in **Appendix C** of this thesis. Additional supporting material related to this publication is provided in **Appendix D**.

Author's contributions: My involvement in this research was to conduct the experiments, deal with data analysis and write the original draft of this paper.
Dr. Nadine Schulze-Kaysers played a crucial role in overseeing the setup and interpreting the design of the experiments, providing valuable assistance in crafting the corresponding content. Prof. Dr. Andreas Schieber, my doctoral dissertation supervisor, played a pivotal role in conceptualizing the work, offering support in supervision, revising the writing, and participating in the editing process.

Summary

The previous chapter aimed to clarify the structure of the pigment and provide a pathway for its formation. However, the physicochemical properties and stability of the pigment require further exploration to fully comprehend its limitations or potential. Additionally, the reaction involving the oxidation of CQA with $NaIO_4$ in the presence of Trp required 72 hours, which is not attractive in terms of production and economic efficiency (Santarcangelo et al., 2023).

The main objective of this chapter is to develop a process for the accelerated generation of the red dye and to characterize its physicochemical properties in order to assess its potential for practical applications.

In an effort to accelerate pigment formation, the influence of various parameters on the color development as described in the following was investigated using the areas of the three peaks detectable at 550 nm as a measured value. The optimization of the red pigment formation process was carried out with the Software Design Expert version 9.0.6.2. To examine the impact of three independent variables, a comprehensive design with three central points (Table S1 appendix D) was evaluated (Trp, $NaIO_4$, temperature). The temperature range of 22–70°C and the reaction duration of 24–72 hours were selected. The quantities of Trp and $NaIO_4$ were employed in a range of 56-140 mM and 1.68-14 mM, respectively. Analysis of the screening results led to the exclusion of temperature increase as a process optimization strategy. This decision was based on two critical observations: a significant decrease in the area of the peaks corresponding to the three target compounds, detected by UHPLC at 550 nm, and the undesirable development of a brown hue in the final product. These negative effects could be attributed to a number of undesirable reactions favored by high temperatures. A preferential oxidation of CQA can be hypothesized, followed by the formation of quinones that further react to produce high molecular weight compounds (Martinez & Whitaker., 1995). These competitive processes would occur before the desired condensation with Trp, preventing optimal formation of the red pigment of interest.

In conclusion, increased temperature, rather than promoting red pigment synthesis, appears to promote side reactions that compromise the yield and quality of the desired product, indicating that the reaction should preferably be conducted at room temperature. Although temperature

tests outside this range may be appealing, it is crucial to evaluate the additional energy costs associated with cooling and the potential impact on the feasibility of the process.

In a next step, the effect of the concentrations of Trp and $NaIO_4$ (56–140 mM and 3.50–14 mM, respectively) was analyzed in a factorial design with four center points (Table S2, Appendix D), resulting in 12 experiments. A modest increase in pigment yield was observed with increasing concentrations of Trp. However, the solubility of Trp was a limiting factor preventing the use of higher concentrations. Therefore, the optimal Trp concentration was maintained at 56 mM. This value represents the best compromise between a good pigment yield and the solubility limits of the amino acid under the reaction conditions studied. The study also showed a significant increase in absorption peaks at 550 nm when $NaIO_4$ concentrations were increased to match those of CQA. However, the reaction was less efficient when a higher amount was used.

The optimization led to the following parameter settings: 14 mM CQA, 56 mM Trp, 14 mM $NaIO_4$, 22 °C and a reaction time of 24 h.

The optimized reaction was tested at various pH values, with pH 9 being optimal for reaction development. Although pH 9 without $NaIO_4$ produced a red pigment with a similar chromatographic profile, the reaction was slower and yielded lower amounts than a combination of pH 9 and $NaIO_4$. In conclusion, the combination of alkaline pH and $NaIO_4$ successfully increased the yield of red pigments while reducing the reaction time from 72 hours to 24 hours. After finding the optimal conditions, a scale-up was performed, and after an initial purification by molecular exclusion chromatography, the pigment was found to have a slightly higher yield (46% *w/w*) than in our previous study (Santarcangelo et al., 2023).

The red pigment was then tested for its solubility and was found to be insoluble in water but completely soluble in ethanol and 0.06 M NaOH. When the pigment dissolved in ethanol was diluted with water, it formed a suspension, likely due to its insolubility in an acidic environment created by the addition of HCl. However, complete solubility was maintained when the dye was dissolved in an alkaline solution and then diluted with water. After prolonged exposure to alkaline solutions, the peak intensity of all three isomers decreased, favoring the formation of a single compound by releasing the quinic acid moiety, which was previously characterized in **Chapter 2**. The three red compounds were completely converted into their hydrolyzed form after 4 hours in 0.06 M NaOH.

The stability of the hydrolyzed CQA-Trp pigment was subsequently characterized by examining its resistance to changes in pH, light, and heat. The hydrolyzed form was preferred for application studies due to the potential impact of isomerization and quinic acid release during processing and storage. The pigment was found to be very stable under alkaline conditions, but tended to precipitate over time when exposed to an acidic environment. In addition, it was found to be particularly stable for 28 days in the dark, while being photosensitive as the period of exposure to light increased. The thermal stability of the hydrolyzed pigment was additionally studied and compared with a commercial preparation of carmine used in food applications; however, only half the concentration of the new pigment was required to obtain similar absorbance values. Mixing the pigment with ascorbic acid/cysteine at pH 3.6 for 24 h showed no change in absorbance, but at pH 7 and with ascorbic acid, the absorbance decreased by 30% during 24 h, probably because of the acidic nature of ascorbic acid. No changes occurred with cysteine (Figure S4, Appendix D), indicating that interactions with the thiol group that could affect color stability did not occur under the conditions investigated.

Food application tests with different matrices yielded more than satisfactory results with all model foods tested (e.g., yogurt, meringue, milk and plant-based alternative) using the pigment in amounts less than 0.01% w/w. With the exception of the meringue, which was only evaluated over a 14-day period because of its limited storage stability, color stability was monitored over a 28-day period in the CIELAB color space. The a* values, which represent the redness value when they are positive, did not show any significant changes during this period (Table S4, Appendix D). The optimization of the pigment formation process described in this study resulted in shorter production times and higher yields, which is a significant advance for the commercial success of the new pigment. Our analysis of the physicochemical properties suggests that it might be a suitable food coloring agent. However, like all food additives, its use is subject to approval by the relevant authorities. Therefore, it is critical to demonstrate the safety of this compound through comprehensive toxicological evaluations and to address regulatory issues related to its inclusion in the list of approved food additives.

Chapter 4

Summary and Outlook

The significance of color in our perception and understanding of the world around us is well-documented (Elliot & Maier, 2014). In the past, its importance was largely related to survival and communication. However, contemporary research indicates that color also influences our daily choices, particularly in the context of food (Viera et al., 2019). The food industry, recognizing the importance of consumer preferences for foods with a vivid color palette, often employs colorants to modify or preserve the visual appearance of products (Saltmarsh & Insall., 2013).

Food dyes can be classified by source, color, or chemical structure (Viera et al., 2019). Another common distinction is natural versus synthetic (Ranaweera et al., 2020). According to Mohamad et al. (2019), natural dyes are produced by nature (obtained from plants, animals, or minerals), although there is no universally accepted definition of "natural" or "artificial" in the context of food additives. While natural flavorings are defined and regulated by the FDA and the EU, the concept of naturalness for food colorings remains more nuanced (Viera et al., 2019). Over time, a growing number of consumers have expressed concern about the use of synthetic additives, including colorants, and have showed increasing interest in more natural alternatives (Mabuza et al., 2023).

Plant sourced pigments, such as anthocyanins, betalains and carotenoids, hold promise for vegetarians and vegans, but present certain limitations due to their sensitivity to external factors such as heat, oxygen, light, and pH (Calva-Estrada et al., 2022; Nabi et al., 2023; Meléndez-Martínez et al., 2022; Prieto-Santiago et al., 2020; Zang et al., 2022). In response to the challenges outlined above, research such as Iacomino et al. (2017) has been directed toward the potential of natural products obtained by oxidative coupling of CQA and amino acids, which are capable of producing colored reaction products. This discovery offers promising avenues for overcoming limitations in the production of natural food dyes.

When CQA is added to egg white, which has an alkaline pH, the acid is oxidized to its quinone, which subsequently reacts with amino acids to form benzacridines (Iacomino et al., 2017). Instead of pure CQA, coffee powder may also be used, which contains CQA as a major component (Moores et al., 1948). Consequently, these green colorants may also be generated in foods without the use of oxidizing agents and be considered a “natural” colorant.

Model studies have demonstrated that the reaction between CQA and Trp is of particular interest due to the production of a bright red pigment in contrast to the commonly observed green color (Bongartz et al., 2016). While the occurrence of this red pigment in nature remains uncertain, it has attracted considerable scientific interest. The exceptional behavior of Trp relative to other amino acids represents an intriguing area of study that required further scientific investigation.

As reported by Moccia et al. (2021), the red coloration can be attributed to the presence of two systems exhibiting a push-pull chromophore. One of these systems was identified as an unusual benzochromene (2,3-b) indole, which was associated with a Trp unit and characterized by a cyanine-type chromophore.

At the time of publication of the previously mentioned article, the author of this thesis was already engaged in the investigation of the interactions between polyphenols and specific amino acids. Preliminary mass spectrometry examinations conducted by the author revealed the presence of additional red reaction products, prompting to further pursue the topic. Consequently, efforts have been directed toward analyzing the dye to investigate its composition (**Chapter 2**).

The CQA oxidation was carried out in accordance with previously published protocols (Iacomino et al., 2017) but using $NaIO_4$ as the oxidizing agent in a TRIS buffer (0.2 M; pH 7.5). UV/Vis spectroscopy and UHPLC-MS were used to regularly evaluate the mixture during the reaction. This reaction produced a number of compounds, three of which were red in color. The red compounds had a molecular ion with a *m/z* value of 735 $[M+H]^+$ with an absorption maximum at 564 nm and identical fragmentation patterns, verified their closely related structures (Table A1 appendix B). Following the addition of 6 M HCl, precipitation of the red pigment was observed, after which it was collected by centrifugation and subsequently lyophilized. The powder obtained was dissolved in a small amount of NaOH and subjected to initial purification by molecular exclusion chromatography, a separation method based on molecule size. The resulting fractions

were combined, acidified, and the precipitate was collected through centrifugation and freeze-dried. The yield obtained was 40 % *(w/w)* based on 5-CQA educt. The pigment thus obtained was then tested for solubility in various solvents such as water, ethanol, DMSO and 0.2 M NaOH. Using UHPLC-MS analysis, it was observed that exposure of the powder to an alkaline environment resulted in a decrease in the area of the three peaks at 550 nm and the development of a new peak at the same wavelength with a molecular ion of *m/z* 561 $[M+H]^+$. The difference in mass of 174 Da was due to quinic acid release.

To determine its molecular structure, additional step of purification was necessary that included the dissolution the pigment in NaOH, in order to completely hydrolyzing it, and purifying it through molecular exclusion chromatography. This resulted in a pure compound with a yield of 15% *(w/w)* based on the 5-CQA educt. The decision to characterize the compound in its hydrolyzed form was made due to the difficulty in separating the three peaks in the nonhydrolyzed form during the purification process, as they exhibited similar retention times.

The final compound was analyzed by UHPLC-DAD-MS, in both positive and negative modes, and then by UHPLC-ESI-ToF-MS, increasing the precision and accuracy in mass determination. Finally, the elucidation of the molecular structure described in **Chapter 2** was achieved by combining the previously obtained information with that obtained by one- and two-dimensional NMR spectroscopy, thus fulfilling one of the main objectives of this thesis. This discovery led to the identification of a novel category of compounds, representing a significant advancement in scientific knowledge.

To gain more information about the behavior of the reaction, several tests were performed with similar compounds. With CQA or Trp alone, the reaction did not produce a red color. CQA alone developed a brown color, probably due to polymer formation (Bongartz et al., 2016; Martinez & Whitaker, 1995). This underscores that the presence of both reactants is a fundamental requirement for the red staining we are interested in to occur. When CQA was replaced with caffeic acid ethyl ester, a slight pink coloration was observed. Additional UHPLC-MS analysis revealed a single peak detected at 550 nm with a *m/z* 589 $[M+H]^+$. Fragmentation patterns of this compound indicated that it had the same core structure as **VIII**. Further attempts to isolate and characterize the compound were not pursued due to its extremely low signal intensity. To confirm the significance of the ester group, an experiment was conducted using simple caffeic acid. In this

case, the brown coloration was observed due to the high reactivity of this compound, which prefers other patterns of formation (Cilliers & Singleton, 1989). In contrast, a slight red coloration was observed when the reaction was performed in diluted solution, which analyzed with HPLC-DAD-MS reported a *m/z* 561 $[M+H]^+$ mass peak that exhibited the same fragmentation pattern as the isolated compound. It has not yet been determined why caffeic acid behaves differently at lower concentrations. When N_α-methyl-L-Trp was used instead of Trp, no red color was observed. However, when 1-methyl-L-Trp was used, a red colored compound was produced.

The use of Trp educts labeled in the reaction enables the identification of two α-amino groups in the structure of the red pigment that remain attached to the core during MS/MS experiments. In addition, the presence of two carboxylic acid groups belonging to Trp in the final structure of the red pigment, which are released during MS/MS, was confirmed. A mechanism for generating red pigment **VIII** was also proposed (Santarcangelo et al., 2023).

The formation of red pigment serves to confirm that quinone reactions continue to present significant gaps in our understanding, with the potential for generating new functional compounds. As evidenced by the findings reported here and in other studies (Vercruysse, 2019), variations in the concentration of reactants influence the formation of different products. In the case of the phenolic compounds studied, three principal reactions can be distinguished. When an excess of phenolic acid is present, a brown polymer is formed. In the presence of an equal amount of Trp and CQA, a greenish color results, which is due to benzacridines (Bongartz et al., 2016; Moccia et al., 2021; Vercruysse, 2019). Conversely, an excess of Trp results in the formation of a red pigment, in accordance with the proposed mechanism.

While an understanding of the chemical structure of a pigment and its formation process is of paramount importance, it is equally crucial to improve its attractiveness from a production standpoint. Despite its simple formation, the generation of the desired pigment required a significant amount of time, 72 hours. To ensure economic efficiency and enhance its appeal to potential consumers, it was necessary to optimize the production process, reducing the time required without compromising yield. To achieve this goal, the protocol needed an optimization that was accomplished with the use of Design Expert Software version 9.0.6.2 **(Chapter 3)**.

A complete design was evaluated with three central points, including Trp concentration, $NaIO_4$ concentration, and temperature (Table S1, Appendix D). The temperature range was set between

22°C and 70°C, and the reaction duration was controlled between 24 and 72 hours with 24 hours intervals. Based on the screening results, it was decided to exclude an increase in temperature as a factor. Higher temperatures resulted in a decrease in the peak area of the three compounds detected by UHPLC at 550 nm and the development of a brown hue. Another set of experiment was conducted with a factorial design with four center points to analyze the effect of Trp and $NaIO_4$ concentrations which resulted in 12 experiments. A positive correlation between increasing concentrations of Trp was demonstrated. However, a new issue arose due to its limited solubility. Therefore, the concentration of Trp was maintained at the previous value of 56 mM. A higher peak area of the red compounds detected by UHPLC was obtained at a $NaIO_4$ concentration of 14 mM. However, higher concentrations were also analyzed (21, 28 and 42 mM), which showed a small peak area of the red compounds when compared to the values obtained from 14 mM. At that point, the reaction parameters were 14 mM CQA, 56 mM Trp, 14 mM $NaIO_4$, 22°C with reaction time of 24 hours.

Finally, the impact of the pH on the progress of the reaction was evaluated. Therefore, the same reaction was conducted at three different pH levels: slightly acidic, medium, and alkaline. The formation of red color was not observed under acidic conditions, supporting the theory that a nucleophilic addition requires at least a neutral and slightly basic environment. The optimal pH for the reaction resulted to be alkaline. A control reaction was performed without $NaIO_4$, which also proceeded, but more slowly and with lower yields. The final result indicated that the combined use of alkaline pH and $NaIO_4$ significantly enhanced the yield of red pigment from 40 % (**Chapter 2**) to 46 ± 2% (**Chapter 3**) *(w/w)* on a CQA basis and reduced the reaction time from 72 to 24 hours. This improvement represents a significant step toward the commercial viability of the new pigment. Thus, another aim of the thesis was achieved.

Once it has been established that the generation of the red pigment can be made more feasible and attractive, it is essential to characterize some of its physicochemical properties, such as solubility in various solvents and stability to heat and light.

After initial purification by molecular exclusion chromatography as described in **Chapter 2**, the pigment obtained was confirmed to be completely soluble in solvents such as ethanol and 0.06 M NaOH, but not in water. However, upon dilution with water, the pigment solution in ethanol formed a suspension, probably due to the insolubility of the pigment in the acidic environment

created by the addition of hydrochloric acid. Nonetheless, the dye was completely soluble when dissolved in an alkaline solution and then diluted with water. After prolonged exposure to alkaline solutions, the peak intensity of all three isomers decreased favoring the formation of compound **VIII** through the release of the quinic acid moiety. The three red compounds were completely converted into their hydrolyzed form, characterized in **Chapter 2**, after four hours in 0.06 M NaOH solution.

The stability of the hydrolyzed CQA-Trp pigment was evaluated through tests for resistance to changes in pH, light and heat. Since isomerization and quinic acid release of CQA may occur during processing and storage, the hydrolyzed form was preferred for application studies. The pigment showed high stability under alkaline conditions, but precipitation occurred in very acidic environments, limiting its performance in certain acidic food matrices. Compared with anthocyanins, this compound offers significant advantages by solving the problem of color variation at different pH levels, making it an ideal candidate for a wide range of food applications. In addition, the pigment was demonstrated to be photosensitive, exhibiting a proportional decrease in color intensity with increasing duration of light exposure. However, when stored in the dark, the pigment remained stable for a period of 28 days. This suggests that when protected from light exposure, the pigment can maintain its properties for a significant period of time. To minimize light exposure and ensure pigment stability during storage or use in beverages, the use of opaque containers or bottles with matte inner linings is an effective methodology to maintain its distinctive characteristics.

The thermal stability of the hydrolyzed pigment was also tested and compared with a commercial preparation of carmine used in food applications. Interestingly, the new pigment required only half the concentration of the commercial preparation to achieve similar absorbance values, and their stability to heat treatment was comparable. This observation is of particular significance as carmine is known to be thermally stable, in contrast to betalains and anthocyanins which, by comparison, tend to be less stable at high temperatures (Calva-Estrada et al., 2022; Müller-Maatsch & Gras, 2016; Oancea, 2021). In addition, the stability of the pigment in the presence of reducing compounds such as cysteine and ascorbic acid needed to be evaluated, as they are commonly found in foods and used as additives. When the pigment was mixed with ascorbic acid/cysteine at pH 3.6 for 24 hours, there was no change in absorbance. However, when mixed

with ascorbic acid at pH 7, the absorbance decreased by 30% over 24 hours, likely due to the acidic nature of ascorbic acid. No interactions with the thiol group that could affect color stability were observed with cysteine under the conditions investigated. These results highlight the importance of the pH value of food matrices intended to be colored with the new pigment.

