IDENTIFICATION OF MOLECUAR INTERACTIONS BETWEEN SYNTHETIC DYES AND FOOD MACROMOLECULES BY FLUORESCENCE SPECTROSCOPY

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ABSTRACT OF THE THESIS

Identification of Molecular Interactions between Synthetic Dyes and Food Macromolecules by Fluorescence Spectroscopy

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Recent studies have demonstrated that in glycerol, glycerol-water, mono and disaccharide solutions, the fluorescence emission intensity of GRAS synthetic colors exhibits a strong sensitivity to the physical properties of the medium, for example, as the viscosity increases, the fluorescence intensity increases. Although molecular mass, structure and intrinsic rigidity of the macromolecule might play a role in probe sensitivity in physical properties of the system, other aspects such as molecular interactions between the probe and the macromolecule may explain these behaviors, such as hydrogen bonding,

electrostatic interactions, hydrophobic interactions, etc. The nature of the effect of macromolecules on the dye molecules are to be identified by fluorescence spectroscopy.

The fluorescence emission intensity was measured by titrating five carbohydrates (alginate, amylopectin, carboxymethyl cellulose, chitosan and methylcellulose) and five proteins (gelatin, gliadin, albumin, whey protein and zein) into three GRAS synthetic dyes (allura red, sunset yellow and fast green) using a Fluoromax-3 (Horiba Scientific Inc.). Temperature (20°C) and dye concentration were kept constant throughout the study. Anisotropy, ionic strength and temperature studies were also carried out to further explore the nature of the molecular interactions.

Results indicate that the fluorescence intensity of fast green is increased by the addition of all water soluble proteins, which are BSA, whey protein and gelatin. It is found that fast green exhibits a very strong binding with the bovine serum albumin molecule, involving electrostatic interactions, hydrogen bonding and hydrophobic interactions. Allura red, as an azo dye, shows less sensitivity to water-soluble protein molecules, while it has hydrophobic interactions with methyl cellulose. Sunset yellow does not show obvious sensitivity to any of the carbohydrates and proteins studied.

These results provide insights into the molecular interactions between synthetic dyes and food macromolecules. Therefore, these findings can be used to enhance the performance of these dyes as intrinsic luminescent probes for the physical state of foods and can also contribute to the enhanced detection of synthetic dyes in food products.

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1. INTRODUCTION

1.1 Fluorescence Spectroscopy

UV-Vis luminescence spectroscopy is a widely-used technique to study the excited states of molecules. The excited state processes can be illustrated by Jablonski diagram, which is named after the famous Polish physicist Aleksander Jablonski (1898-1980). The Jablonski diagram (Figure 1.1.1) is an energy diagram for the electronic transition of molecules. Firstly, the photons may absorb energy from light, causing the electronic transition from ground state (lower energy level S_0) to excited singlet state (higher energy levels S_1 and S_2). Then through vibrational relaxation, the molecule will quickly transit to the lowest excited state energy level. Internal conversion, which is a similar process with vibrational relaxation, causes an electron to transit from a higher excited state energy level to a lower one when there are overlapping of energy levels. Finally, the de-excitation process will take place either by emission of photons, which is called fluorescence, or by non-radiative pathways with no photons emitted, called non-radiative decay. For certain molecules under specific conditions, they will undergo intersystem crossing from the singlet state S_1 to the triplet excited state T_1. Then the molecule can

also go through vibrational relaxation to its lowest excited triplet state energy level. The de-excitation will occur either by non-radiative pathways with no photons emitted, or by the radiative pathway called phosphorescence. The energy of triplet excited states is lower than that of the singlet excited states.