Food color application tests have also been conducted using a variety of matrices to assess feasibility, and all of the model foods tested gave satisfactory results when the pigment was used at levels of less than 0.01% *w/w*. Color stability was monitored over a 28-day period in the CIELAB color space, with the exception of the meringue, which was only evaluated for 14 days due to limited storage stability. These results provide valuable information on the pigment's ability to maintain its color and integrity under different food conditions and suggest its potential use in a variety of food applications (Santarcangelo et al., 2024).

The future use of this colorant is, of course, contingent upon the necessary approvals by the relevant authorities. Detailed toxicological studies are essential to ensure its safety for human consumption and to address regulatory issues related to its inclusion in the list of approved food additives.

Although the classification of the pigment as "natural" remains an open question due to the lack of clear regulations (Viera et al., 2019), its derivation from natural products may promote its acceptance by consumers, especially those who are concerned about the naturalness of ingredients.

In summary, the potential of the red compound as a future food colorant can be considered promising (Santarcangelo et al., 2024). However, technical and regulatory challenges need to be overcome before it can be effectively commercialized. Optimizing the stability of the compound, as well as compliance with current regulations, will be the focus of future investigations.

In conclusion, the author of this thesis believes that the participation in this discovery has helped to expand the actual knowledge about the formation of red colorant based on the reaction of plant phenols and amino acids and understand its characteristic and potential use as food coloring. Furthermore, the author suggests that further research be conducted on this topic in order to facilitate its inclusion in the list of approved additives.

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Appendix

A. Publication 1

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Generation and structure elucidation of a red colorant formed by oxidative coupling of chlorogenic acid and tryptophan

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ABSTRACT

In view of the poor acceptance of synthetic food colorants by consumers, there is intense interest in novel natural compounds, preferably from plant-derived sources. We oxidized chlorogenic acid using $NaIO_4$ and reacted the resultant quinone with tryptophan (Trp) to obtain a red-colored product. The colorant was precipitated, freeze-dried, purified by size exclusion chromatography, and subsequently characterized using UHPLC-MS, high-resolution mass spectrometry, and NMR spectroscopy. Additional mass spectrometric studies were performed on the reaction product generated with Trp educts labeled with ^{15}N and ^{13}C. The data obtained from these studies allowed the identification of a complex compound consisting of two Trp and one caffeic acid moieties, and the proposition of a tentative pathway of its formation. Thus, the present investigation expands our knowledge about the formation of red colorants based on the reaction of plant phenols and amino acids.

1. Introduction

Color is an extremely important quality trait of foods and has a huge impact on consumer decision whether or not to purchase a food item. Synthetic food colorants are cost efficient, impart attractive color hues, and are stable toward heat and light and over a wide pH range. However, like many other synthetic food additives, they are suffering from poor consumer acceptance, especially since the "Southampton Study" suggested an association between the intake of artificial food dyes and hyperactivity of children (McCann et al., 2007). Although scientifically debatable, as evidenced by the EFSA Scientific Opinion, regulation EC 1333/2008 was amended such that foods containing certain dyes must be labeled "may impair activity and alertness of children".

As a result of the above challenges, the food industry is continuously looking for alternate colorants from natural sources. These should preferably originate from plants because animal-derived compounds such as carmine are not accepted by vegetarians and vegans (Meléndez-Martínez et al., 2022; Müller-Maatsch & Gras, 2016). Although such alternatives are already available and approved for food use, for example, anthocyanins, betalains, and carotenoids, their application is not without problems, due to their sensitivity to oxidation, enzymatic degradation, heat, light, and changes in pH values (Zang et al., 2022; Calva-Estrada et al., 2022; Meléndez-Martínez et al., 2022).

Oxidative conversion of plant phenols with an *o*-dihydroxy structure may lead to the formation of colored compounds. After oxidation of caffeic acid with polyphenol oxidase, a red reaction product was obtained (Weber et al., 2019). Chlorogenic acid quinone generated either chemically or enzymatically may react with certain amino acids to form substituted benzacridines (Schieber, 2018; Iacomino et al., 2017), which are intensely green at pH 9 but are converted into yellow compounds at lower pH values. In addition, they show color changes in the presence of ascorbic acid (Yabuta et al., 1996). Interestingly, while the reaction with some other amino acids yields brown or faint greenish compounds, only the reaction of chlorogenic acid quinone with tryptophan leads to a red color (Bongartz et al., 2016). Subsequent investigations into the underlying compounds demonstrated the formation of cyanine-type structures (Moccia et al., 2021). Mass spectrometric studies performed in our lab indicated the presence of additional red reaction products

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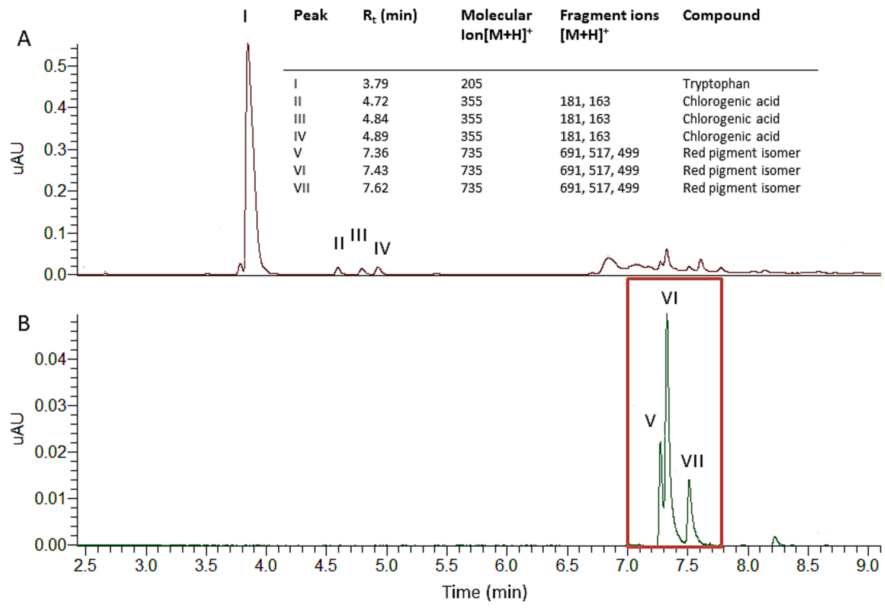

Fig. 1. UHPLC-DAD chromatogram (280 nm (A) and 550 nm (B)) of the model solution containing Trp and CQA after 72 h; 1:50 v/v dilution in water.

which did not match the data reported by Moccia et al. (2021). Therefore, we aimed to isolate and elucidate the structure of a red colorant in an effort to expand the database of compounds that may potentially be used as future natural food colorants. For this purpose, chlorogenic acid was oxidized with $NaIO_4$ in the presence of L-tryptophan. The red fraction was dissolved in an alkaline solution and subsequently purified by size exclusion chromatography. The repetition of this process allowed the isolation of the target compound. Its structure was elucidated by mass spectrometry and NMR spectroscopy, with additional mass spectrometry studies being carried out using ^{15}N- and ^{13}C-labeled Trp.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid (PubChem CID: 1794427) (CQA; ≥95 %) was obtained from BLDpharm (Shanghai, China). L-Tryptophan (PubChem CID: 6305), caffeic acid (PubChem CID: 689043) (≥95 %), ethyl caffeate (PubChem CID: 5317238) (≥90 %), 1-methyl-L-tryptophan (PubChem CID: 676159), N_α-methyl-L-tryptophan (PubChem CID: 160511), ($^{15}N_\alpha$)-L-tryptophan (PubChem CID: 12209732), (1-^{13}C)-L-tryptophan (PubChem CID: 71309644) and sodium metaperiodate ($NaIO_4$) (PubChem CID: 23667635) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Hydrochloric acid was purchased from VWR International GmbH (Darmstadt, Germany) and used further without purification.

2.2. Small-scale preparation of the red colorant

The oxidation of CQA was performed according to protocols reported previously (Iacomino et al., 2017). However, in the present study, $NaIO_4$ was used as the oxidizing agent. Solutions of Trp (112 mM), $NaIO_4$ (7 mM), and CQA (28 mM) buffered in Tris buffer (0.2 M; pH 7.5) were mixed (1:1, *v/v*). The mixture was reacted under vigorous stirring (40 rpm, 72 h) using a Variomag Thermomodul 40 ST (HP Labortechnik, Oberschleißheim, Germany) and periodically analyzed by UV/Vis spectroscopy and UHPLC-MS in 24 h intervals after dilution with water (1:50, *v/v*). In additional experiments the reaction was performed under the following settings: i) in the absence of either Trp or CQA; ii) replacement of CQA with ethyl caffeate or caffeic acid; iii) replacement of Trp with 1-methyl-L-Trp, N_α-methyl-L-Trp, ($^{15}N_\alpha$)-L-Trp, or (1-^{13}C)-L-Trp. The reaction with caffeic acid was carried out also in dilute solution (1:10, *v/v*).

The red fraction formed from the reaction between CQA and Trp was precipitated by acidification with 6 M HCl to pH 1, centrifuged (10000 rpm, 15 min, 4 °C) and freeze dried. The pigment was hydrolyzed in a minimum volume of 0.2 M NaOH for 2 h and analyzed by UHPLC-MS before and after hydrolysis.

2.3. Preparation and isolation of the red pigment by reaction of CQA and Trp

In a second set of experiments, the reactions were performed as described in Section 2.2 but on a larger scale. For this purpose, 1 M equivalents of CQA (0.25 g) and 4 M equivalents of Trp (0.57 g) reacting in the presence of $NaIO_4$ (0.04 g; 0.25 M equivalents) were used. For the isolation of the red compound, the precipitate obtained after the hydrolysis with 0.2 M NaOH for 2 h was purified on a Sephadex G-10 column using water as the eluent. Fractions of 10 mL were collected, diluted with water (1:50, *v/v*), and analyzed spectrophotometrically. The fractions showing an absorption maximum at approximately 560 nm were combined and the solution was acidified to pH 1 using 6 M HCl to precipitate the colorant. After centrifugation, the precipitate was collected and freeze-dried. In total, 0.1 g of a red powder was obtained, corresponding to a 40 % (*w/w*) yield based on CQA. Because subsequent analyses still revealed the presence of impurities, the purification step was repeated. Finally, 36 mg (15 % *w/w* yield based on CQA) of the purified red powder was obtained, which was used for structure elucidation by MS and NMR spectroscopy. The protocol described here was highly reproducible, with comparable results being obtained in three replications, and was used in subsequent experiments when additional material was required for structural characterization.

2.4. Instrumental analysis

UV/Vis absorption spectra were recorded on a V-730 JASCO double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany) between 400 and 800 nm in a 1 mm path-length glass cuvette

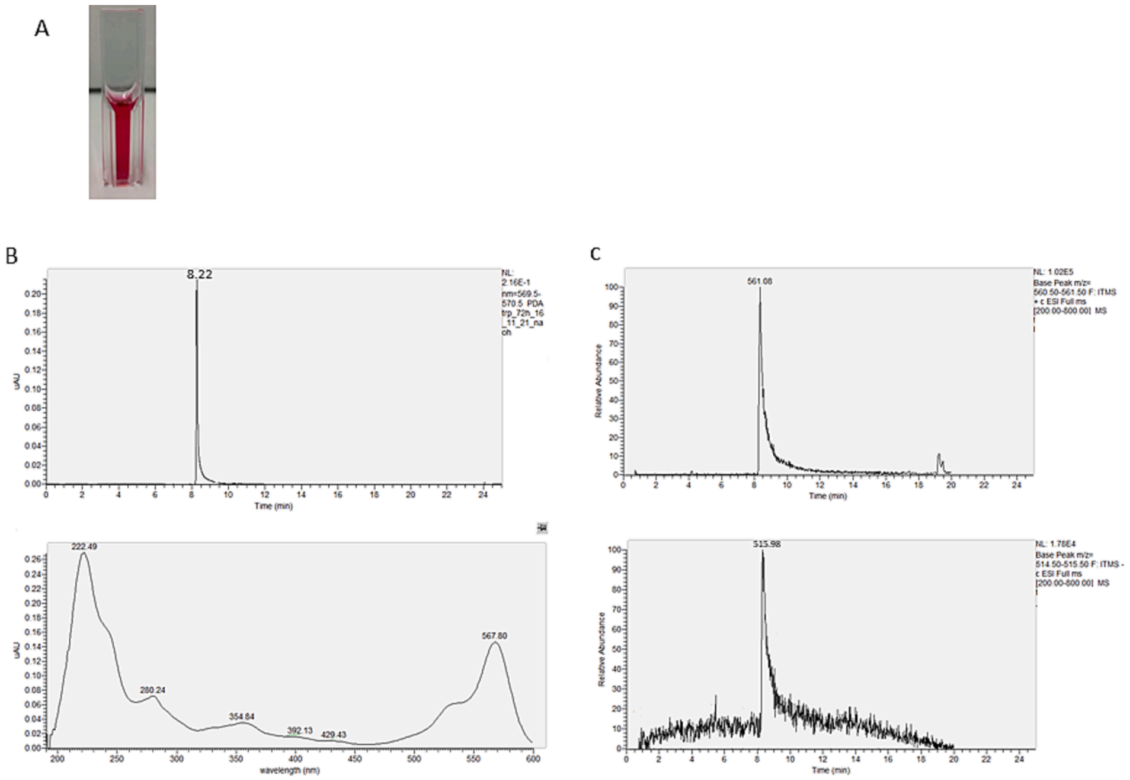

Fig. 2. Solution of the purified compound (0.18 mg/ml in DMSO) (A). UHPLC-DAD chromatogram (570 nm) of the purified compound A diluted in water (1/10; *v/v*) (top) and its UV–vis spectrum (bottom) (B). ESI/MS spectrum in the positive (top) and negative (bottom) ionization mode of the compound eluting at 8.22 min (C).

(Hellma GmbH & Co. KG, Müllheim, Germany).

UHPLC analysis of the reaction products was performed on an Acquity UPLC I-Class system (Waters, Milford, MA, USA). The apparatus consisted of a sample manager cooled at 10 °C, a binary pump, a column oven, and a diode array detector scanning from 200 to 600 nm. The column oven temperature was set at 40 °C. An Acquity HSS-T3 RP18 column (150 mm × 2.1 mm; 1.8 μm particle size) was combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm × 5 mm, 1.8 μm), both from Waters (Milford, MA, USA). The separation was performed with water (A) and acetonitrile (B) as eluents, both acidified with 0.1% (*v/v*) formic acid.

A gradient elution program at a flow rate of 0.4 mL/min was used as follows (min/%B): 0/2; 3/14; 5/15; 6/30; 12/40; 20/100; 23/100; 23.1/2; 25/2. The injection volume was 5 μL.

For MS analysis, the UHPLC was coupled with an LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Waltham, MA, USA), which was equipped with an electrospray interface operating in positive and negative ion mode. Mass spectra were recorded in the range of *m/z* 300–800. For positive mode the source voltage was kept at 4.5 kV at a current of 100 μA, and the tube lens was adjusted to 50 V. The capillary temperature was set at 350 °C with a spray voltage of 4.5 V. Nitrogen was used as the sheath, auxiliary, and sweep gas at a flow of 70, 10, and 1 arbitrary units, respectively. Xcalibur software (2.2SP1.48, Thermo Scientific, Inc., Waltham, MA, USA) was used to evaluate the data.

For the determination of the exact molecular mass, the UHPLC was connected to a Vion IMS QTOF mass spectrometer (Waters, Milford, MA, USA) operating in the positive ion mode. The capillary voltage was 0.8 kV, the source temperature was 120 °C, the cone voltage was 50 V, the desolvation gas temperature was 550 °C and the desolvation gas flow was 700 L/h. The measurements were conducted with automatic lock correction. Nitrogen was used as the drift and collision gas and the MS mode was high definition with low collision energy of 6 eV and a high collision energy ramp of 20 eV. Data were acquired and processed using UNIFI software v1.9.2.045 (Waters, Milford, MA, USA).

NMR experiments (^{1}H, ^{13}C, DEPT-135 and two-dimensional COSY, ROESY, HMBC, and HSQC) were carried out on a Bruker Ascend 600 spectrometer equipped with Prodigy cryoprobe (Rheinstetten, Germany). The sample was dissolved in d_6-DMSO (99.8% D, Deutero, Kastellaun, Germany). Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 2.50/39.5.

3. Results and discussion

The oxidation of CQA with $NaIO_4$ in the presence of Trp for 72 h led to the formation of several products, three of them showing a red color. The separation by UHPLC of the compounds present in the reaction mixture is illustrated in Fig. 1. Compound **I** represents unreacted Trp, whereas compounds **II-IV** were identified as CQA isomers. Although only 5-caffeoylquinic acid had initially been used for oxidation, this compound is known to be susceptible to isomerization reactions (Xie et al., 2011). The red compounds **V**, **VI** and **VII** showed an identical absorption maximum at 564 nm and a molecular ion at *m/z* 735 $[M + H]^+$ in the positive ionization mode. All compounds had identical fragmentation patterns (Table A1), which indicated that their structures are closely related. Dissolution experiments showed that the red pigments are only partially soluble in water but completely soluble in 0.2 M NaOH solution, ethanol, and DMSO. However, UHPLC analyses revealed that exposure of the pigments to alkaline solutions led to a decrease in the peak intensity of all three products and to the concomitant formation of a single compound **VIII**, which showed a molecular ion at *m/z* 561 $[M + H]^+$. The mass difference of 174 Da corresponds to the loss of one quinic acid moiety. For structure elucidation of the colored moiety, this hydrolysis product was purified on a Sephadex G-10 column. The purification protocol proved to be highly reproducible and led to yields of

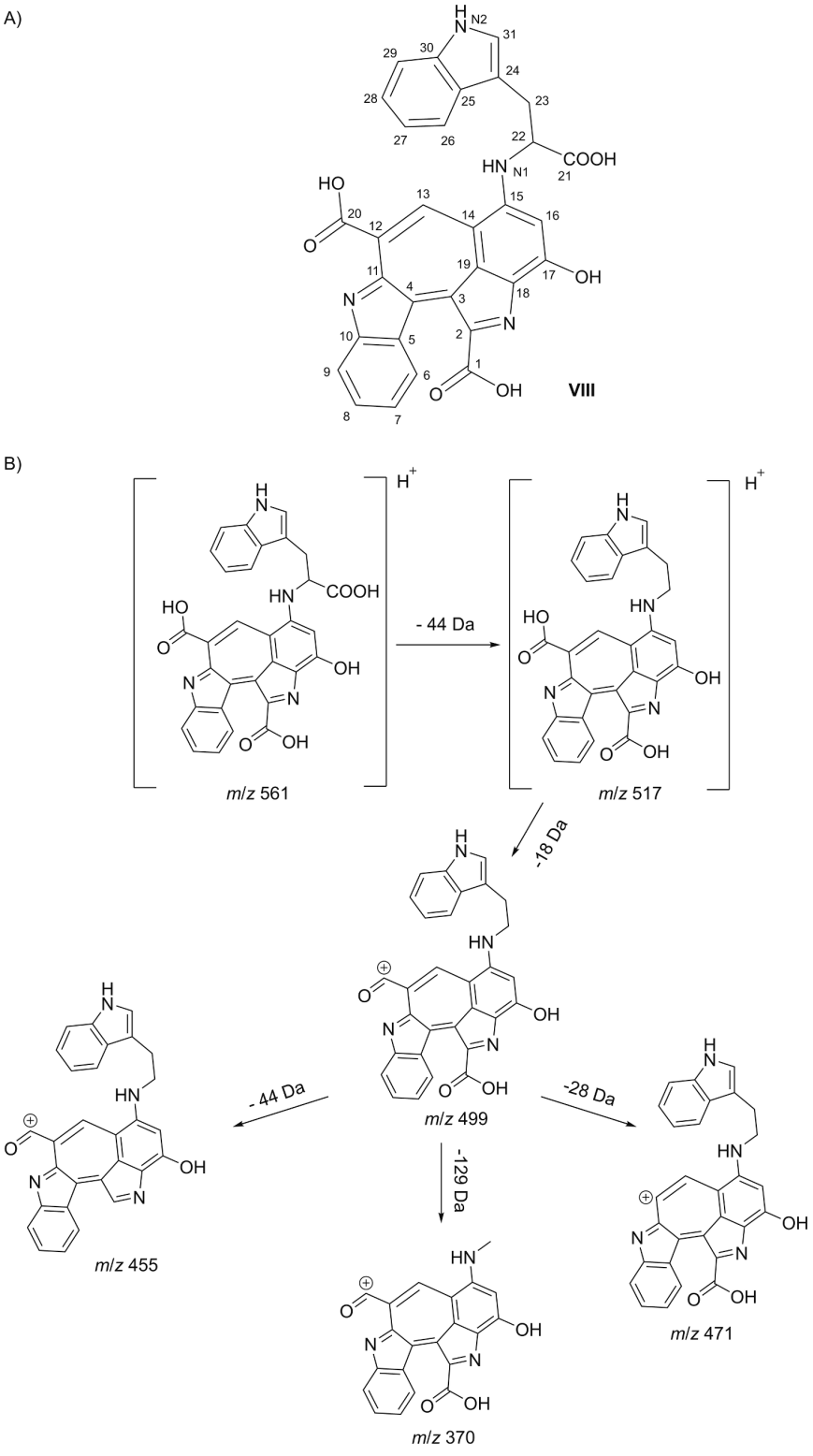

Fig. 3. Structure of the isolated compound **VIII** (A) and its proposed MS fragmentation pattern (B).

Table 1
NMR data (DMSO-d_6) of red compound **VIII**.

Position	type	^{13}C	^{1}H	HMBC
C1	C_q	169.3	–	
C2	C_q	123.3	–	
C3	C_q	107.9	–	H13
C4	C_q	125.2	–	H6, H9
C5	C_q	116.8	–	H6, H7, H9
C6	CH	125.0	8.57 (br d, J = 8.0 Hz)	H11
C7	CH	126.3	7.78 (td, J = 8.0, 1.2 Hz)	H9
C8	CH	133.0	8.00 (td, J = 8.0, 1.2 Hz)	H6, H7
C9	CH	120.9	8.33 (br d, J = 8.0 Hz)	H7
C10	C_q	135.6	–	H6, H8
C11	C_q	121.9	–	H13
C12	C_q	143.9	–	H13
C13	CH	136.1	9.94 (s)	
C14	C_q	106.5	–	H16, NH1
C15	C_q	157.5	–	H13, H16, H22, NH1
C16	CH	99.4	7.00 (s)	NH1
C17	C_q	165.5	–	H16, NH1
C18	C_q	131.8	–	H13, H16
C19	C_q	142.4	–	H13, H16
C20	C_q	169.0	–	H13
C21	C_q	171.9	–	H22, H23
C22	CH	58.1	4.90 (br q, J = 8.0 Hz)	H23
C23	CH_2	26.8	3.60 (d, J = 8.0 Hz, 2H)	H22, H31, NH1
C24	C_q	109.7	–	H22, H23, H26, H31, NH2
C25	C_q	127.0	–	H23, H26, H27, H29, H31, NH2
C26	CH	118.2	7.74 (br d, J = 7.7 Hz)	H28
C27	CH	118.5	6.97 (td, J = 7.7, 1.2 Hz)	H29
C28	CH	121.0	7.01 (td, J = 7.7, 1.2 Hz)	H26
C29	CH	111.4	7.27 (dt, J = 7.7, 1.2 Hz)	H27
C30	C_q	136.7	–	H26, H28, H31, NH2
C31	CH	123.9	7.30 (d, J = 2.2 Hz)	H23, NH2
N1	NH	–	9.85 (d, J = 8.0 Hz)	
N2	NH	–	10.90 (br d, J = 2.2 Hz)	

15 % (*w/w*) based on the 5-CQA educt. Considering the initial molar concentration of CQA, the final compound was obtained in a molar yield of 9 %. Although this might be considered low, it should be kept in mind that quinones are unstable entities that may readily react to form high-molecular condensation products (Cheynier et al., 1988). After purification, UHPLC-DAD-MS analysis showed a single peak with an absorption maximum at 568 nm, a molecular ion at *m/z* 561 $[M+H]^+$ in the positive mode, and a fragment at *m/z* 515 $[M-CO_2-H]^-$ in the negative mode (Fig. 2). UHPLC-ESI-ToF-MS revealed an accurate mass of *m/z* 561.1400 $[M + H]^+$, suggesting the molecular formula of $C_{31}H_{20}N_4O_7$ (calc. *m/z* 561.1405 $[M + H]^+$). From these data, a tentative composition consisting of one caffeic acid moiety and two Trp moieties could be derived. NMR spectroscopic experiments led to the suggested structure **VIII** given in Fig. 3. The ^{1}H and ^{13}C NMR data are summarized in Table 1 and the interpretations from correlation experiments are shown in Fig. 4.

The structure elucidation of **VIII** turned out to be particularly challenging because of the large number of 18 quaternary carbons. The ^{1}H spectrum revealed signals of 11 aromatic methines in addition to one aliphatic CH group, two NH groups and one aliphatic CH_2 group, the latter being confirmed by the DEPT135 signal at 26.8 ppm. Two sets of triplets of doublets (H-7/H-8; H-27/H-28) can be explained by two indole rings from two Trp moieties that were further supported by ^{1}H, ^{1}H-COSY and HMBC correlations, establishing the spin systems H-6-7-8-9 and H-26-27-28-29 and their connections to quaternary carbons. One Trp moiety still bears the aliphatic side chain, as indicated by the upfield signals at 3.60 and 4.90 ppm. HMBC correlations from H-31 to the aliphatic carbons C-22 and C-23 and to the aromatic carbons C-25 and C-30, from NH-2 to C-24, C-25, C-29, C-30 and C-31, from H-23 to C-24, C-25 and C-31, from H-22 and H_2-23 to the carboxylic acid carbon C-21, and from NH-1 to H_2-23 completed the Trp substructure. The second indole moiety did not include any other protons, hence, the former aliphatic side chain of the second Trp may be included in the aromatic core of the target molecule containing ten quaternary carbons, two aromatic CH groups and one sp^2 nitrogen. Fortunately, pronounced HMBC correlations including several ^{4}J couplings from H-6 to C-4, from H-13 to C-3, C-11, C-15, C-18, C-19 and (very weak) to C-4, from NH-1 to C-14, C-15 and C-16, and from H-16 to C-14, C-15, C-17, C-18 and C-19 allowed to construct the central part of the molecule and its connection to the above-mentioned indole/Trp moieties. Two links at C-3 and C-18 remained open that must carry the last quaternary carbon, the sp^2 nitrogen and a carboxylic acid function. These were placed based on chemical shift considerations. The structure was further supported by a ROESY experiment that revealed correlations between the central protons H-13, H-26 and NH-1.

The assigned structure and its fragmentation pattern of **VIII** are illustrated in Fig. 3. In the MS^2 and MS^3 experiments, the successive loss of 44 Da and 18 Da was observed, corresponding to the loss of CO_2 and H_2O. Subsequent fragmentations were identical to those obtained from the parent compound (*m/z* 735; $[M + H]^+$), which indicates that the core structure of the molecule is not altered. Fragmentations of the intermediate ion at *m/z* 499 consisted in the loss of 44 Da (CO_2), 28 Da (CO), and 129 Da (indole moiety). When the reaction was conducted with only Trp or CQA, the formation of a red color was not observed, proving that the presence of both compounds is essential for the reaction. Replacing CQA with caffeic acid ethyl ester resulted in a slight pink color. Subsequent analysis revealed a compound absorbing at 550 nm and showing a molecular ion at *m/z* 589 $[M + H]^+$ (Table A3). The fragmentation experiments demonstrated that it contained the same core as in **VIII**. Because of the very low intensity of the signals, no further attempts were made to isolate and characterize this compound.

When caffeic acid was oxidized under the conditions reported above, no red pigment was formed and no peak was detected during the analysis. However, when the reaction was performed in dilute solution (1:10 *v/v*), a slight red hue was observed and a small peak was detected with *m/z 561* $[M + H]^+$ showing the same fragmentation as compound **VIII**. The reason for the deviating behavior of caffeic acid when reacted at lower concentration remains to be clarified. When Trp was replaced with N_α-methyl-L-Trp, no red color was observed. In contrast, the use of 1-methyl-L-Trp as an educt led to a red colored compound. These results suggest that an unsubstituted α-amino group of Trp is a prerequisite for the reaction to occur. Important structural information of the red colorant was obtained also through the use of the labeled compounds, ($^{15}N_\alpha$)-L-Trp and (1-^{13}C)-L-Trp: 1) The red pigment contains two α-amino groups, which remain attached to the core during fragmentation in the MS/MS experiments; 2) The two carboxylic acid groups of Trp are positioned in the red pigment such that they constitute good leaving groups that are released during MS/MS. The complete fragmentation data are shown in Tables A6 and A7.

A tentative mechanism for the formation of the red pigment **VIII** is presented in Fig. 5. The reaction starts with the $NaIO_4$ induced oxidation of CQA to the 1,4-quinone **2** (Le Bourvellec & Renard, 2012), which forms Schiff bases with two units of Trp (**3**) (Cordes & Jencks, 1962). Subsequent oxidation leads to a conjugated electron system in **4** and enables cyclization via the enolate **5** to yield **6**. After tautomerization promoted by re-aromatisation of the central ring to **7** and oxidation to **8**, the seven-membered ring is formed by electrophilic aromatic substitution via **9** to result in **10**. Further oxidation leads to the formation of the core structure of the red pigment (**11**), from which the quinic acid moiety is cleaved by hydrolysis to yield **VIII**.

The detection of the red pigment is remarkable because it confirms the view that reactions of quinones are still poorly understood and that the potential of these reactions to generate novel functional compounds

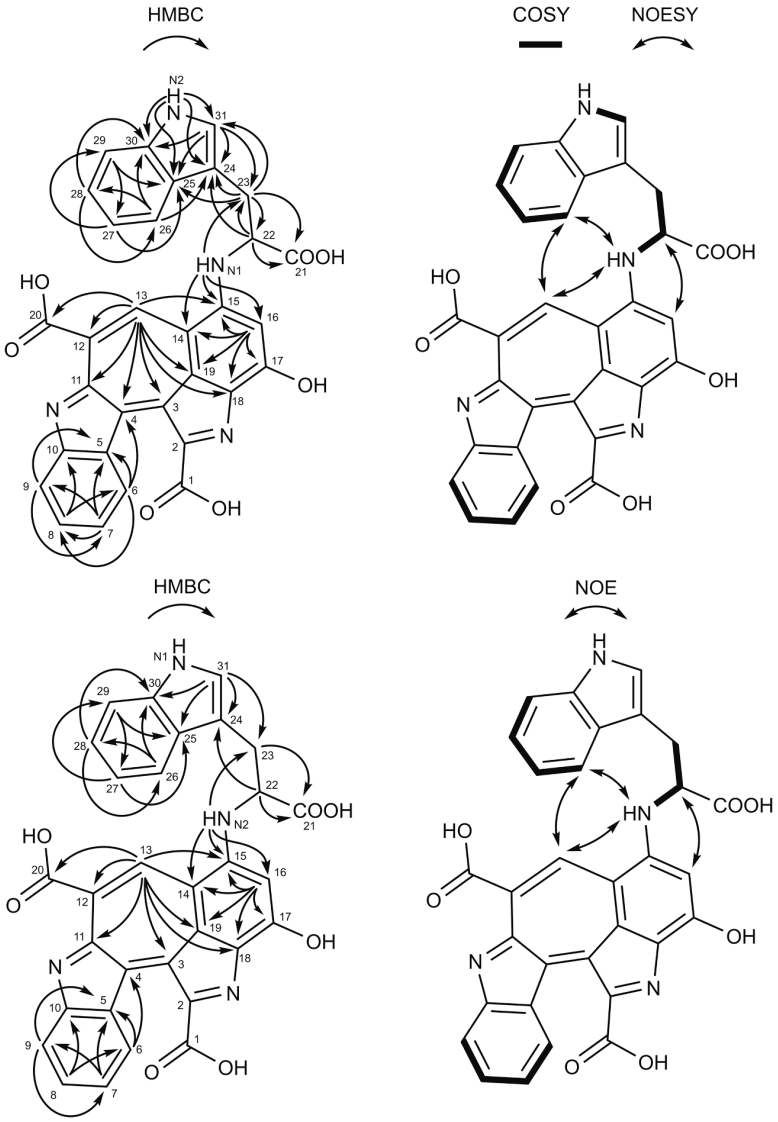

Fig. 4. Structure elucidation of the isolated compound **VIII** with highlighted correlations obtained from $^{1}H,^{1}H$-COSY, HMBC and NOESY experiments.

is tremendous (Schieber, 2018). As shown in this study for caffeic acid and by others (Vercruysse 2019), even variations in the concentration of the reaction partners may lead to different product profiles. Three main reactions of the phenolic compounds studied can be identified: The formation of a brown polymer is observed when the phenolic acid is present in excess. When Trp and CQA are present in similar quantities, the reaction proceeds to the formation of a greenish color, most likely due to benzacridines (Bongartz et al., 2016; Moccia et al., 2021; Vercruysse 2019). In excess of Trp, however, a red pigment is formed, which is in agreement with the mechanism proposed in Fig. 5.

4. Conclusions

A novel red compound was generated from the reaction between chlorogenic acid quinone and tryptophan and structurally elucidated using mass spectrometry and NMR spectroscopy. Chlorogenic acid is a plant phenol and thus fully complies with the current trend to use plant source materials for the production of food ingredients. Tryptophan, like many other amino acids, is obtained mainly by biotechnological means. The red compound might have the potential to expand the list of approved food colorants in the future. While its formation from two natural educts may provide interesting prospects for coloring foodstuff, a number of challenges still exist on the way to a new food additive. First, the protocol for the generation of the red colorant needs to be optimized, aiming at short reaction times, food grade reaction conditions, and high yields in order to make the process economically feasible. Second, methods for downstream processing need to be established that allow for the isolation of the compound. Third, comprehensive studies need to be carried out to determine the physicochemical properties of the colorant. Such studies should include also solubility and spectral characteristics as well as stability tests in various matrices and under conditions that reflect real food systems. Toxicological assessments will urgently be required to ensure the safety of the colorant. Finally, regulatory aspects need to be clarified to include the compound in the list of additives approved for food use.

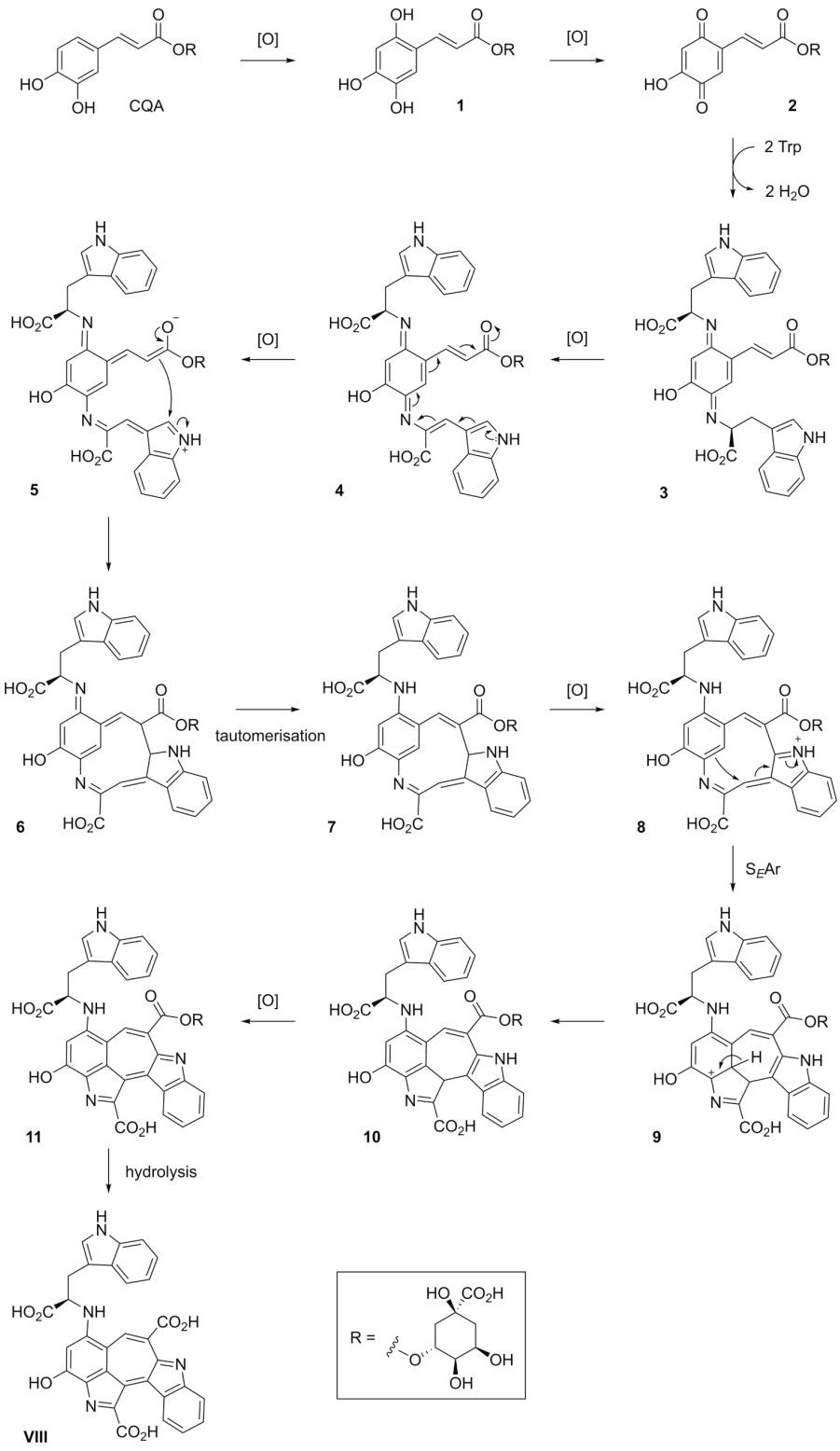

Fig. 5. Tentative mechanism for the formation of the red product **VIII** (R = quinic acid).