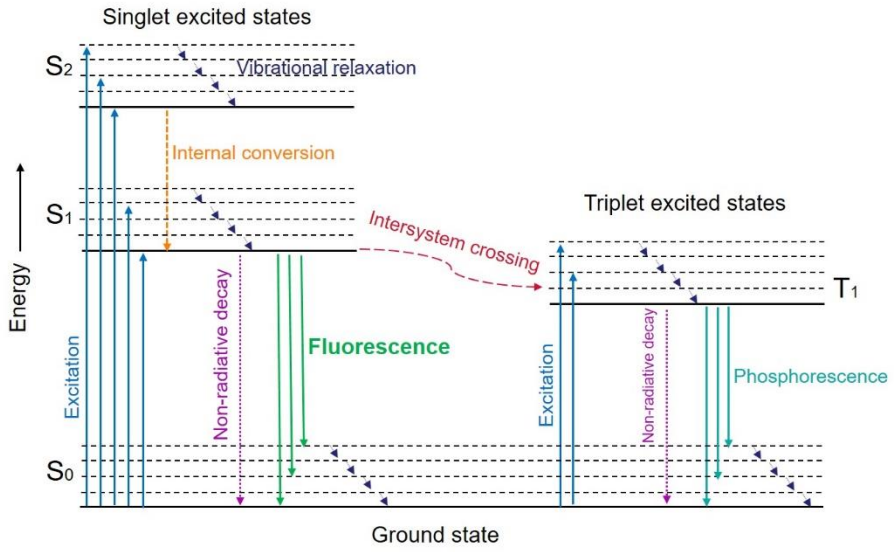


Figure 1.1.1 Jablonski diagram

The time it takes for the different processes of electronic transitions shown in the Jablonski diagram also differs. Absorption takes about 10^{-15} seconds, internal conversion and vibrational relaxation needs 10^{-14} to 10^{-11} seconds, and non-radiative decay takes 10^{-7} to 10^{-5} seconds. Fluorescence takes 10^{-9} to 10^{-6} seconds, while phosphorescence can take 10^{-3} to 10^{2} seconds. Usually the faster the transition, the more likely that process will happen. Therefore fluorescence is a much more common phenomenon than phosphorescence.

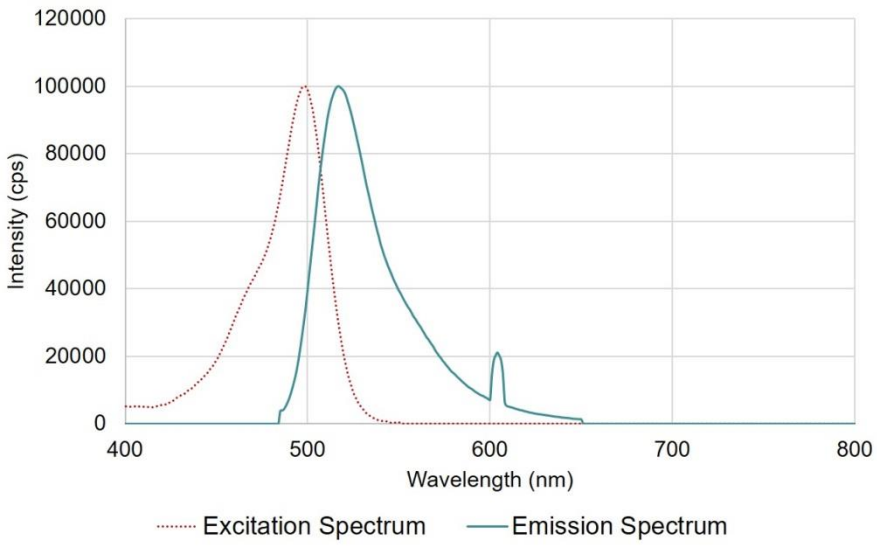


Figure 1.1.2 Fluorescence spectra

The fluorescence spectrum (Figure 1.1.2) is a figure that explains the fluorescence behavior of the sample measured by a spectrofluorometer. X-axis shows the wavelength in nanometers, while y-axis represents the measured fluorescence intensity in cps (counts per second). There are two types of spectra, one is excited spectra obtained by scanning wavelengths at a constant emission wavelength, the other, on the contrary, is emission spectra obtained by scanning wavelengths at a constant excitation wavelength. The excitation spectra and the emission spectra are independent of each other. For most fluorescent molecules, the excitation and emission spectra can be considered as “mirror” images because they have similar vibrational structures in the excited state and ground state. The major fluorescence peak represents the properties of the fluorescent molecule, while the minor peaks may suggest presence of impurities or light scattering. Some molecules may have two or more peaks on their emission spectra depending on molecular

structures. The energy loss of the molecule during the lifetime of the excited state will lead to a difference between the wavelengths of the excitation maximum and the emission maximum, which is called Stokes Shift, as a physical constant of a fluorescent molecule.

The basic equation for fluorescence intensity is shown as follows:

$$F = \Phi I_0(1 - e^{-\varepsilon bc})$$

Where F is the fluorescence intensity, Φ stands for quantum efficiency, I_0 is the incident radiant power, ε refers to molar absorptivity, b represents the path length of the cell, and c is the molar concentration. The quantum efficiency depends on wavelength, temperature and molecule itself. The incident radiant power depends on the light source. The molar absorptivity is dependent on the molecule itself. Usually, the higher concentration of the molecule, the intensity becomes higher. However, there is a specific phenomenon in fluorescence where the intensity will decrease with increasing concentrations. This is called inner filter effect, which happens when the high concentration of fluorophore molecules block the light passage. The fluorescent probe absorbs part of the excitation light (and maybe part of the emitted light as well) so more light is located at the front of the cuvette than the back of the cuvette, thus reducing the fluorescence intensity.