CRediT authorship contribution statement

Ardemia Santarcangelo: Investigation, Methodology, NMR interpretation, Writing – original draft. **Fabian Weber:** Conceptualization, NMR interpretation, Writing – original draft. **Stefan Kehraus:** NMR spectroscopy, Measurement and interpretation. **Jeroen S. Dickschat:** Mechanism hypothesis, NMR interpretation, Writing. **Andreas Schieber:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136473.

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B. Supporting material publication 1

Generation and structure elucidation of a red colorant formed by oxidative coupling of chlorogenic acid and tryptophan

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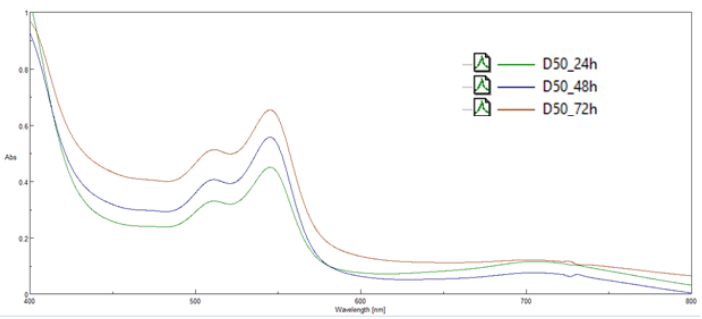

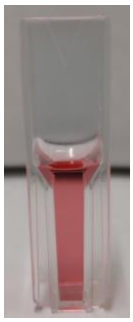

Figure A1: Left: UV-vis absorption spectrum of the reaction mixture of CQA (14 mM) and Trp (56 mM) at pH 7.5 after 24, 48 and 72 h diluted in water (1/50; *v/v*); right: red color obtained after 72 h diluted in water (1/50; *v/v*).

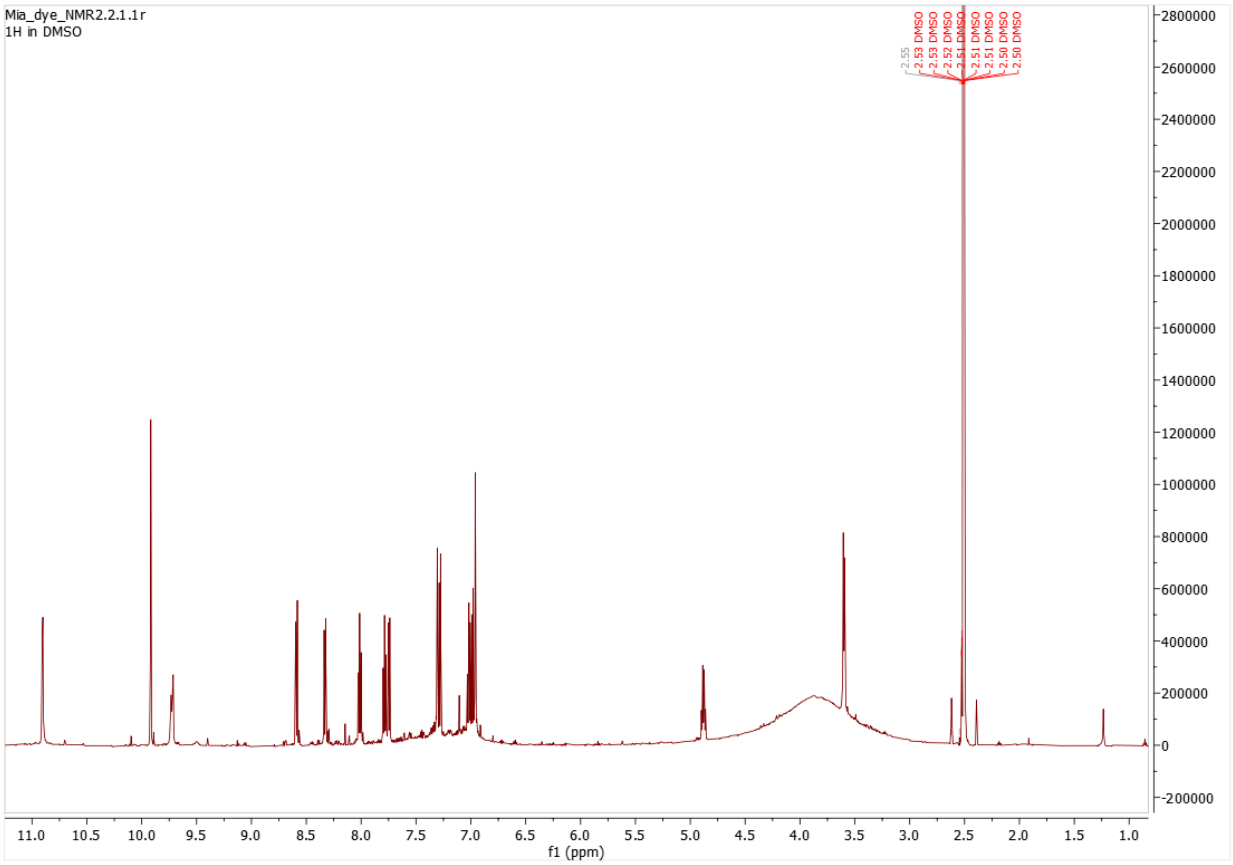

Figure A2: ^{1}H NMR spectrum of compound **VIII** in DMSO-d_6.

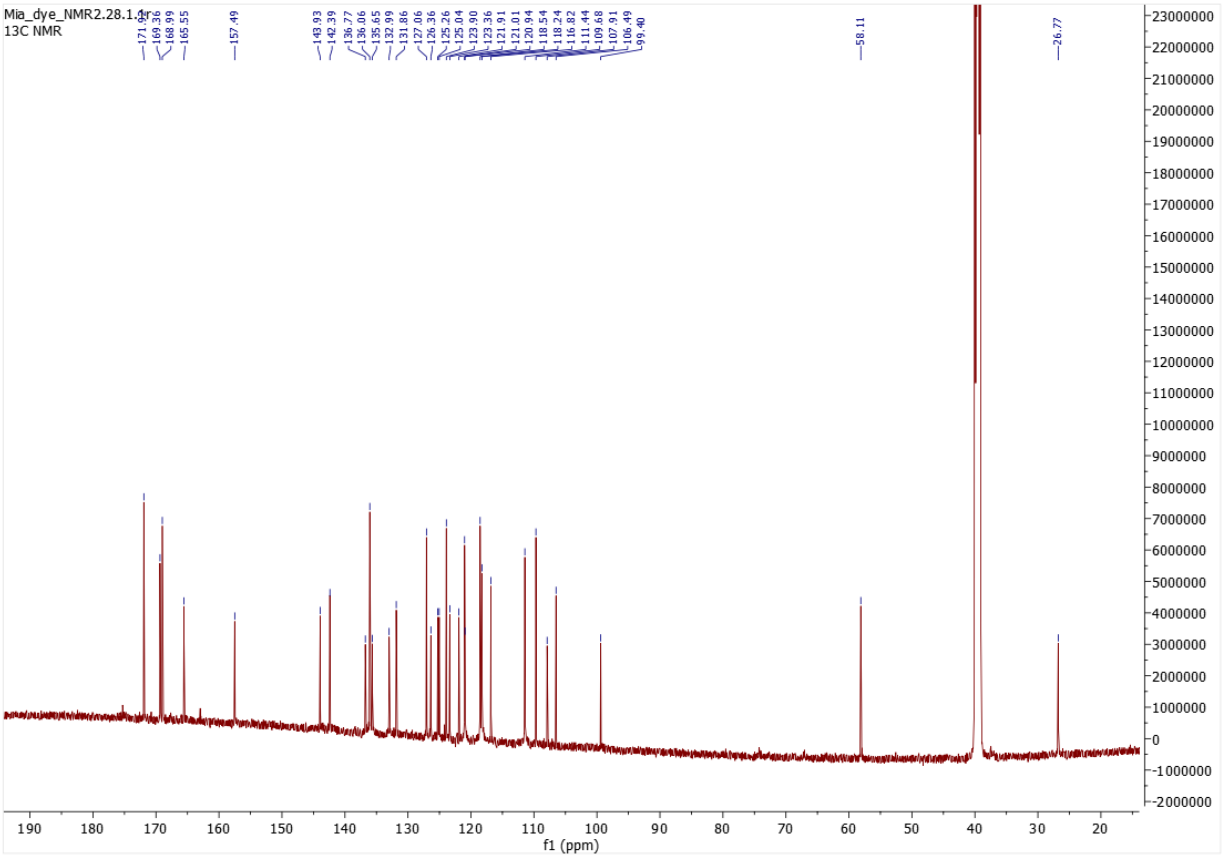

Figure A3. ^{13}C NMR spectrum of compound **VIII** in DMSO-d_6.

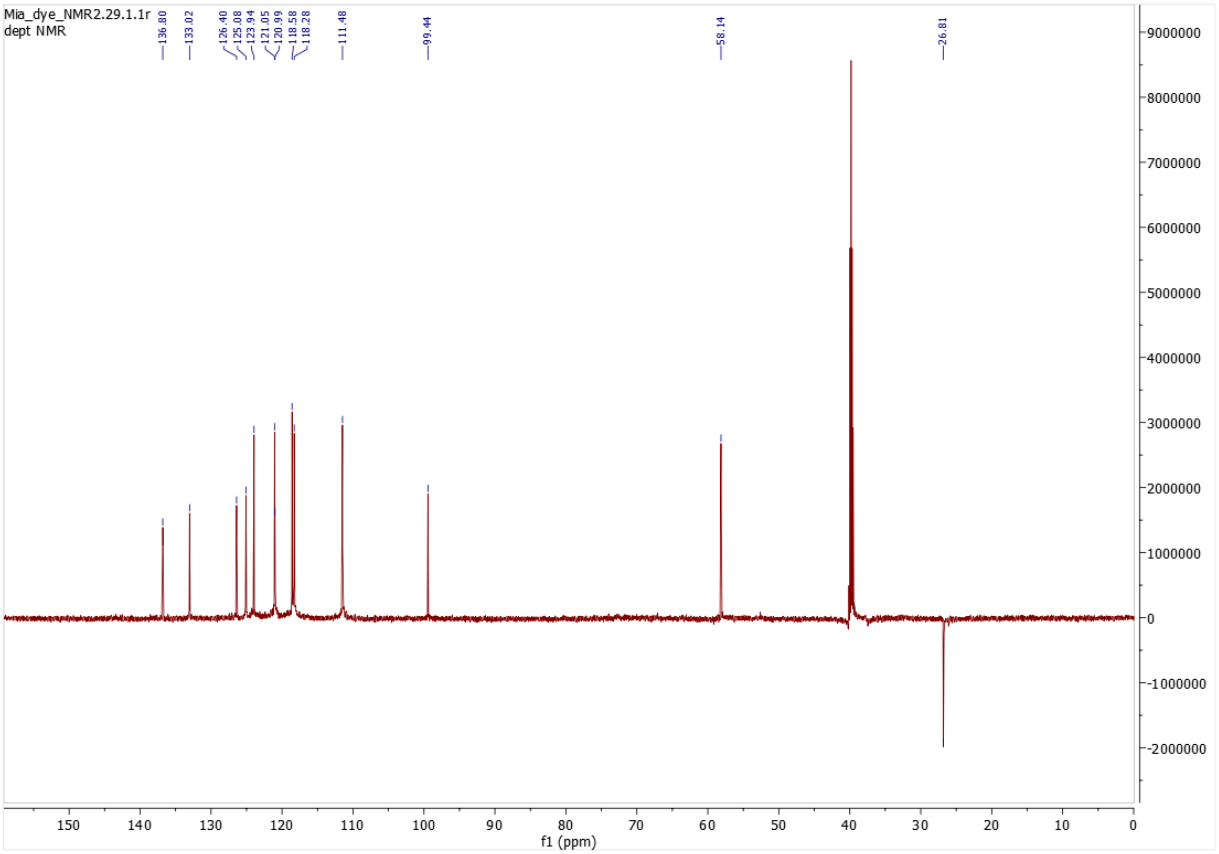

Figure A4. DEPT spectrum of compound **VIII** in DMSO-d_6.

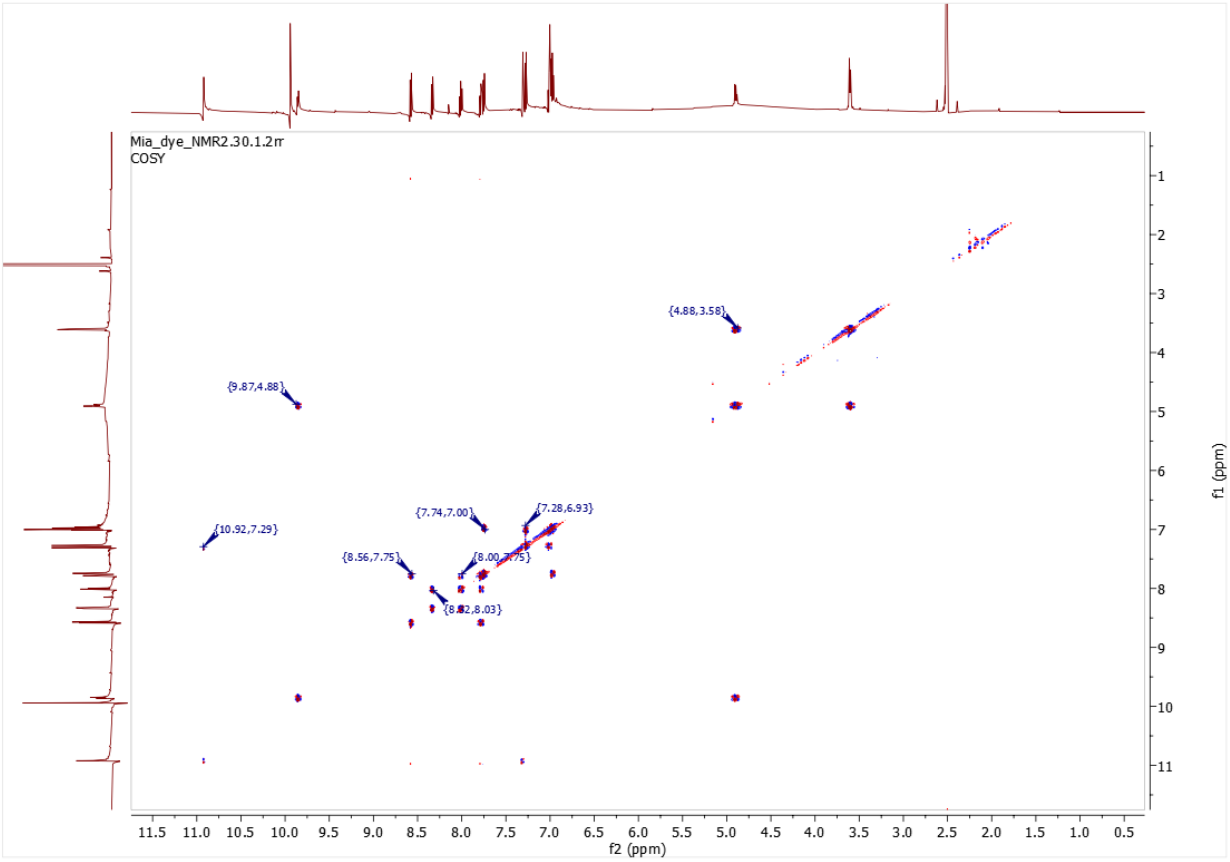

Figure A5. ^{1}H,^{1}H COSY spectrum of compound **VIII** in DMSO-d_6.

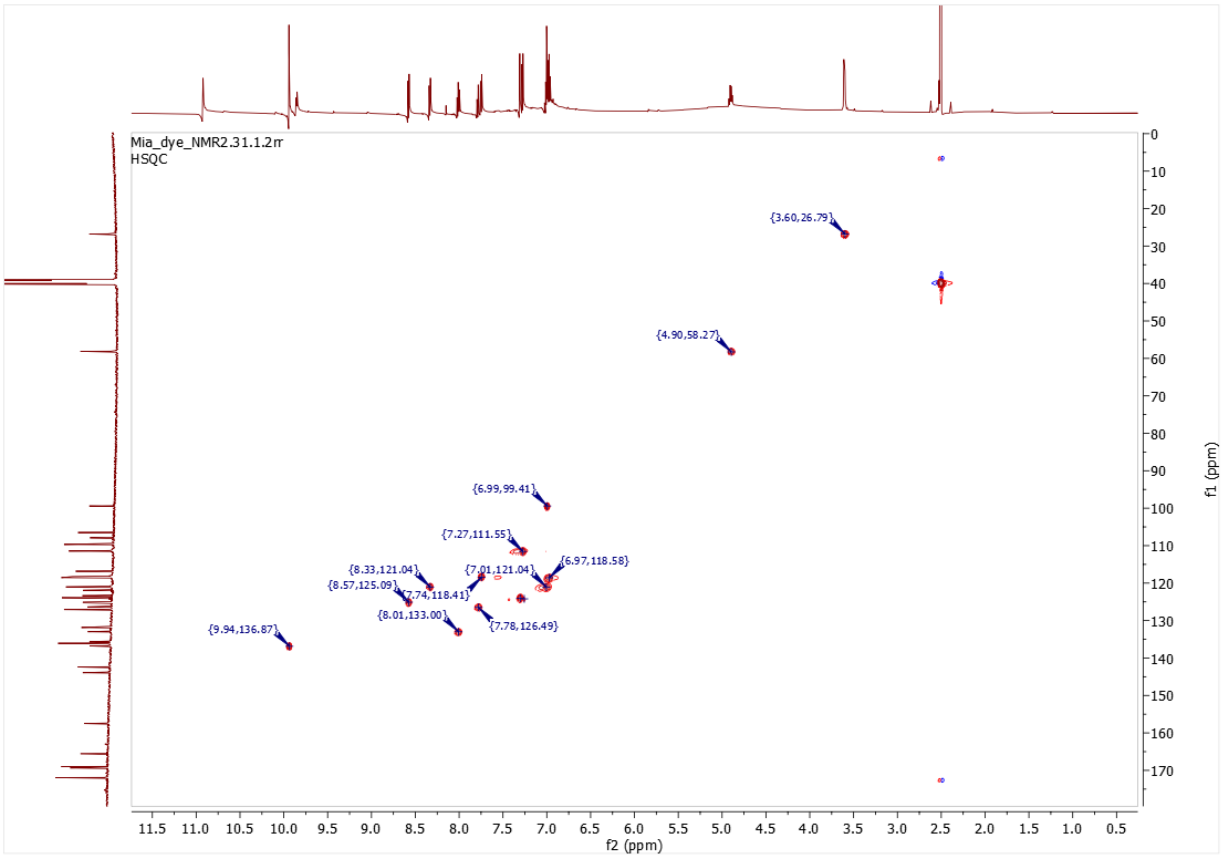

Figure A6. $^{1}H,^{13}C$ HSQC spectrum of compound **VIII** in DMSO-d_6.

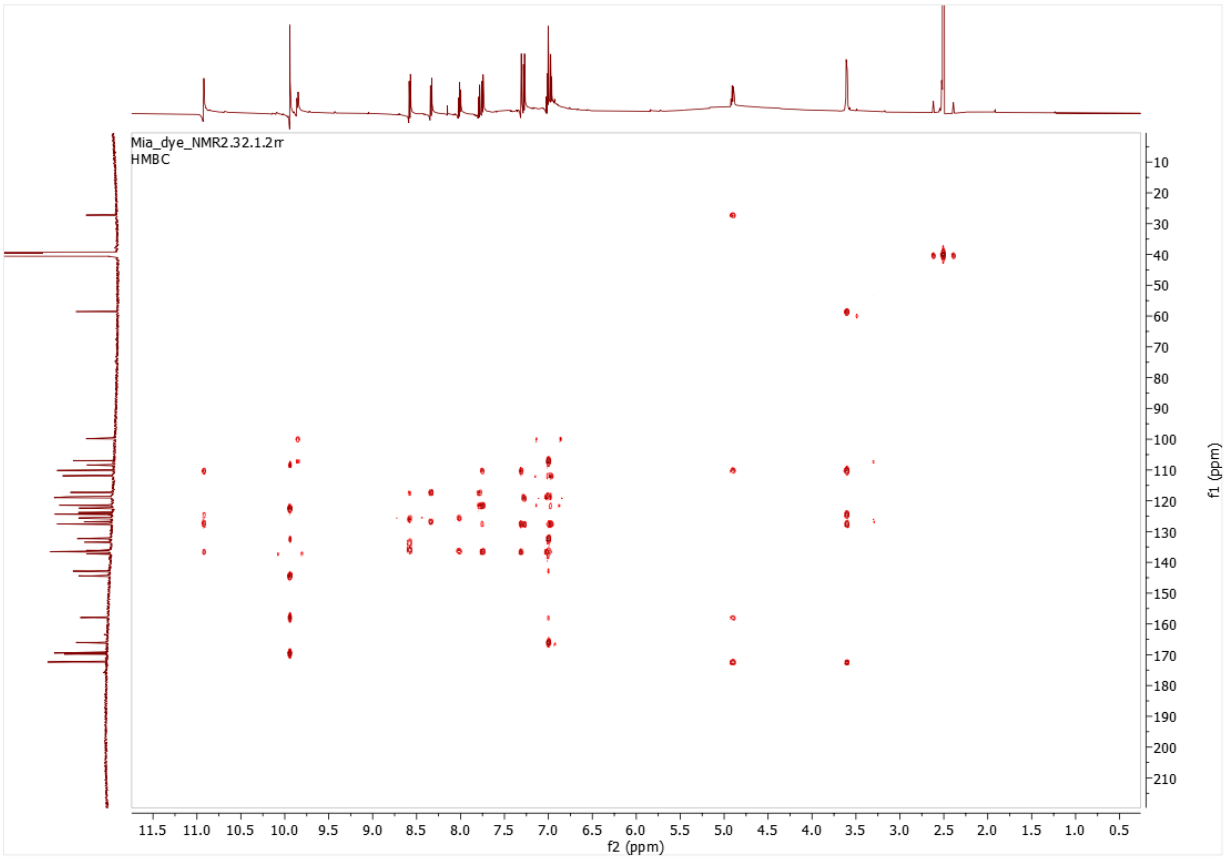

Figure A7. $^{1}H,^{13}C$ HMBC spectrum of compound **VIII** in DMSO-d_6.

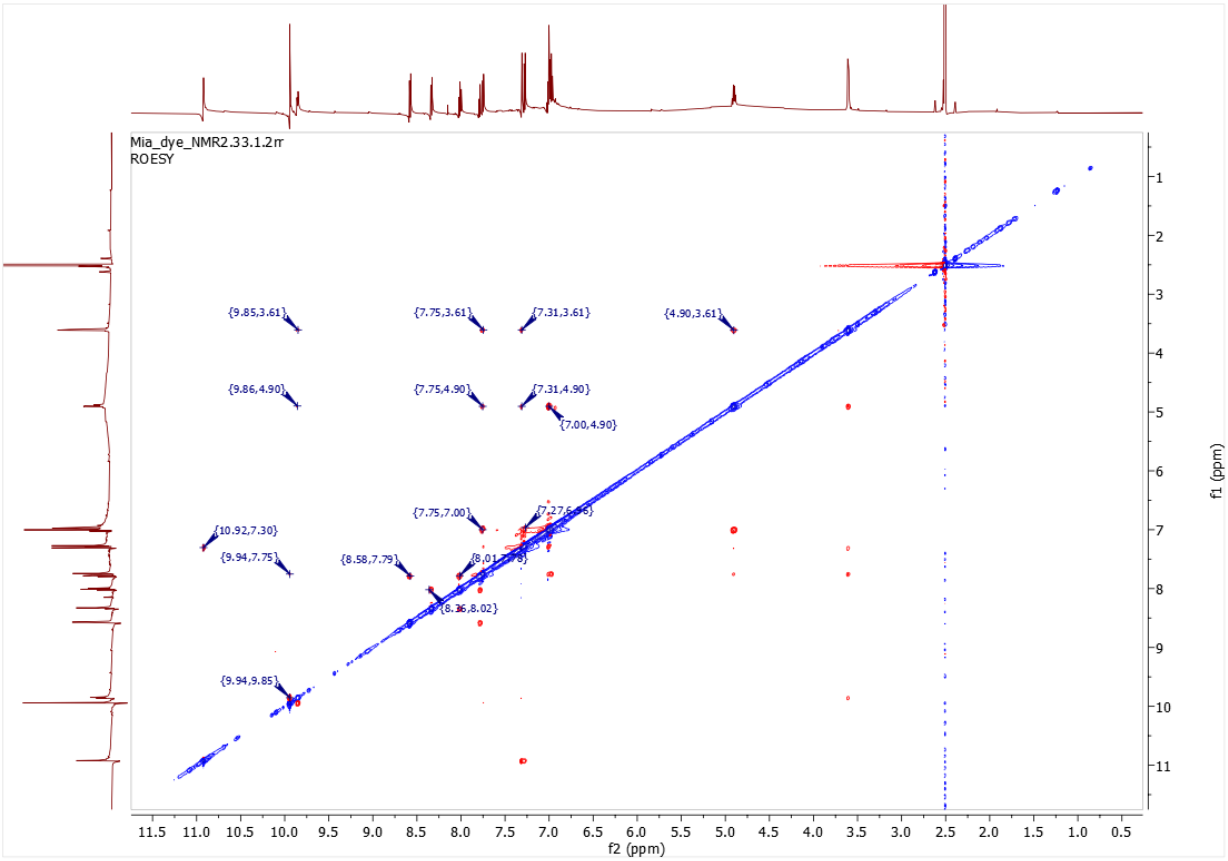

Figure A8. ROESY spectrum of compound **VIII** in DMSO-d_6.

Table A1. Mass spectrometric characteristics of the molecule with *m/z* 735 $[M+H]^+$.

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m/z* 735 (% base peak)
7.36	**735**	MS2 [735]: 691 (100) MS3 [735→691]: 499 (100), 517 (86), 645 (45), 530 (8), 674 (8), 338 (6) MS4 [735→691→499]: 370 (100), 338 (79), 312 (78), 368 (77), 298 (77), 427 (47), 324 (46), 453 (39), 455 (29), 342 (19), 326 (14), 271 (10), 481 (9), 296 (9), 471 (9), 310 (6), 297 (5), 336 (4)
7.43	**735**	MS2 [735]: 691 (100) MS3 [735→691]: 499 (100), 517 (86), 645 (45), 530 (8), 674 (8), 338 (6) MS4 [735→691→499]: 370 (100), 338 (79), 312 (78), 368 (77), 298 (77), 427 (47), 324 (46), 453 (39), 455 (29), 342 (19), 326 (14), 271 (10), 481 (9), 296 (9), 471 (9), 310 (6), 297 (5), 336 (4)
7.62	**735**	MS2 [735]: 691 (100) MS3 [735→691]: 499 (100), 517 (86), 645 (45), 530 (8), 674 (8), 338 (6) MS4 [735→691→499]: 370 (100), 338 (79), 312 (78), 368 (77), 298 (77), 427 (47), 324 (46), 453 (39), 455 (29), 342 (19), 326 (14), 271 (10), 481 (9), 296 (9), 471 (9), 310 (6), 297 (5), 336 (4)

Table A2. Mass spectrometric characteristics of compound **VIII** with *m/z* 561$[M+H]^+$ and *m/z* 515 $[M-H-CO_2]^-$.

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m/z* 561 (% base peak)
8.22	**561**	MS2 [561]: 517 (100) MS3 [561→517]: 499 (100) MS4 [561→517→499]: 370 (100), 312 (81), 338 (75), 298 (74), 368 (66), 427 (46), 324 (38), 453 (29), 455 (24), 342 (21), 326 (16), 471 (11), 481 (11), 296 (8), 271 (6), 440 (5)

Rt[min]	$[M-H-CO_2]^-$	HPLC/ESI($^-$)-MS^n experiment *m/z* 515 (% base peak)
8.22	**515**	MS2 [515]: 471 (100) MS3 [515→471]: 427 (100), 297 (41)

Table A3. Mass spectrometric characteristics of the molecule with *m*/*z* 589 $[M+H]^+$.

Rt[min]	$[M+H]^+$	HPLC/ESI(+)-MS^n experiment *m*/*z* 589 (% base peak)
9.21	**589**	MS2 [589]: 545 (100) MS3 [589→545]: 499 (100) MS4 [589→545→499]: 370 (100), 312 (88), 338 (82), 298 (77), 368 (66), 427 (47), 324 (44), 453 (30), 455 (29), 342 (26), 326 (16), 481 (14), 471 (11), 296 (8), 271 (5), 440 (4)

Table A4. Mass spectrometric characteristics of the molecule with *m*/*z* 561 $[M+H]^+$ obtained by reaction with caffeic acid (diluted conditions).

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m*/*z* 561 (% base peak)
8.22	**561**	MS2 [561]: 517 (100) MS3 [561→517]: 499 (100) MS4 [561→517→499]: 370 (100), 312 (79), 338 (75), 298 (70), 368 (70), 427 (39), 324 (37), 455 (25), 453 (24), 326 (18), 342 (15), 471 (11), 481 (11), 296 (8), 271 (8), 440 (4)

Table A5. Mass spectrometric characteristics of the molecule with *m*/*z* 763 $[M+H]^+$ obtained by reaction with N_α-methyl-L-tryptophan.

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m*/*z* 763 (% base peak)
8.02	**763**	MS2 [763]: 719 (100) MS3 [763→719]: 674 (100), 544(88), 503(25), 529(14),574(13) MS4 [763→719→674]: 545(100)
8.19	**763**	MS2 [763]: 719 (100) MS3 [763→719]: 674 (100), 544(88), 503(25), 529(14),574(13) MS4 [763→719→674]: 545 (100)
8.48	**763**	MS2 [763]: 719 (100) MS3 [763→719]: 674 (100), 544(88), 503(25), 529(14),574(13) MS4 [763→719→674]: 545(100)

Table A6. Mass spectrometric characteristics of the molecule with *m/z* 563 $[M+H]^+$ and *m/z* 517 $[M-H-CO_2]^-$ with ($^{15}N_\alpha$)-L-tryptophan.

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m/z* 563 (% base peak)
8.21	**563**	MS2 [563]: 519 (100) MS3 [563→519]: 501(100) MS4 [563→519→501]: 372 (100), 314 (85), 340 (82), 300 (77), 370 (65), 429 (44), 326 (37), 455 (31), 457 (28), 344 (19), 328 (16), 473 (10), 483 (12), 298 (7), 272, (6), 442 (3)

Rt[min]	$[M-H-CO_2]^-$	HPLC/ESI($^-$)-MS^n experiment *m/z* 517 (% base peak)
8.21	**517**	MS2 [517]: 473 (100) MS3 [517→473]: 429(100), 299 (37),300 (18), 343(7)

Table A7. Mass spectrometric characteristics of the molecule with *m/z* 563 $[M+H]^+$ and *m/z* 516 $[M-H-{}^{13}CO_2]^-$ with (1-^{13}C)-L-tryptophan.

Rt[min]	$[M+H]^+$	HPLC/ESI($^+$)-MS^n experiment *m/z* 563 (% base peak)
8.21	**563**	MS2 [563]: 518 (100) MS3 [563→518]: 500 (100) MS4 [563→518→500]: 371 (100), 312 (82), 338 (81), 298 (78), 369 (66), 427 (43), 324 (29), 453 (36), 455 (25), 343 (24), 326 (14), 472 (11), 481 (13), 296 (7), 271 (6), 440 (3)

Rt[min]	$[M-H-{}^{13}CO_2]^-$	HPLC/ESI($^-$)-MS^n experiment *m/z 516* (% base peak)
8.21	**516**	MS2 [517]: 471 (100) MS3 [517→471]: 427(100), 297 (37), 298 (18), 341(9)

Table A8: Observed ROESY correlations of the red product. For position labels refer to Figure 3.

Position	Δ(ppm)	**H6 8.57**	**H7 7.78**	**H8 8.00**	**H9 8.33**	**H13 9.94**	**H16 7.00**	**H22 4.90**	**H23 3.60**	**H26 7.74**	**H27 6.97**	**H28 7.01**	**H29 7.27**	**H31 7.30**	**N1 9.85**	**N2 10.9**
H6	**8.57**		X													
H7	**7.78**	X		X												
H8	**8.00**		X		X											
H9	**8.33**			X												
H13	**9.94**									X					X	
H16	**7.00**							X								
H22	**4.90**						X		X	X				X	X	
H23	**3.60**							X		X				X	X	
H26	**7.74**					X		X	X		X					
H27	**6.97**									X						
H28	**7.01**												X			
H29	**7.27**											X				
H31	**7.30**							X	X							X
N1	**9.85**					X		X	X							
N2	**10.9**													X		

C. Publication 2

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Improved Generation, Physicochemical Characteristics, and Food Application Studies of a Red Colorant Obtained from Oxidative Coupling of Chlorogenic Acid and Tryptophan

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Abstract: Due to a widespread consumer reluctance toward synthetic food dyes, the interest in natural compounds from plants has increased. This study aimed to optimize the oxidation process between chlorogenic acid (CQA) and tryptophan (Trp) using sodium periodate ($NaIO_4$) to obtain a red-colored pigment. The impact of temperature and different ratios of Trp to $NaIO_4$ on the reaction progress was investigated. After the best conditions for the reaction were established, three pH values were tested. The reaction time could be reduced from 72 to 24 h with a yield of 46 ± 2% *w*/*w* based on the quantity of CQA. After the first purification step of the product by size exclusion chromatography, the pigment obtained was characterized for its solubility, and its hydrolyzed form was used for investigations into the stability at different pH values, storage under light and in the dark (period of 28 days), in the presence of reducing agents, and for heat resistance. Finally, several food matrices were successfully colored with the natural pigment in amounts from 0.005 to 0.01% (*w*/*w*). In conclusion, the present study provides new insights into the feasible production and comprehensive characterization of a red pigment derived from oxidative coupling of CQA and Trp, as well as its application in food systems.

Keywords: chlorogenic acid; red pigment; optimized synthesis; stability; design of experiment

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1. Introduction

There is a marked reservation among consumers about synthetic additives, including artificial colors [1–3]. Therefore, great efforts are currently being made to find natural colorants for use in foods. Since colorants of animal origin are not acceptable to vegetarians and vegans, substances from plant sources, in particular, such as anthocyanins, betalains, and carotenoids, are at the forefront of interest [4]. However, these classes of pigments are characterized by a more or less high sensitivity to external influences such as thermal treatment, exposure to oxygen and light, and changes in pH values [5–9].

In view of the aforementioned challenges, it is noteworthy that the oxidative coupling of natural substances, namely chlorogenic acid and amino acids, results in the formation of colored reaction products [10–12]. While in these reactions, the majority of amino acids form green pigments, representing substituted benzacridines, a red colorant is obtained with tryptophan [13,14].

After Moccia et al. [15] had described two red compounds based on cyanines, our own studies demonstrated the formation of different red products consisting of one equivalent of chlorogenic acid with the quinic acid still attached and two equivalents of tryptophan [16]. The structure of the predominant compound is shown in Figure 1. Because the focus of that study was on the structural elucidation of the pigment and the proposal of a formation pathway, no investigations into the physicochemical properties and stability were performed. Furthermore, the formation of the pigment required a reaction time of 72 h, which is not acceptable in terms of economic production. Therefore, the aim of the

present study was to develop a process for the accelerated generation of the red colorant and to characterize its physicochemical properties, such as solubility in various solvents and stability against heat and light. In addition, the colorant was used in select food systems to evaluate its application potential.

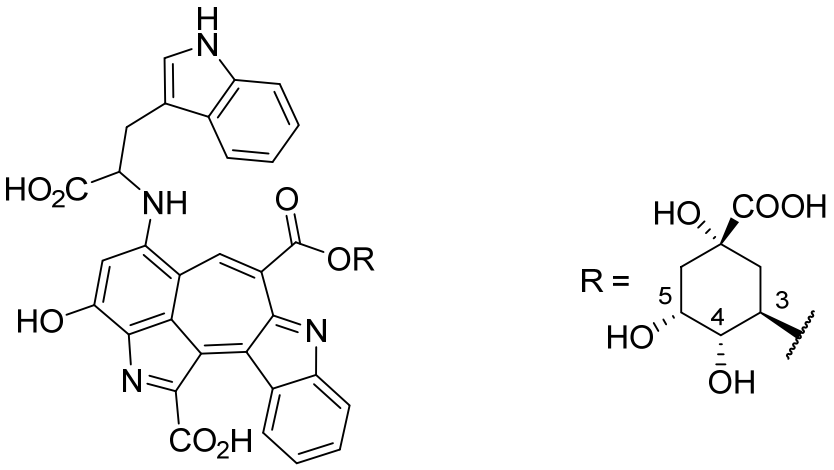

Figure 1. Structure of a red compound consisting of a chlorogenic acid moiety, in which the caffeic group is esterified with a hydroxyl group of quinic acid, attached to two tryptophan moieties, one of which is embedded in the chromophore core. While the caffeic acid moiety can be attached to positions 1, 3, 4, and 5, only one isomer is shown in this representation.

2. Materials and Methods

2.1. Chemicals

Chlorogenic acid (CQA; ≥95%) was obtained from BLDpharm (Shanghai, China). L-Tryptophan (≥98%), sodium metaperiodate ($NaIO_4$), sodium hydroxide, L-cystein (≥97%), and ascorbic acid (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carmine (E 120, ≥43%) was acquired from Symrise AG (Holzminden, Germany). Hydrochloric acid was purchased from VWR International GmbH (Darmstadt, Germany).