Anisotropy, which stands for different properties in different directions, is also an important concept in fluorescence spectroscopy. If the electric field of the light is oriented in a particular axis of the excited molecule, the excitation of the molecule by a photon will occur. Excited molecules are mostly oriented within a range of angles to the

polarized light applied on the fluorophores. This phenomena is called photoselection. With vertical polarization, the molecules will move from vertical polarized configuration to unpolarized configuration, leading to the loss of fluorescence. With horizontal polarization, there will be more excited molecules turning depolarized from vertically polarized. The ratio of the intensity difference and total intensity is defined as fluorescence anisotropy. The observed anisotropy equation for the relationship between anisotropy and probe rotation is:

$$r = \frac{r_0}{1+\tau/\phi}$$

Where r stands for observed anisotropy, r_0 is the intrinsic anisotropy only dependent on the molecule itself, τ refers to the fluorescence lifetime, and ϕ stands for the rotational time constant. Fluorescence anisotropy can be used to study the interactions of small probes to large molecules. Given vertically polarized light, a subset of fluorophores will be excited according to the orientation with the light. The molecular interaction affects the rotational diffusion of the fluorophore, thus leading to changes in anisotropy.

A spectrofluorometer is the instrument used to measure the fluorescence and phosphorescence properties of liquid or solid samples. It consists of six parts. The first part is light source which provides the energy for excitation. Xenon lamp and mercury lamp are two most common light sources used in the spectrofluorometer. The second part is the primary monochromator as the excitation wavelength selector, followed by the sample compartment as the third part. The sample is usually placed in a clear quartz cuvette which will not interfere with the light path. Then comes the fourth part, which is

the second monochromator as the emission wavelength selector. Photomultipliers serve as the detector and the fifth part of the spectrofluorometer with a good sensitivity in a wide range of UV-Vis wavelengths. Then the signals will be collected as data through the sixth part, a computer software. There is a 90° angle between the excitation and emission monochromators mainly to eliminate the background noise caused by the light source.

Not all organic molecules can fluoresce. The basic structure for a fluorescent molecule usually contains at least one π-electron system. And within all the fluorescent molecules the ones with aromatic hydrocarbon structures typically show better fluorescence sensitivity. That is because the conjugated π-bonding system requires lower energy for the promotion of an electron from a π-bonding (π) to a π-antibonding (π*) orbital transition. It is harder for saturated bonds such as σ bonds to fluoresce because higher energy will be needed for an σ-bonding (σ) to σ-antibonding (σ*) transition. The substituents on the aromatic hydrocarbon group may either reduce the π→π* transition, which leads to lower fluorescence sensitivity, or increase the π→π* transition causing a higher sensitivity. However, energy transfer is not the only factor that determines the rate of radiative decay. The excited molecule can be de-excited through various processes which have their own specific rate. When the rates of non-radiative decays are faster than radiative-decay process, the fluorescence emission will not occur. When radiative-decay rate is the fastest, it will lead to the fluorescence emission of the excited molecule.

There are a wide variety of organic molecules that exhibit good fluorescence sensitivity, and they have been well studied for the applications in analytical chemistry. Listed below are the main categories of fluorescent natural organic molecules. (1) Amino acids, such as tryptophan (Trp), phenylalanine (Phe) and tyrosine (Tyr). (2) Proteins containing Phe, Tyr and Trp groups. (3) Coenzymes and vitamins, such as NADH (nicotinamide adenine dinucleotide), NADPH (NADH phosphate), vitamin A (retinol), vitamin B_2 (riboflavin), vitamin E (tocopherol), and folic acids. (4) Nucleic acid compounds, such as pyrimidines and purines. (5) Alkaloids, such as indole alkaloids, quinolone alkaloids, carboline alkaloids, etc. (6) oxygen ring compounds, such as coumarins and flavonoids. (7) Dyes and pigments, such as porphyrins, chlorophylls, noncyclic pyrroles, quinoids, etc. (8) Other compounds such as phenols, aldehydes, ketones, etc. Some of these molecules also exhibit good phosphorescence sensitivity under certain conditions (usually at low temperature) so that they can be used to study the molecular mobility of targeted chemical reactions.