2.2. Optimization of Pigment Production by Means of Experimental Design

The oxidation of CQA was performed according to protocols reported previously [16], in which a solution of Trp (112 mM) with $NaIO_4$ (7 mM) and CQA (28 mM) buffered in Tris buffer (0.2 M; pH 7.5) was mixed (1:1, *v*/*v*), obtaining final concentrations of 56 mM Trp, 3.5 mM $NaIO_4$ and 14 mM CQA.

The process optimization for an increase in red pigment yields was conducted using the software Design Expert version 9.0.6.2 (Stat-Ease, Inc., Minneapolis, MN, USA). First, a full factorial design with 3 center points (Table S1) was employed to screen the effects of three independent variables (concentrations of Trp and $NaIO_4$, temperature) on the dependent variables (peak area at 550 nm after 24, 48, and 72 h). A temperature range of 22–70 °C and a range of 24–72 h for the reaction time was chosen. Trp and $NaIO_4$ were used in ranges of 56–140 mM and 1.68–14 mM, respectively (referred to as the final solution). Based on the findings of this screening, an increase in temperature was no longer considered since higher temperatures led to a decrease in the peak area of the three compounds detected by UHPLC at 550 nm. In the next step, the effect of the concentrations of Trp and $NaIO_4$ (56–140 mM and 3.50–14 mM, respectively) was analyzed in a factorial design with 4 center points (Table S2), resulting in 12 experiments.

Based on the results of an initial set of experiments, $NaIO_4$ at a starting concentration of 3.5 mM was used because it was found that larger quantities correlate with a larger peak area for the three compounds at 550 nm. This concentration also corresponds to that used in our previous study [16] and thus served as a reference point for the optimization process. Due to the significance of the curvature in the adjusted model, this design was augmented with face-centered axial points for optimization (Table S3). In all screening and

optimization experiments, the reaction was carried out under vigorous stirring (40 rpm) using a Variomag Thermomodul 40 ST (HP Labortechnik, Oberschleißheim, Germany) for 72 h in a volume of 10 mL. Samples were taken after 24, 48, and 72 h, diluted with water (1:10; v/v), and analyzed by UHPLC.

Because the optimization showed an increase in the amount of red pigment with increasing $NaIO_4$ concentrations, additional experiments were conducted with 21, 28, and 42 mM $NaIO_4$. Based on the optimal parameter settings, the reaction was subsequently performed in 0.1 M acetate buffer solution at pH 5 or in 0.2 M Tris buffer solution at pH 7.5 and 9. The resulting red pigment was precipitated by acidification to pH 1 with 6 M HCl and freeze-dried after centrifugation (10,000 rpm, 15 min, 4 °C). As a control, the reaction was carried out under optimal conditions but without $NaIO_4$. All reactions were performed in duplicate.

2.3. Scale up and Isolation of the Red Pigment

In another set of experiments, the reactions were performed as described in Section 2.2. under the optimized conditions (14 mM CQA, 56 mM Trp, 14 mM $NaIO_4$, 22 °C, pH 9 in 0.2 M tris buffer) but on a larger scale. For this purpose, CQA (0.25 g) was reacted with Trp (0.57 g) in the presence of $NaIO_4$ (0.15 g) in a final volume of 50 mL Tris buffer (pH 9, 0.2 M). After acidification to pH 1 with 6 M HCl, a precipitate was formed, which was separated by centrifugation (10,000 rpm, 15 min, 4 °C) and subsequently lyophilized. The precipitate was dissolved in 0.2 M NaOH and then purified on Sephadex G-10 (46 × 3.5 cm) using water as an eluent. Fractions of 10 mL were collected, diluted with water (1:50, v/v), and analyzed spectrophotometrically. The fractions showing an absorption maximum at approximately 550 nm were combined, and the solution was acidified to pH 1 using 6 M HCl. After centrifugation, the precipitate was collected and freeze-dried. In total, 0.114 ± 0.004 g of a red powder was obtained, corresponding to a yield of 46 ± 2% (w/w) based on CQA. The above-described protocol was highly reproducible and was therefore used when additional material was required for application studies.

2.4. Solubility Tests

The solubility of the colorant in water, alkaline solution (0.06 M NaOH), and ethanol was determined using 5 mg of the pigment. It turned out that it could be solubilized only in sodium hydroxide and ethanol, whereas its water solubility was poor. For subsequent analysis by HPLC, the pigment was dissolved first in NaOH or in ethanol and afterwards diluted (1:100; v/v) in water or ethanol.

2.5. Stock Solution of the Hydrolyzed Pigment

A stock solution of the hydrolyzed pigment, that is, the core molecule without the quinic acid moieties initially attached to chlorogenic acid, was obtained by dissolving 5 mg of pigment in 0.06 M NaOH (1.7 mL) for 4 h. This stock solution was used for subsequent investigations.

2.6. Stability at Different pH Values

The stock solution prepared in Section 2.5 was diluted (1:100; v/v) in 0.1 M phosphate buffer at pH 7 and 8 and in 0.1 M acetate buffer (pH 3 and 5). The absorbance of the solutions was determined by U$V/V$is spectrophotometry in the range from 400 to 800 nm.

2.7. Storage Stability

The samples prepared as described in Section 2.6 were capped, sealed with parafilm, and divided into two groups. One sample was exposed to direct daylight, whereas the other was stored in darkness. Both groups were kept at room temperature, and the absorbance was measured after 1, 7, 14, and 28 days in the range from 400 to 800 nm.

2.8. Thermal Stability

For the determination of the thermal stability, the stock solution prepared as described in Section 2.5 was diluted 1:100 (*v*/*v*) in 0.1 M phosphate buffer at pH 7 and in 0.1 M acetate buffer at pH 3.6 and 5 (0.029 mg/mL) and placed in capped vials. These were sealed with parafilm, kept at 90 °C for 30 min, and periodically analyzed by UV-Vis spectroscopy at 10-min intervals. For comparison, the thermal stability of a commercial carmine colorant (0.059 mg/mL) was determined using the same procedure.

2.9. Stability in the Presence of Reducing Agents

Aliquots of 10 µL of the stock solution (Section 2.5) were added to 880 µL of 0.1 M phosphate buffer (pH 7), to 0.1 M acetate buffer (pH 3.6), or to 880 µL water, to which 100 µL of a solution of ascorbic acid or cysteine (10 mg/mL) was added subsequently. A solution prepared with 100 µL water instead of the reducing agents served as a control. The samples were kept in the dark at room temperature, and their absorbance was determined by comparing the absorbance values obtained from the UV-Vis spectrum after 0, 2, 5, and 24 h.

2.10. Application as a Food Colorant

The pigment was used as a coloring agent in several model food applications. For this purpose, 40 mg of the colorant was dissolved in 12.8 mL NaOH (0.06 M), agitated for 4 h, and subsequently stored in a brown glass bottle at room temperature. Aliquots of this stock solution were added to the model foods as described below.

Milk and plant-based alternatives: 500 µL of the stock solution was mixed with 15 mL of cow´s milk or oat drink and compared with the respective controls without added colorant.

Yogurt: 500 µL of the solution was blended with 30 g of natural yogurt (3.5% fat) and compared with the respective control without adding colorant.

Meringue: 1500 µL of the solution and a pinch of salt were added to 60 g of egg white and whisked. Subsequently, 110 g of sucrose was gradually added until stiff peaks occurred. The meringues were shaped with a piping bag and dried at 90 °C in an oven for 2 h.

The colored samples were stored in a refrigerator (4 °C). Color analysis on all samples except meringue was carried out using a chromometer at 0, 7, 14, and 28 d. The meringue was stored in a tightly closed container in the dark and analyzed after a period of 14 days. Longer times were not considered because of the limited shelf life.

Alcoholic beverage: 500 µL of the solution was added to 20 mL water and 6 g sucrose, heated, and stirred until the sugar was completely dissolved and then allowed to cool. Subsequently, 5 mL ethanol was added and stirred. The beverage was stored at room temperature in the dark, and samples were taken for color analysis as described above.

2.11. Instrumental Analysis

U*V*/*Vis* absorption spectra were recorded on a V-730 JASCO double-beam spectrophotometer (JASCO Deutschland GmbH, Pfungstadt, Germany) between 400 and 800 nm in a 1 cm path-length glass cuvette (Hellma GmbH & Co. KG, Müllheim, Germany).

UHPLC analysis was conducted on a Prominence UFLC system (Shimadzu, Kyoto, Japan) equipped with two Nexera X2 LC-30AD high-pressure gradient pumps, a Prominence DGU-20A5R degasser, a Nexera SIL-30AC Prominence autosampler (15 °C, injection volume 5 µL), a CTO-20AC Prominence column oven at 40 °C, and an SPD-M20A Prominence diode array detector. An Acquity HSS-T3 RP18 column (150 mm × 2.1 mm; 1.8 µm particle size) was combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm × 5 mm, 1.8 µm), both from Waters (Milford, MA, USA). The separation was performed with water (A) and acetonitrile (B) as eluents, both acidified with 0.1% (*v*/*v*) formic acid. A gradient elution program at a flow rate of 0.4 mL/min was used as follows (min/%B): 0/2; 3/14; 5/15; 6/30; 12/40; 12.1/100; 15/100; 15.1/2; 17/2.

UHPLC-MS analysis of the pigment was performed on an Acquity UPLC I-Class system (Waters, Milford, MA, USA) coupled with an LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Waltham, MA, USA), which was equipped with an electrospray interface operating in positive and negative ion mode. The apparatus consisted of a sample manager cooled at 10 °C, a binary pump, a column oven, and a diode array detector scanning from 200 to 600 nm. The column oven temperature was set at 40 °C. An Acquity HSS-T3 RP18 column (150 mm × 2.1 mm; 1.8 µm particle size) was combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm × 5 mm, 1.8 µm), both from Waters (Milford, MA, USA). The separation was performed with water (A) and acetonitrile (B) as eluents, both acidified with 0.1% (*v*/*v*) formic acid. A gradient elution program at a flow rate of 0.4 mL/min was used as follows (min/%B): 0/2; 3/14; 5/15; 6/30; 12/40; 12.1/100; 15/100; 15.1/2; 17/2. The injection volume was 5 µL.

Mass spectra were recorded in the range of *m*/*z* 300–800. For positive mode, the source voltage was kept at 4.5 kV at a current of 100 µA, and the tube lens was adjusted to 50 V. The capillary temperature was set at 350 °C with a spray voltage of 4.5 V. Nitrogen was used as the sheath, auxiliary, and sweep gas at a flow of 70, 10, and 1 arbitrary units, respectively. Xcalibur software (2.2SP1.48, Thermo Scientific, Inc., Waltham, MA, USA) was used for data analysis.

Color parameters according to CIELAB color metrics were determined using a CR-400/410 chromometer with illuminant D65 (Konica Minolta, Langenhagen, Germany). For this purpose, 15 mL of milk, oat drink, alcoholic beverages, and 15 g of yogurt were subjected to color measurements in a glass cuvette with a diameter of 60 mm. Color loss during storage was calculated as the overall color difference ΔE* and Δa obtained from the color parameters: L* indicates brightness, a* is the red (positive value)/green (negative value) coordinate, and b* is the yellow (positive value)/blue (negative value) coordinate.

3. Results and Discussion

As reported in our previous paper [16], the oxidation of CQA with $NaIO_4$ in the presence of Trp for 72 h led to the formation of several products, three of them showing a red color. In an effort to accelerate pigment formation, the influence of various parameters on color development, as described in the following, was investigated using the areas of the three peaks detectable at 550 nm as a measured value.

3.1. Parameters Influencing the Development of the Red Pigment

3.1.1. Temperature Effect

The one-factor plot of the factorial screening design shows that temperature has a significant linear and negative effect on the peak area of the red pigments (Figure 2). Concomitantly, the formation of a brown color hue at the cost of the red color was observed when the reaction was performed at higher temperatures. This may be explained by the preferred oxidation of chlorogenic acid and subsequent reactions of the quinone to high-molecular compounds before condensation with Trp to form the red pigment takes place [17]. From these findings, it can be concluded that in a range of 22–70 °C, there is a negative effect and, therefore, that the reaction should preferably be carried out at room temperature. While it might be of interest to test temperatures below this range, it needs to be considered that cooling would be required, which would entail additional energy costs and might compromise the feasibility of the process.

3.1.2. Effect of Tryptophan Concentration

The effect of the Trp concentration is slightly positive (Figure S1). At the same time, we observed that Trp could not completely be dissolved when used in higher quantities. Therefore, it was decided to use Trp at a concentration of 56 mM.

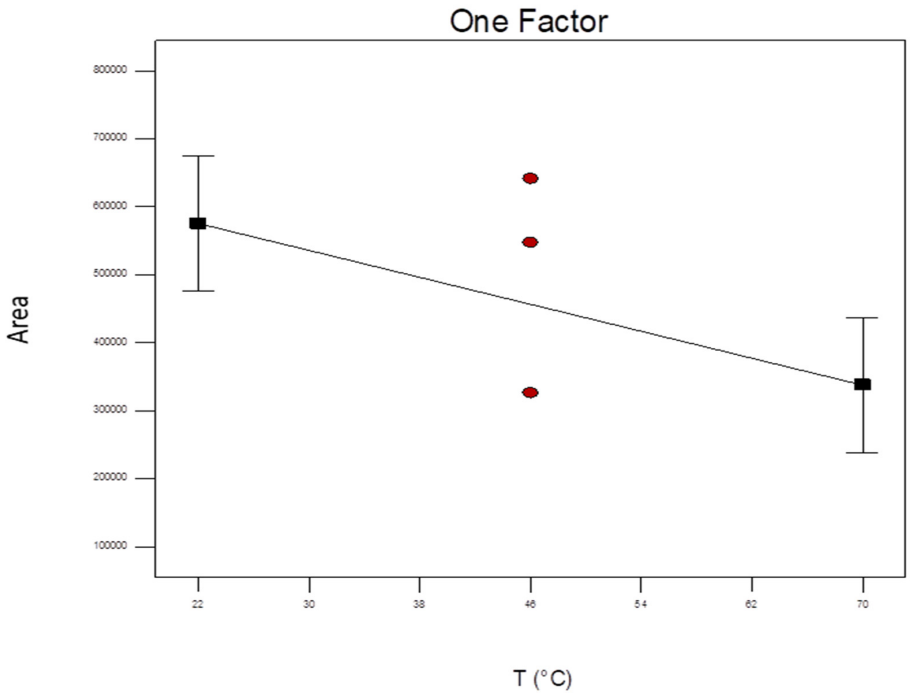

Figure 2. Variation of total peak area at 550 nm as a function of temperature after 24 h reaction time, with Trp and $NaIO_4$ concentration on medium level (98 mM and 7.84 mM respectively). The red points represent the center points.

3.1.3. Effect of $NaIO_4$ Concentration

With increasing concentrations of $NaIO_4$, a significant increase in the total area of the peaks absorbing at 550 nm was observed (Figure S2). Since no maximum could be identified, additional tests with higher $NaIO_4$ concentrations (21, 28, and 42 mM) were conducted (Figure 3). However, as the increase did not lead to an improvement, the reaction seems to be less efficient. The highest yields of red pigments were observed when the concentration of $NaIO_4$ was equal to that of CQA (14 mM, 1 M equivalents). The reaction with 3.5 mM (0.25 M equivalents) $NaIO_4$ reacting at room temperature corresponds to that reported previously [16].

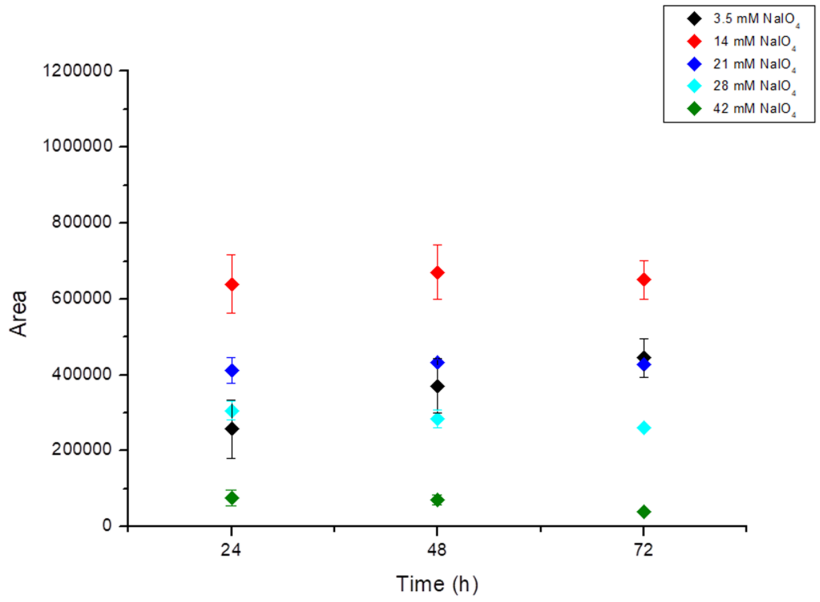

Figure 3. Variation of total peak area at 550 nm as a function of concentration of $NaIO_4$ after 24, 48, and 72 h reaction time.

Statistical analysis of the central composite design resulted in a linear model ($p = 0.0011$ with a significant positive effect of $NaIO_4$ ($p \leq 0.0003$), satisfying regression coefficients (0.6–0.7), and no significance of the lack of fit. The optimization led to the following parameter settings: 14 mM CQA, 56 mM Trp, 14 mM $NaIO_4$, 22 °C, and a reaction time of 24 h.

3.1.4. Effect of pH Value

The optimized reaction was subsequently conducted at different pH values (Figure 4). At pH 5, no red product was generated, whereas pH 9 was considered optimal for the development of the reaction. Although it was found that the reaction at pH 9 without $NaIO_4$ also led to the formation of the red pigment with a chromatographic profile identical to that of the reaction with $NaIO_4$, the reaction proceeded slower, and the yield after 72 h was comparable to that with 14 mM $NaIO_4$ at pH 7.5 and even lower than that resulting from a combination of pH 9 and 14 mM $NaIO_4$ (1 M equivalents) after 24 h.

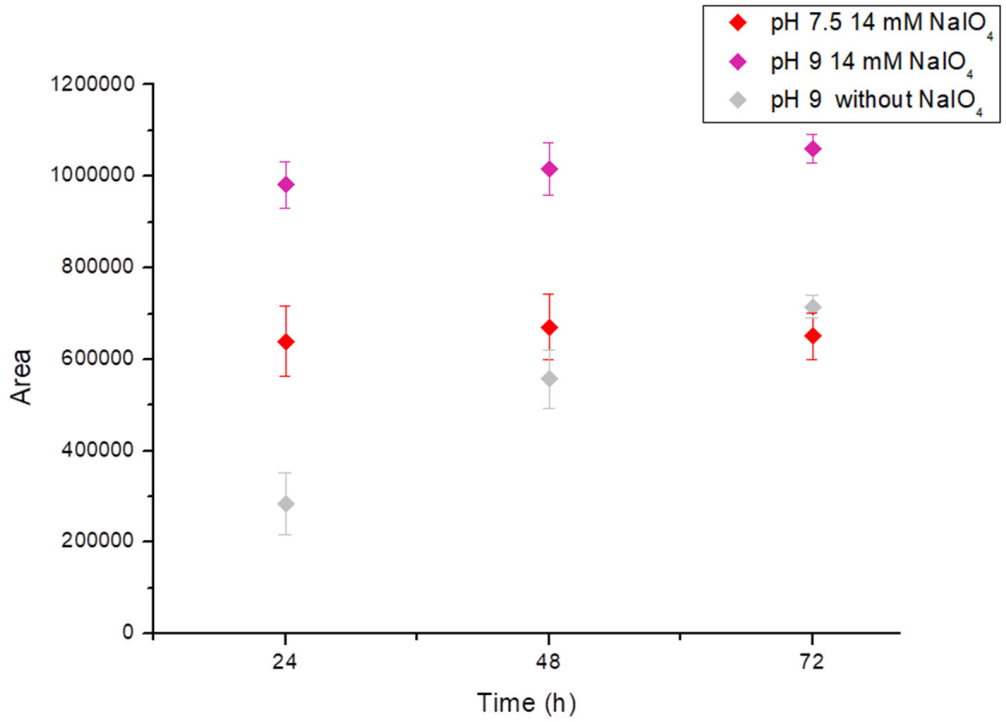

Figure 4. Total peaks area at 550 nm after 24, 48, and 72 h in the presence of 14 mM of $NaIO_4$ (1 M equivalent) in Tris buffer 0.2 M at pH 7.5, at pH 9, and at pH 9 without the use of $NaIO_4$.

In conclusion, the yield of red pigments was successfully increased by a combination of alkaline pH and the addition of $NaIO_4$, while concomitantly, the reaction time was tremendously reduced from 72 h to 24 h. Compared to our previous study [16], this optimized method even achieved a slight increase in yield under these conditions.

3.2. Optimized Process for the Production of the Red Pigment

Based on the results of the above studies, the optimum reaction conditions were found to be room temperature (22 °C), Tris buffer 0.2 M (pH 9), and a ratio of 56 mM (4 M equivalents) of Trp and 14 mM (1 M equivalents) of 5-CQA in the presence of 14 mM (1 M equivalent) of $NaIO_4$. To obtain a complete view of pigment formation, the reaction was carried out under these conditions, and peak areas were determined after 3, 6, 12, 24, 48, and 72 h. From Figure S3, it can be seen that after 24 h, significantly higher peak areas were obtained than after shorter reaction times, whereas a further increase to 48 h and 72 h did not lead to a considerable improvement. Therefore, a reaction time of 24 h can be considered optimal.

The scale-up of the reaction had no significant effect on its performance. After 24 h of reaction, a consumption of 89 ± 2% CQA and 48 ± 1% Trp was observed. The residual amount of CQA and Trp was determined by comparing the peak areas in the HPLC trace with those obtained by standard solutions of the compound at a known concentration. After the precipitation step with 6 M HCl, the pigment was centrifuged and lyophilized. HPLC analysis of the pigment (dissolved in an alkaline medium) showed the presence of 3% Trp, while no residual CQA was detected.

For the first purification process, the pigment was dissolved in a minimum amount of alkaline solution and directly subjected to purification on a Sephadex G-10 column. The purification protocol conducted in triplicate proved to be reproducible, leading to yields of 46 ± 2% (*w*/*w*) with respect to the initial 5-CQA, and no residual Trp was detected by HPLC.

3.3. Solubility of the CQA-Trp Pigment

The pigment obtained after the first purification step by size exclusion chromatography was not soluble in water but completely soluble in ethanol and 0.06 M NaOH. When the pigment dissolved in ethanol was further diluted with water, a suspension formed after a few minutes. It is assumed that after precipitation of the colorant with hydrochloric acid, the acidic environment still renders the compound insoluble when water is added. In contrast, when the colorant is first dissolved in an alkaline solution and then diluted with water, complete solubility is achieved. Prolonged exposure of the pigment to alkaline solutions decreased the peak intensity of all three isomers, favoring the formation of a single compound resulting from the release of the quinic acid moiety [16]. This conversion of the three red compounds to their hydrolyzed form was complete after 4 h.

3.4. Characterization of pH, Light, and Thermal Stabilities of the Hydrolyzed CQA-Trp Pigment

The hydrolyzed pigment stock solution was subsequently used to investigate its chemical and physical properties and thus assess its potential applications in the food industry. Because processing and storage may cause the isomerization and even the release of the chlorogenic acid moiety, which might, in turn, lead to a change in the physicochemical characteristics of the pigment, we preferred to use its hydrolyzed form in the application studies.

However, the authors concede that in some applications, the use of the pigment with the quinic acid moiety attached may be preferable because of its higher solubility. As shown in Figure 5, a pH dependence can be observed with a hypsochromic shift of 15 nm (from 577 to 562 nm) in the maximum absorption between pH 3 and pH 8. At pH 3, the color intensity decreased. In addition, when the pigment was exposed to acidic conditions (pH 3), it tended to form a precipitate over time, which confirms the previously reported low solubility of the colorant in an acidic solution. Remarkably, the precipitate could be redissolved by shaking and did not occur at higher pH values. Considerable differences in absorbance were not observed between pH values 7 and 8. The behavior of the pigment at higher pH values was not studied because alkaline conditions rarely occur in foods.

The stability of the hydrolyzed CQA-Trp pigment during storage in the dark and when exposed to light was also tested. From Figure 6, it can be seen that the pigment was particularly stable for 28 days in the dark, whereas it was photosensitive as the period of light exposure increased. Data for pH 8 are not presented because no significant difference to pH 7 was found.

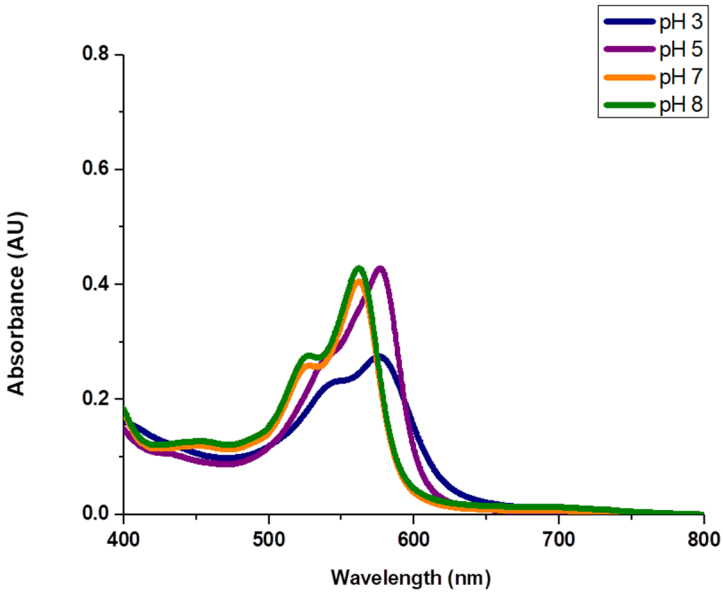

Figure 5. UV-Vis absorption spectra of solution 0.029 mg/mL of the red pigment in the hydrolyzed form in 0.1 M acetate buffer (pH 3 and 5) and in 0.1 M phosphate buffer (pH 7 and 8).

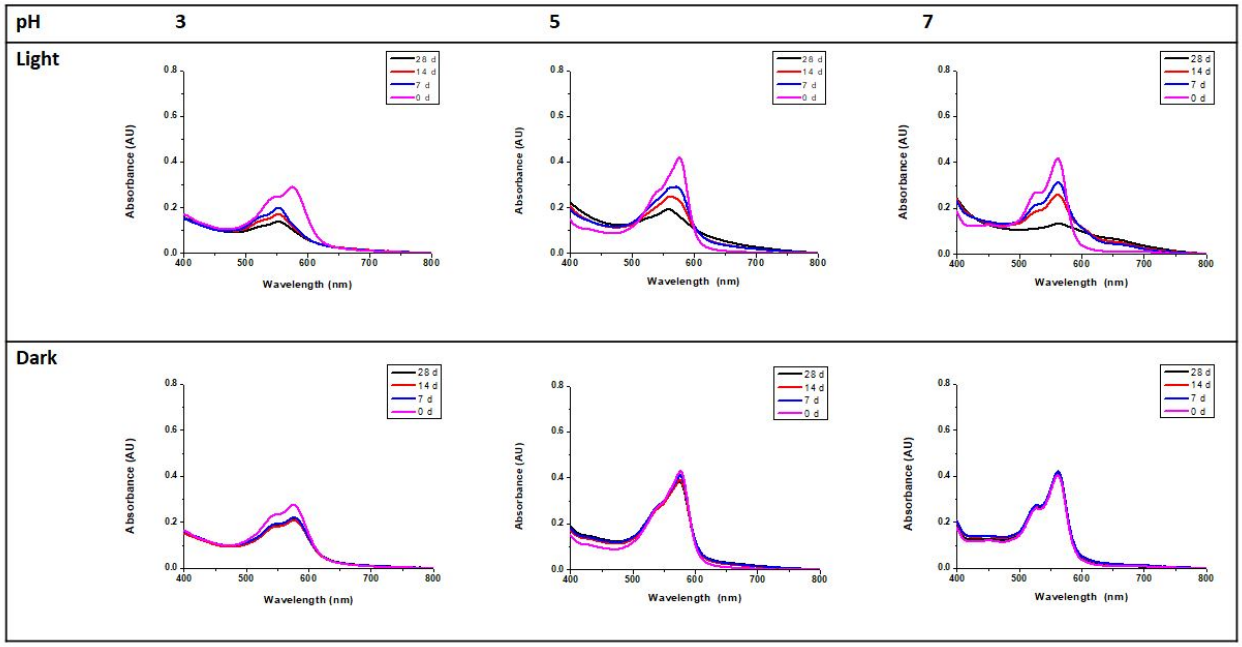

Figure 6. UV-Vis absorption spectra of solutions of the hydrolyzed pigment at different pH values and times (0/7/14/28 d) after exposure to light or dark.

The thermal stability of the hydrolyzed pigment was studied and compared to a commercial carmine preparation used in food applications. The pigment solutions were kept at 90 °C and analyzed by UV–Vis spectroscopy after 0, 10, 20, and 30 min (Figure 7). Their stability to thermal treatment was comparable. However, only half the concentration of the novel pigment was needed to obtain similar absorbance values. Remarkably, only at pH 3.6, the red pigment showed an increase in absorbance after 10 min. The reasons for this behavior are not entirely clear. However, an explanation might be an increase in the–initially limited–solubility under acidic conditions due to the effect of the temperature. Higher pH values did not lead to a remarkable difference in absorbance over time. The good stability of the pigment to heating under different pH conditions is an important

advantage for a wide range of food applications, as this is one of the main limitations of natural pigments, which tend to degrade when subjected to heat [9].

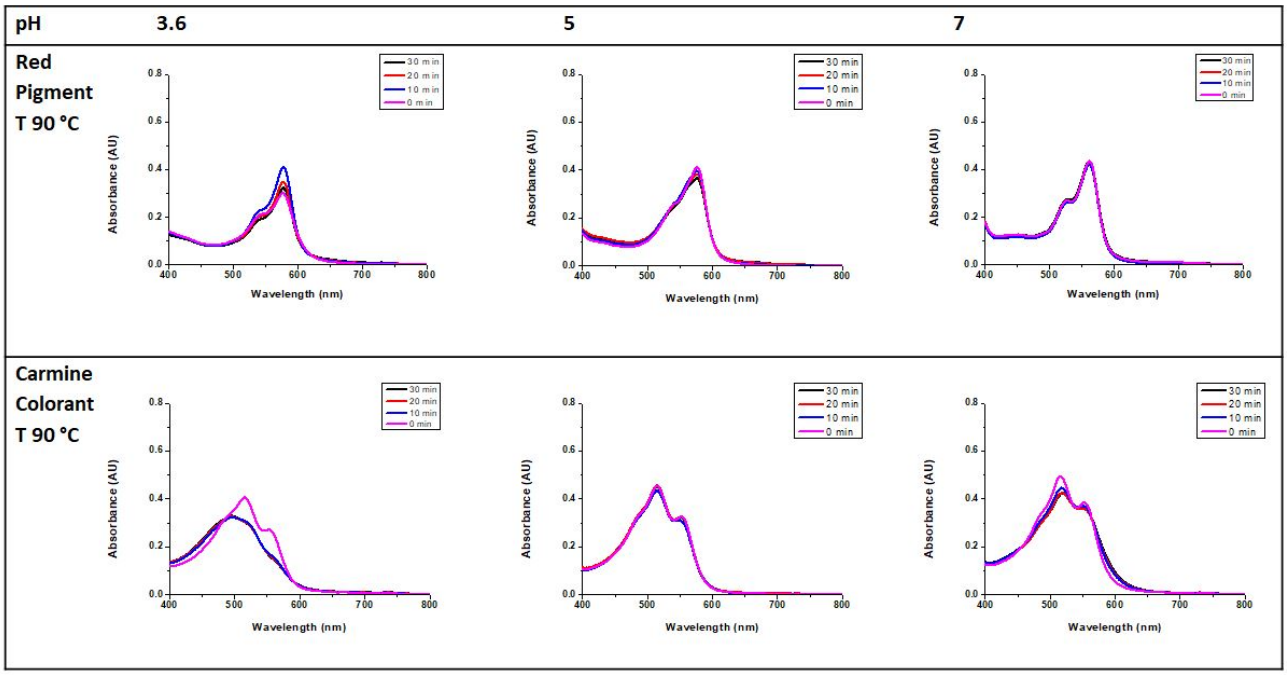

Figure 7. UV–Vis absorption spectra of solutions of the hydrolyzed pigment (0.029 mg/mL) and carmine (0.014 mg/mL) at different times in 0.1 M acetate buffer (pH 3.6 and 5) and 0.1 M phosphate buffer (pH 7).

3.5. Stability of Hydrolyzed Pigment against Reducing Compounds

No changes in absorbance were observed after mixing the pigment with ascorbic acid or cysteine at pH 3.6 for a period of 24 h. However, at pH 7 and in the case of ascorbic acid, a decrease in absorbance of 30% during 24 h was found, probably due to the acidity of ascorbic acid. With cysteine, no changes in absorbance occurred (Figure S4), which indicates that interactions with the thiol group that might affect color stability did not take place under the conditions investigated. This finding is extremely important because both ascorbic acid and cysteine are frequently found as natural components in foods and are also approved as additives. Furthermore, our results highlight the importance of the pH value of food matrices intended to be colored with the novel pigment.

3.6. Application as a Food Colorant

The potential of the pigment as a food colorant was explored using several food matrices. However, because of its insolubility in most of these matrices, it was necessary to dissolve the pigment in NaOH 0.06 M. This step also ensures total conversion to the hydrolyzed form.

Food application trials yielded more than satisfactory results with all model foods tested using the pigment in amounts less than 0.01% *w*/*w* (Figure 8).

Cow's milk was selected as a model system because of its high commercial importance and tendency to be accepted with a reddish color, for example, when red berries such as strawberries are added. Plant-based drinks such as oat drinks are considered milk alternatives and have attracted increasing attention from consumers who wish to avoid animal-derived foods. Our studies revealed that the novel pigment produced excellent results for these specific applications, inducing a homogeneous color hue. Also, yogurt proved to be a suitable matrix because no precipitation occurred, and good pigment stability was observed under moderately acidic conditions. Finally, a potential use for an alcoholic beverage was proposed in light of the increased demand for drinks with vivid colors

achieved by adding pigments, which appeal to a broad consumer base. Color stability in the samples was monitored for 28 days with CIELAB color space, with the exception of meringue, which was evaluated only for 14 days because of its limited storage stability. During this period, no significant changes in the a* values were observed (Table S4).

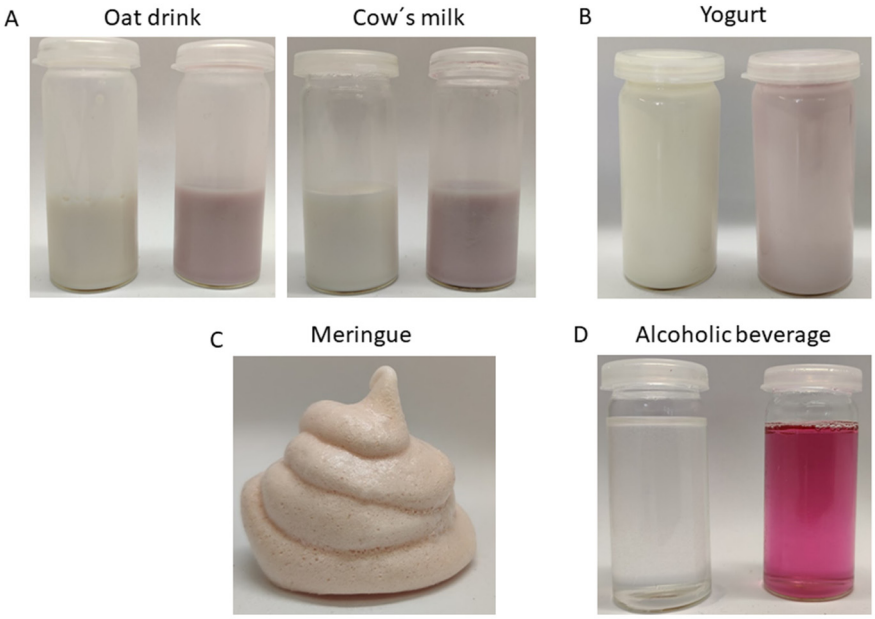

Figure 8. Food applications of the novel pigment. (**A**) Pure oat drink and oat drink with 0.01% *w*/*w* of colorant (left); Pure cow´s milk and cow´s milk with 0.01% *w*/*w* of dye. (**B**) Pure natural yogurt and natural yogurt with 0.005% *w*/*w* of colorant. (**C**) Meringue containing 0.007% *w*/*w* of colorant. (**D**) Alcoholic beverage with 20% ethanol containing 0.01% *w*/*w* of colorant.

4. Conclusions

The optimization of the pigment formation process reported in the present study has led to shorter production times and higher yields. This represents an important step for the commercial success of the novel pigment. Our investigations into the physicochemical properties indicate its potential suitability as a food colorant. However, as with all food additives, its use is subject to approval by the relevant authorities. Therefore, it is crucial to demonstrate its safety through comprehensive toxicological assessments and to address regulatory issues related to the inclusion of this compound in the list of approved food additives.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods13050686/s1, Table S1: Experimental conditions for plan 1; Table S2: Experimental conditions for plan 2; Table S3: Experimental conditions for plan 3; Table S4: Values of color space for different model food applications. C represents a control without the addition of dye, while +P refers to the sample with the addition of colorant. ΔE* and Δ a were calculated using values from day 0 and day 28, with the exception of the meringue, which was calculated using values from day 0 and day 14; Figure S1: Variation of total peak area at 550 nm as a function of Trp concentration after 24 h reaction time; Figure S2: Variation of total peak area at 550 nm as a function of NaIO4 concentration after 24, 48 and 72 h reaction time; Figure S3: Area of the total peaks at 550 nm for the optimal reaction measured at 3, 6, 12, 24, 48, and 72 h; Figure S4: UV-Vis absorption spectra of red solution 0.029 mg/mL in 3.6 pH acetate buffer 0.1 M, pH 7 phosphate buffer 0.1 M or H_20 in presence of 1 mg/mL ascorbic acid, cysteine, or plain buffer solution.