When a specific interaction between a fluorescent molecule and another substance or environment in the system occurs, the excited molecule may prefer to return to ground state by non-radiative pathways rather than the radiative pathway, leading to the reduction of fluorescence intensity. This phenomena is defined as quenching. There are four types of quenching. First is impurities. If other molecules are present at certain concentrations, they may cause interference by collisional quenching, energy transfer, electron transfer and heavy-atom effect. Second is temperature. As the temperature

increases, the fluorescence intensity decreases due to the increasing molecular motions and collisions which cause the energy loss. So it is better to keep all the samples at a constant temperature during the measurements. The third type is oxygen quenching. Singlet oxygen can easily react with excited molecules and oxidize the chromophore. Triplet states are more vulnerable to oxygen quenching than singlet excited states at normal tension. Ideally oxygen should be completely removed for luminescence measurements. Sometimes inert gas such as nitrogen is bubbled into the sample to eliminate oxygen quenching. Fourth type is concentration quenching. At dilute concentrations, the fluorescence intensity increases with increasing concentration. However, at high concentrations, there are more chances of molecule collisions, leading to the direct quenching of the excited molecules. In order to avoid inner cell effect, a pre-measurement of the intensity-concentration relation of the fluorescence molecule must be conducted. Generally speaking, dilute solutions are preferred in fluorescence measurements. To sum up, in order to obtain more accurate results of fluorescence intensity, the four types of quenching must be taken in consideration and be controlled to have minimum effect on the fluorescent molecules.

There are plenty of advantages of fluorescence spectroscopy which contributes to its potential as an analytical method to be widely used in the food industry. Firstly and most importantly, the excellent sensitivity has made it possible to detect concentration as low as 1 ppb. Secondly, it has high specificity that each fluorescent molecule has its unique excitation and emission wavelengths. Thirdly, fluorescence spectroscopy has a larger

linear range than transmittance spectrophotometric method. In addition, it is highly efficient and rapid since the scanning usually takes about less than a minute. Furthermore, this method yields reproducible results with little variation. Last but not least, it is a non-destructive method as it will not affect the physical and chemical properties of the samples. On the other hand, there have been obstacles for the development of fluorescence spectroscopy. For example, low concentration samples and clear solutions without the effect of light scattering are preferred for fluorescence measurements, while food systems are usually concentrated mixtures of carbohydrates, proteins and lipids. Nonetheless, the development of modern techniques of fluorescence spectroscopy will definitely help both fundamental researches and analytical applications in the field of food science.

1.2 Food Dyes

Food colorants, or food dyes / pigments, are edible additives in food and drinks mainly to enhance or change the color of the product and thus making it more appealing to customers. Food dyes can also be used in other industries such as cosmetics, pharmaceuticals, plastics, etc. if they are considered safe in these related applications. From a scientific standpoint, food colorants can make up the loss of natural colors due to exposure to air, moisture, high temperature, light and extreme storage conditions, enhance the colors that occur naturally, and correct the natural color variations. From a

marketing perspective, food dyes can make the food product more attractive to consumers and deliver easy identification and vivid information. Therefore food dyes have been massively used in commercial food and drinks as a functional additive and they have become an essential part of the food industry.

Table 1.2.1 Typical Natural Food Dyes

Dyes	**Source type**	**Typical source**	**Color**
Annatto	plant	achiote tree	orange
Anthocyanin	plant	blueberry	red, purple, blue
Betanin	plant	beet	red
Caramel	plant and animal	sugar	brown
Carmine	animal	cochineal insect	red
Carotenoid	plant, bacteria and fungi	carrots	yellow, orange, red
Chlorophyll	plant	leaves	green
Lycopene	plant	tomato	red
Paprika	plant	chili pepper	orange, red
Turmeric	plant	ginger	yellow

Food dyes can be divided into two major categories: natural food dyes and synthetic food dyes. Natural food dyes refer to organic compounds or mixtures extracted mainly from plant source, some from animal or microbial sources. Examples of typical natural food dyes are listed in Table 1.2.1. Synthetic food dyes, or artificial colorants, are produced by means of chemical synthesis from products of the coal and oil industry. Examples of typical synthetic dyes can be seen from Table 1.2.2.

Table 1.2.2 Typical Synthetic Food Dyes

Common name	**FD&C name**	**Type**	**Color**
Brilliant Blue FCF	FD&C Blue No.1	triarylmethane dye	bright blue
Indigotine	FD&C Blue No.2	indigo carmine	royal blue
Fast Green FCF	FD&C Green No.3	triarylmethane dye	sea green
Erythrosine	FD&C Red No.3	organoiodine dye	cherry red
Allura Red AC	FD&C Red No.40	azo dye	orange-red
Tartrazine	FD&C Yellow No.5	azo dye	lemon yellow
Sunset Yellow FCF	FD&C Yellow No.6	azo dye	orange
Amaranth	FD&C Red No.2	azo dye	dark red
Scarlet GN	FD&C Red No.4	azo dye	red
Sudan II	FD&C Red No.32	azo dye	orange-red
Orange I	FD&C Orange No.1	azo dye	orange
Orange B	/	azo dye	orange
Citrus Red 2	/	azo dye	orange-yellow
Brilliant Black	/	azo dye	black
Azorubine	/	azo dye	red
Ponceau 4R	/	azo dye	red
Lithol Rubine BK	/	azo dye	red
Quinoline Yellow	/	quinophtalone dye	greenish yellow
Patent Blue V	/	triarylmethane dye	blue
Green S	/	triarylmethane dye	green

The society has gone through four stages for the usage of food colorants. The first stage was the application of natural food colorants in ancient times. For instance, ancient Egyptian candy makers were able to add wine during processing to enhance colors, the Mayans knew how to extract carmine from female ground bugs, and the Chinese people acquired the skills to ferment meat and wine by Monascuspurpureus, a fungi with a characteristic red color. Then the second stage came with the development with a wide variety of synthetic dyes during the industrial revolution. The low-cost, stability and easy-production of synthetic food dyes quickly led to the replacement of natural dyes in

the food industry. However, problems gradually occurred with the massive use of synthetic colorants, such as adulteration, heavy metals, toxicity, etc. For the past few decades we have evolved to the third stage, where natural food dyes are again applied in the food production, while synthetic dyes still account for the major part of color additives. With the development of modern analytical techniques, people have begun to understand the properties and possible adverse effects on health of synthetic dyes. As several synthetic dyes have become concerns or doubts to the public, plenty of government regulations have been enforced on the usage of synthetic dyes to ensure food safety. Finally, the fourth stage may start in the near future when natural colors will prevail over synthetic ones in the food industry due to the freshness, healthiness, safety and sustainability people are pursuing nowadays. Though, there is still a long way to go since natural dyes are not well-studied compared to synthetic ones, and the techniques of massive production of natural food colorants also need improvements.

The current regulations on food colorants vary from country to country. In the United States, color additives that are permitted by FDA consist of two parts, one is certified color additives and the other is exempt from certification color additives. There are 7 certified food colorants in the US, including FD&C Yellow No.6 (Sunset Yellow FCF), FD&C Yellow No.5 (Tartrazine), FD&C Blue No.1 (Brilliant Blue FCF), FD&C Red No.40 (Allura Red AC), FD&C Blue No.2 (Indigotine), FD&C Red No.3 (Erythrosine) and FD&C Green No.3 (Fast Green FCF). Exempt-from-certification color additives refer to artificial colorants derived from natural sources or are synthetic variations of natural

colorants, including astathanxin, caramel, carmine, riboflavin, beta carotene, titanium dioxide, fruit juice, vegetable juice, etc. These exempt colorants still have certain limitations, which can be found on 21 CFR Section 73. Each approved colorant has its usage limits in different kinds of products. All color additives are required to be labeled by FDA, and it is prohibited to declare the additives as "natural" in the ingredient statement, although the food industry usually advertise them as natural colors.

The European Union has a different regulation system on food colorants than the US. All colorants are named by an E-classification system, such as fast green as E143 and allura red as E129. Colorants are classified into three groups in the EU, which are color additives, flavors and coloring foods. There are more artificial dyes approved in the EU than in the US, including Green S (E142), Ponceau 4R (E124), Quinoline yellow (E104), Patent blue V (E131), Carmoisine (E122), etc. In Canada, the certified colors are the same with the US, while amaranth is also allowed (amaranth is banned in the US). Color additives in Mexico follow the regulations of Official Mexican Standard, as well as Codex, EU or U.S. regulations for certain colorants. China has its own standard for food additives, which is under GB2760-2011-National Food Safety Standard. Australia and New Zealand have created the Australia New Zealand Food Standards Code for regulations of food colorants. Japan and Korea also have regulatory guidance specific to the use of color additives in their countries. According to global regulations, the food colors mostly permitted worldwide are allura red, brilliant blue, sunset yellow and tartrazine, which are commonly added into beverage products (McAvoy, 2014).

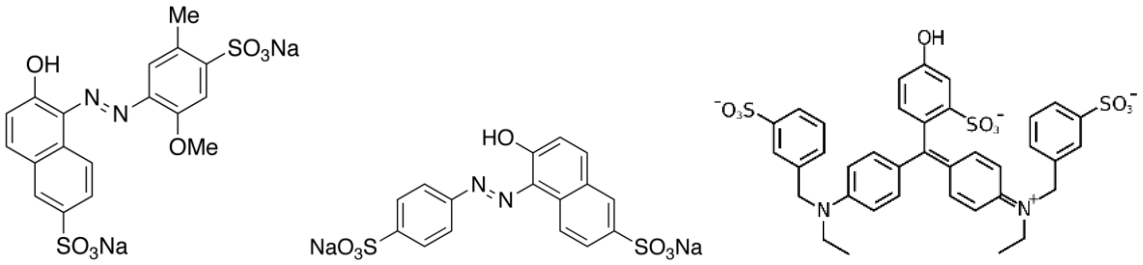


Figure 1.2.1 Molecular structures of allura red (left), sunset yellow and fast green (right)