Author Contributions: A.S. (Ardemia Santarcangelo); Investigation, methodology, data analysis, writing, and editing; N.S.-K.; Design of Experiments (Set-up and Interpretation), writing; A.S. (Andreas Schieber); Conceptualization, supervision, writing, editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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D. Supporting material publication 2

Improved generation, physicochemical characteristics, and food application studies of a red colorant obtained from oxidative coupling of chlorogenic acid and tryptophan

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Run-Order	CQA (mM)	Trp (mM)	$NaIO_4$ (mM)	T (°C)
1	14	98	7.84	46
2	14	56	1.68	70
3	14	56	14	70
4	14	98	7.84	46
5	14	56	1.68	22
6	14	140	1.68	22
7	14	140	1.68	70
8	14	56	14	22
9	14	140	14	70
10	14	98	7.84	46
11	14	140	14	22

Table S1: Experimental Conditions for plan 1.

Run-Order	CQA (mM)	Trp (mM)	$NaIO_4$ (mM)
1	14	56	3.5
2	14	140	3.5
3	14	56	3.5
4	14	56	14
5	14	98	8.75
6	14	98	8.75
7	14	140	14
8	14	98	8.75
9	14	56	14
10	14	140	3.5
11	14	140	14
12	14	98	8.75

Table S2: Experimental Conditions for plan 2.

Run-Order	CQA (mM)	Trp (mM)	$NaIO_4$ (mM)
1	14	140	8.75
2	14	98	14
3	14	140	14
4	14	98	8.75
5	14	56	3.5
6	14	140	3.5
7	14	98	8.75
8	14	56	14
9	14	98	8.75
10	14	56	8.75
11	14	98	8.75
12	14	98	8.75
13	14	98	3.5

Table S3: Experimental Conditions for plan 3.

Sample	*time*	*L**	*a**	*b**	*Δ E**	*Δa*
***M*ilk**	O d	46.30	-0.70	4.63		
	28 d	47.17	-0.49	4.72	0.90	0.21
MilK + P	0 d	35.03	8.37	-1.32		
	7 d	41.36	8.41	-2.02		
	14 d	41.22	8.20	-1.94		
	28 d	40.92	8.28	-1.96	0.85	-0.09
Oat Drink C	0 d	42.77	0.91	5.66		
	28 d	46.11	0.86	6.63	3.48	-0.05
Oat Drink+ P	0 d	35.68	9.40	-0.11		
	7 d	38.93	9.40	-0.34		
	14 d	38.65	9.09	-0.25		
	28 d	38.06	8.97	-0.19	2.42	-0.43
Yogurt C	0 d	67.06	-2.16	6.95		
	14 d	53.37	-1.55	5.48		
	28 d	58.87	-2.20	5.83	8.27	-0.04
Yogurt + P	0 d	42.39	6.08	-0.37		
	7 d	40.77	7.14	-0.67		
	14 d	40.10	6.81	-0.62		
	28 d	40.80	6.63	-0.60	1.70	0.55
20 % alcoholic beverage C	O d	50.25	-0.61	0.39		
	24 d	51.5	-0.30	0.55	1.30	0.3
20 % alcoholic beverage+ P	0 d	32.30	29.25	-2.24		
	7 d	35.64	25.26	-4.75		

	14 d	33.79	30.47	-3.27		
	28 d	33.54	29.61	-3.53	1.83	0.36
Meringue + P	O d	73.58	6.85	11.61		
	14 d	78.45	6.09	9.79	5.25	-0.76

Table S4: Values of CIELAB color space for different model food applications. C represents a control, without the addition of dye, while +P refers to the sample with addition of colorant. ***Δ E**** and ***Δ a*** were calculated using values from day 0 and day 28, with the exception of the meringue, which was calculated using values from day 0 and day 14.

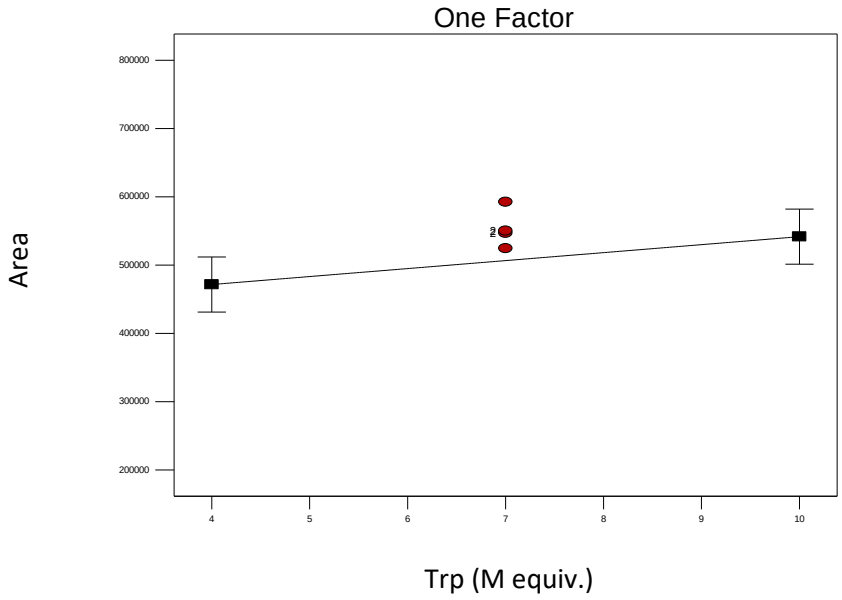
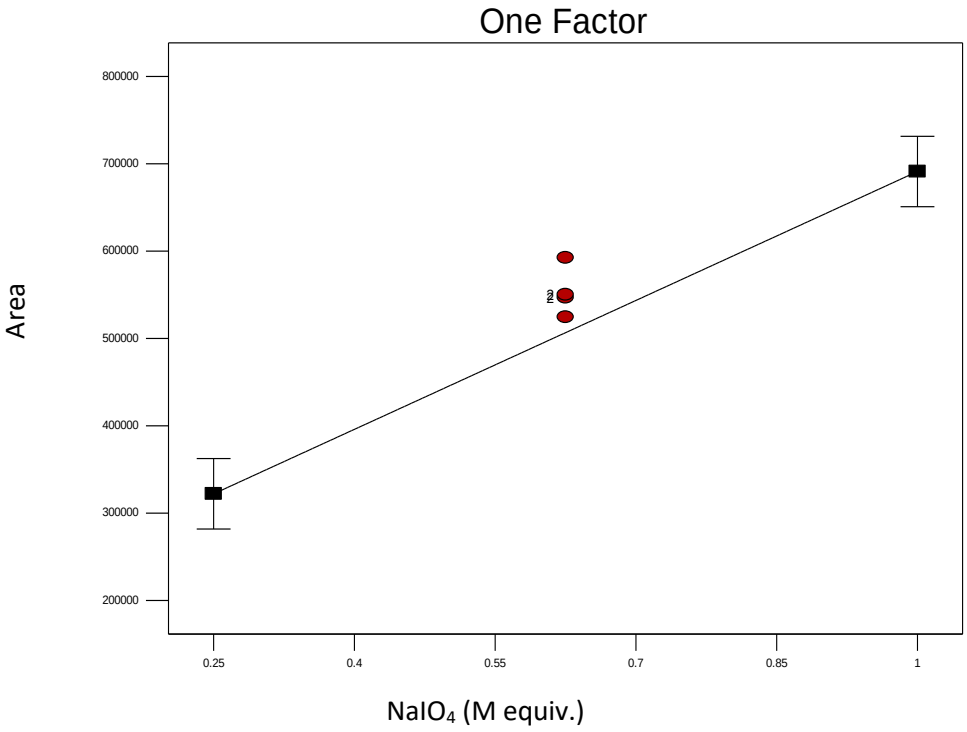
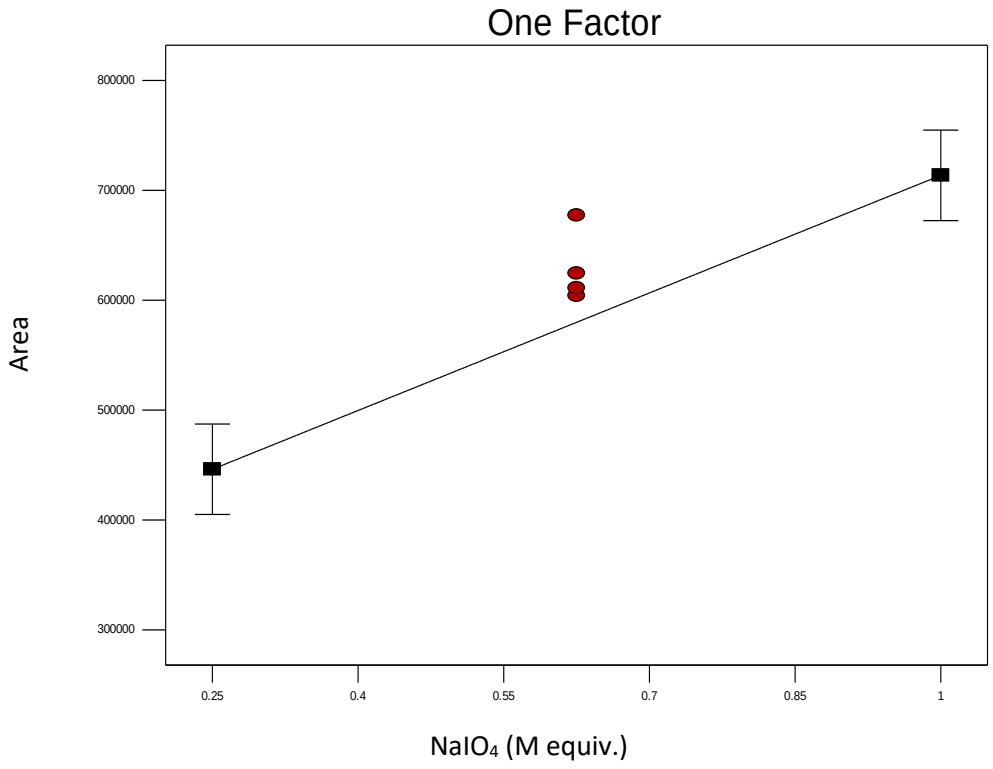


Figure S1: Variation of total peak area at 550 nm as a function of Trp concentration after 24 h reaction time with $NaIO_4$ concentration on medium level (8.75 mM). The red points represent the center points and 1M equivalent corresponds to 14 mM.

24 h


One Factor
Area
800000
700000
600000
500000
400000
300000
200000
0.25
0.4
0.55
0.7
0.85
1
NaIO4 (M equiv.)

48 h


One Factor
Area
800000
700000
600000
500000
400000
300000
0.25
0.4
0.55
0.7
0.85
1
NaIO4 (M equiv.)

72 h

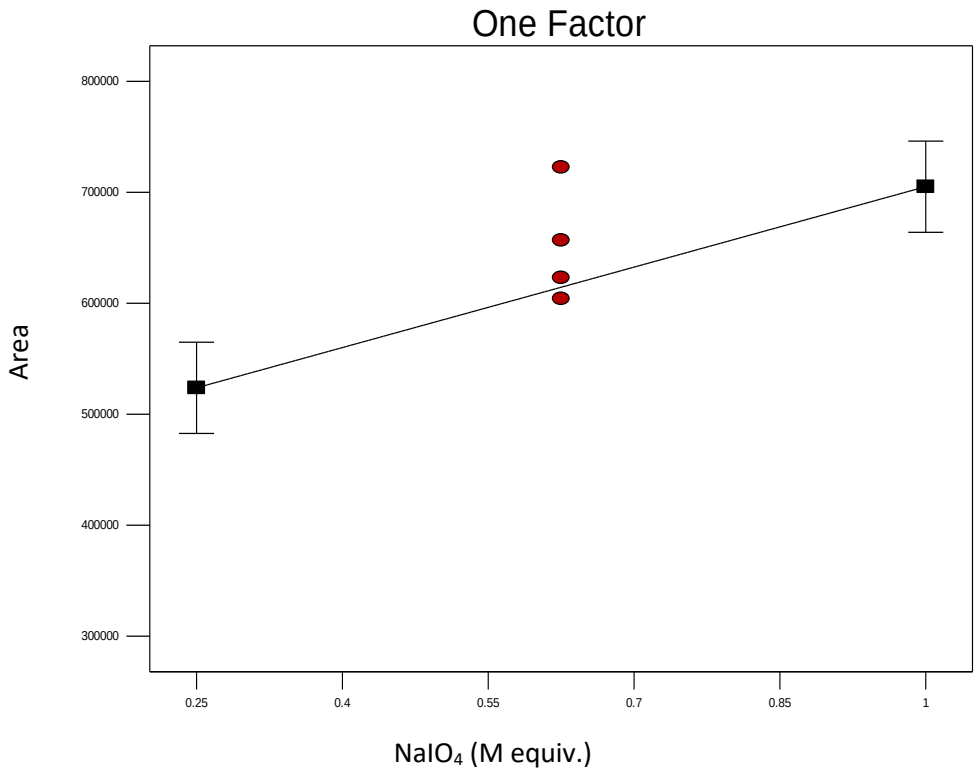


Figure S2: Variation of total peak area at 550 nm as a function of $NaIO_4$ concentration after 24, 48 and 72 h reaction time with Trp concentration on medium level (98 mM). The red points represent the center points and 1M equivalent corresponds to 14 mM.

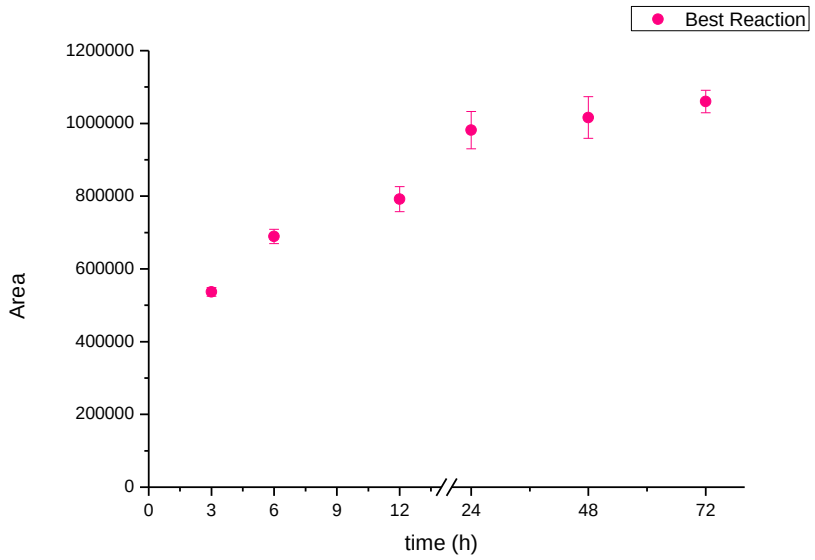


Figure S3: Area of the total peaks at 550 nm for the optimal reaction measured at 3, 6, 12, 24, 48, and 72 h.

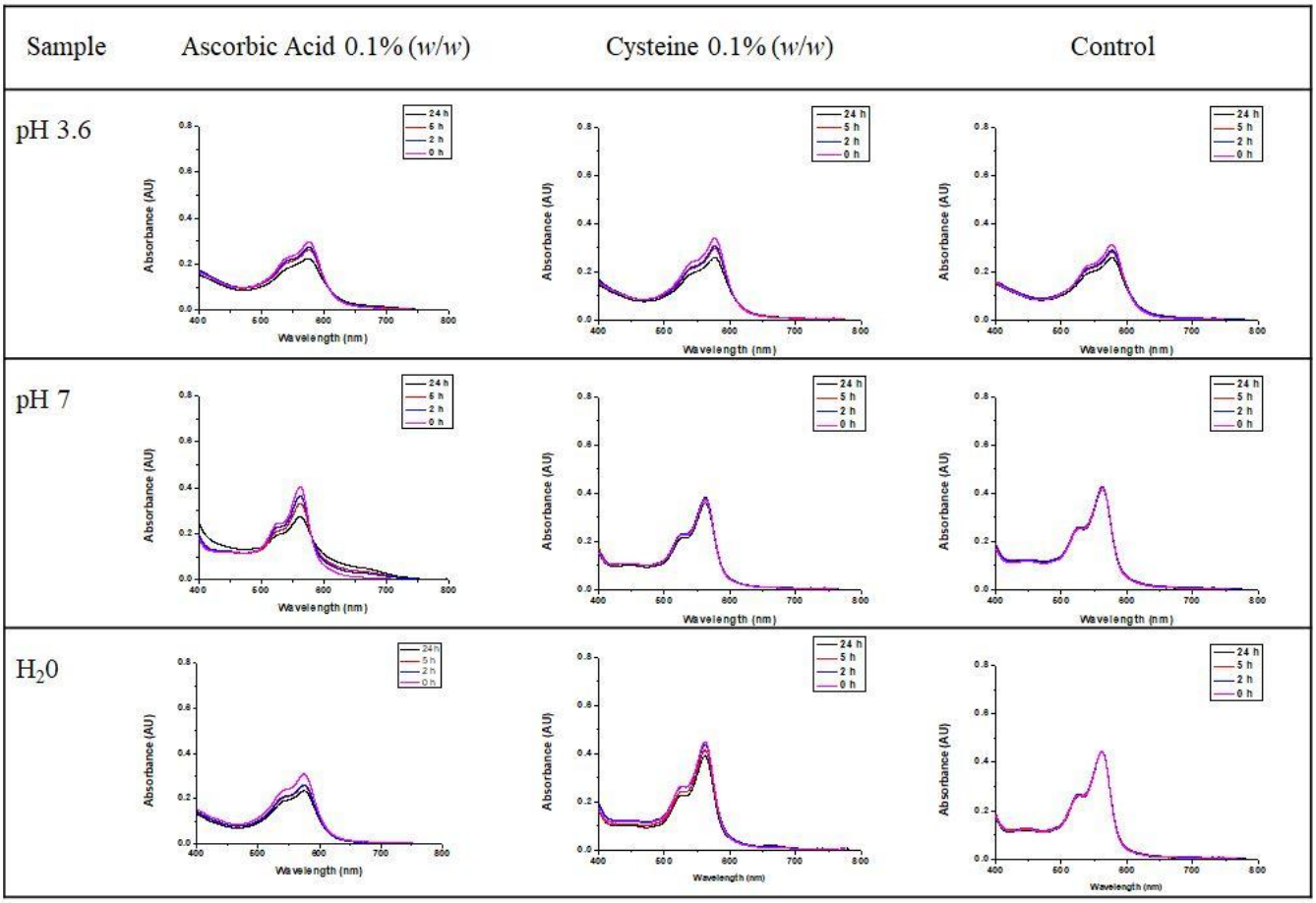


Figure S4: UV-Vis absorption spectra of red solution 0.029 mg/mL in 3.6 pH acetate buffer 0.1 M, pH 7 phosphate buffer 0.1 M or H_20 in presence of 1 mg/mL ascorbic acid, cysteine, or plain buffer solution.

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