Table 1.2.3 General information of allura red, sunset yellow and fast green

Items	**Allura Red AC**	**Sunset Yellow FCF**	**Fast Green FCF**
FD&C name	FD&C Red No.40	FD&C Yellow No.6	FD&C Green No.3
Formula	$C_{18}H_{14}N_2Na_2O_8S_2$	$C_{16}H_{10}N_2Na_2O_7S_2$	$C_{37}H_{37}N_2O_{10}S_3^+$
CAS number	25956-17-6	2783-94-0	2353-45-9
EC number	247-368-0	220-491-7	219-091-5
CI	16035	15985	42053
Structure type	azo	azo	triarylmethane
Molecular weight (g/mol)	496.43	452.37	765.89
Solubility	Soluble in water, slightly soluble in ethanol	Soluble in water, slightly soluble in ethanol	Soluble in water and ethanol
Appearance	dark red powder	orange powder	green powder
Regulation status	Approved in the U.S. and EU	Approved in the U.S. and EU	Approved in the U.S., banned in EU
Absorbance λ_{Max} (nm)	506	482	624
Fluorescence λ_{Exc} (nm)	540	480	580
Fluorescence λ_{Emi} (nm)	585	565	650

Allura red AC, chemical name disodium

6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonate, is an azo

dye synthesized from petroleum, and it is also named as Food Red 17, C.I. 16035, FD&C Red No.40 or E129. As the mostly used red dye in the US, allura red has been gradually replacing amaranth and erythrosine due to the adverse health effects caused by these two dyes. Allura red is prevalent in beverages, ice cream, yogurt, cereals and snacks to create an attracting red color. However, in the 1970s studies have shown that allura red may cause side effects such as hyperactivity in children so it was once banned in several countries in Europe (McCann, 2007). While it is approved of use in most countries worldwide, the safety of allura red still remains controversial.

Sunset yellow FCF, chemical name disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate, is also an azo dye synthesized from petroleum, and it can be named as Orange Yellow S, FD&C Yellow No.6, or C.I. 15985 or E110. It is often added into confectionary, desserts, sauces and snacks to make an orange-yellow shade. Tartrazine, another widely used orange-yellow azo dye having a similar molecular structure with sunset yellow, has been proved to cause allergies and intolerance reactions among 0.1% of population in the U.S. In comparison with tartrazine, no toxicity or carcinogenicity has been found for sunset yellow, and the U.S. approves its use with an ADI (acceptable daily intake) at 3.75 mg/kg (Hashem, 2010).

Fast green FCF, chemical name ethyl - [4 - [[4 - [ethyl -[(3 - sulfophenyl) methyl] amino] phenyl] - (4 - hydroxy - 2 - sulfophenyl) methylidene] - 1 - cyclohexa - 2, 5 - dienylidene] - [(3 - sulfophenyl) methyl] azanium, is classified as a triarylmethane dye with three aryl methane groups. Other common names for fast green are FD&C Green No.

3, Solid Green FCF, Food green 3, C.I. 42053, Green 1724, or E143. Studies have indicated that fast green may cause mutagenic and tumorigenic effects on experimental animals (Mali, 2000). It is also not well-absorbed by human intestines. Therefore fast green is the least used dye among the certified synthetic dyes in the U.S., and it is prohibited in EU and some other countries. Typical products that contain fast green as a color additive include salad dressings, candies, cereals, yogurt, etc.

Molecular structures and other basic information of the three synthetic dyes used in this project are shown in Figure 1.2.1 and Table 1.2.3. Allura red, sunset yellow and fast green are chosen as luminescent probes in this study for the following reasons. Firstly, they all exhibit excellent fluorescence sensitivity at very low concentration while they have different excitation and emission wavelengths. Secondly, they are GRAS (generally recognized as safe) colorants and are widely used in food production globally. Thirdly, they are water soluble compounds and have higher light, oxygen and pH stability than other synthetic and natural dyes. Fourthly, these dyes are inexpensive, allowing them to become potential probes that can be massively used in fluorescence spectroscopy. Last but not least, differences in molecular structures of these dyes may lead to different results of the study of interactions between the dye molecule and food macromolecules.

1.3 Previous Researches

1.3.1 Food dyes as luminescent probes for physical properties

Studies indicate that there is a strong connection between the fluorescence behavior of food dye molecules and the environmental physical properties. For example, in the excited state, the rotation of the aromatic groups through the azo group (R-N=N-R') in an azo dye will be restricted in a high-viscosity solution, causing them to predominantly decay through a radiative pathway rather than a non-radiative decay. Research has found that in glycerol-water solution, the fluorescence emission intensity of allura red increases with viscosity, which follows by an empirical model (Waxman, 2016). The fluorescence intensity of azo dyes also shows an increase with lower water activity, suggesting the restriction of rotation of the azo group in rigid environment.

Azo dyes, triarylmethane dyes and anthocyanins all exhibit this kind of behavior as so-called "molecular rotors". Studies show that the fluorescence emission intensity of azorubine, a molecular rotor azo dye, also increases with increasing viscosity of the solvents. And when using hydrocolloid solutions to modulate the viscosity, the fluorescence intensity increase is at a lesser extent compared with glycerol solutions (Kashi, 2015). This result is caused by the penetration of sucrose and glycerol solutions in the hydration cell of the fluorescent dye, while less molecular crowding occurs around the fluorescent probe in hydrocolloid such as MC and CMC when they are used to modulate the bulk viscosity. However, besides from the structure and size of the

hydrocolloid molecule, the other factor that may affect the fluorescence sensitivity of the dye probe is the interactions between the probe and the hydrocolloid, which needs to be further explored.

Food dyes can also be a probe for the micro viscosity in lipid systems. For instance, Citrus Red 2, a lipo-soluble azo dye, has been studied to monitor the micro viscosity of liquid oil entrapped in colloidal fat crystal networks (Du, 2014). Another possible application of food dyes is to understand the microstructural organization of colloidal systems. The binding capacity of sunset yellow and a surfactant, cetyltrimethylammonium bromide, has been studied by fluorescence spectroscopy to discover the microstructure change during micelle formation (Nazar, 2014). In addition, food dyes can be applied in phosphorescence as probes for matrix molecular mobility in solid and semi-solid amorphous foods. One example is that the synthetic pigment Erythrosine B can be used to monitor the α-relaxations of amorphous sugars and proteins with its phosphorescence intensity and lifetime (Draganski, 2010). The significance of food dyes as molecular rotors is that they can be used as probes for measuring bulk and micro viscosity of food systems, and may someday replace viscometers as a more rapid, specific and intrinsic analytical method.

Though studies discovered that food dyes can serve as luminescent probes for monitoring physical properties of foods, further researches still need to focus on the understanding of the molecular interactions between the dye molecule and the

environment which is majorly composed of macromolecules such as carbohydrates, proteins and lipids.

1.3.2 Interactions between food dyes and proteins

Researches have shown that several synthetic food dyes are capable of binding macromolecules, especially with protein molecules. For example, using fluorescence spectroscopy and isothermal titration calorimetry, allura red has been found to have hydrophobic and hydrogen bonded binding sites with BSA (bovine serum albumin) (Lelis, 2017). Allura red can also bind to HSA (human serum albumin) by similar kinetics with BSA (Wang, 2014). Electrophoresis studies using SDS-PAGE method indicate that allura red binds with a variety of proteins at a wide range of temperature and pH, which makes it a highly sensitive probe in detecting the proteins in food samples, such as peanut, rice bran and garlic (Abdullah, 2008).

As to sunset yellow, its interactions with DNA molecules have been revealed to be driven by hydrogen bonding and Van der Waals forces, and the reaction is exothermic and enthalpy favored ($\Delta H<0$, $\Delta S<0$) (Kashanian, 2012). Tartrazine, a similar azo dye with sunset yellow, has also been found to have interactions with HSA and BSA, and the binding affinity of tartrazine with BSA is higher than HSA (Basu, 2015). The binding kinetics differ with change of ionic strength. In low salt concentration, electrostatic interactions mainly contribute to the binding, while in high salt concentrations, hydrogen

bonding and van der Waals forces dominate (Bolel, 2012). The interactions of three food colorants, fast green, patent blue V and acid blue 1 with BSA have been compared by fluorescence quenching studies and results have shown that electrostatic force mainly contributes to the colorant binding with BSA. Fast green, among these three food dyes, has the strongest binding ability with BSA (Li, 2016).

However, most fluorescence studies of interactions between food dyes and proteins are based on fluorescence quenching technique, where the fluorescence emission intensity of the protein molecule (mainly from the amino acid tryptophan) is measured. No researches have been carried out focusing on the fluorescence intensity change of food dyes, which are highly sensitive fluorescent probes themselves.

A computational method called molecular docking has been used in order to identify the binding sites of food dyes and macromolecules, which can better explain the kinetics of interactions. The docking analysis is performed by a computer software designed to predict 3D structures of proteins and score protein-small molecule complexes through energy calculations. It is found that HSA (human serum albumin) has several hydrophobic interaction sites and hydrogen bonding sites with allura red, tartrazine, and some natural dyes, cyanidin-3-glucoside and curcumin. And the binding affinity of synthetic dye-protein H-bonds are much higher than that of natural dye-protein H-bonds (Masone, 2015). By molecular docking, the residual amino acids which form the protein-dye binding site can be identified. For example, allura red is mainly bound to HSA in site I with Trp214 – Glu250 residues, while allura red can also form two

hydrogen bonds with another residual amino acid site in the HSA molecule (Wu, 2015). These recent studies have provided insights on the binding properties of synthetic dyes and proteins on a molecular level, thus helping the development of synthetic dyes as probes in monitoring food safety and quality.

1.4 Objectives

This study aims to see if there are any increases in the fluorescence sensitivity of the probes due to molecular interactions between synthetic dyes (allura red, sunset yellow and fast green) and common food macromolecules, and thus identify the molecular interactions between them. There have been not many related studies on this topic, especially targeting the fluorescent properties of synthetic food dyes. Five carbohydrate molecules and five protein molecules are chosen for this research since they are macromolecules typically existing in food systems, while they have different physical and chemical properties due to structural differences. The carbohydrates are methyl cellulose, carboxymethyl cellulose, amylopectin, alginate and chitosan, and the proteins are bovine serum albumin, whey protein isolate, porcine gelatin, wheat gliadin and corn zein. By using the technology of luminescence spectroscopy, the fluorescence emission sensitivity of different food dyes in different macromolecule solutions will be compared. Therefore, the molecular interactions between food dyes and macromolecules may be found out according to the corresponding fluorescence behavior. Then by conducting the

anisotropy, ionic strength and temperature study, the nature of these molecular interactions will be further discovered and explained, which will provide a deeper understanding of the behaviors at a molecular level.

Based upon the achievements of this research, the potential of synthetic food dyes as luminescent probes in monitoring food quality can be assessed. The findings will also contribute to the development of novel fluorescence technology which may one day be massively applied in the food industry as a highly efficient analytical method. Examples of future applications include rapid detection of food ingredients such as carbohydrates, proteins and colorants, detection of physical properties such as viscosity and water activity, detection of chemical adulteration, detection of microbial activity and shelf life study of food products, automation in food processing, portable fluorometer, etc.

2. METHODS

2.1 Materials

Allura red AC (MW 496.43 g/mol), sunset yellow (MW 452.36 g/mol), fast green FCF (MW 808.85 g/mol), methyl cellulose (viscosity 400cP, 2% in H_2O, 20°C), sodium carboxymethyl cellulose (viscosity 470cP, 2% in H_2O, 20°C), sodium alginate, chitosan (low molecular weight), amylopectin (from corn), albumin (bovine serum, ≥ 98%), gelatin (type A, from porcine skin), gliadin (from wheat), zein (from corn) and sodium chloride (≥ 99%) used in the project are from Sigma Aldrich (St. Louis, MO, USA).

BiPro whey protein isolate (≥ 95%) used in the study is provided by Davisco Foods (Eden Prairie, MN, USA).

Reagent alcohol 90% and Acetic acid (≥ 99.7%) used are from BDH VWR Analytical (Radnor, PA, USA).

2.2 Instruments

A Fluoromax-3 spectrophotometer (Horiba Scientific Inc., Edison, NJ, USA) is used to measure fluorescence intensity and anisotropy for all samples. A Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) is used for absorbance measurements. 10mm path length quartz cuvettes (NSG Precision Cells, Farmingdale, NY, USA) are used as the sample holder.

2.3 Sample Preparation

Stock solutions of 1mM allura red, 1mM sunset yellow, and 1mM fast green are prepared in distilled water. After the fluorescence sensitivity – dye concentration study, the stock solutions are diluted respectively to 20μM allura red, 20μM sunset yellow and 5μM fast green to be used throughout all the following measurements. Stock solutions of all dyes are stored at 4°C.

Stock solutions of all macromolecules are prepared to be 1%, which is a low concentration in order to reduce the impact of turbidity and viscosity. Solvents for methyl cellulose, carboxymethyl cellulose, amylopectin, alginate, gelatin, whey protein and albumin are distilled water. Amylopectin is gelatinized by heating during the dissolving process. Chitosan is dissolved in 0.1M acetic acid solution, while zein and gliadin are dissolved in 80% ethanol solution to achieve complete dissolution. All macromolecule solutions except gelatin are stored at 4°C. Gelatin solution is stored at room temperature and is dissolved for 5 minutes in ultrasonic bath before measurements to eliminate any possible gel formation.

2.4 Measurements

Preliminary runs are carried out to choose the optimal excitation wavelength, emission and excitation slits for each dye. For this study, excitation wavelength used for allura red is 540nm, sunset yellow 505nm, and fast green 580nm. Both emission and excitation slits for all dyes are chosen to be 5nm. Scanning emission wavelengths for allura red is 560-800nm, sunset yellow 525-800nm, and fast green 600-800nm. Fluorescence excitation spectra of blank and deionized water sample are usually collected before measurements to check if the light source (xenon lamp) works well, as ~467nm should be the excitation wavelength of the xenon light source where the peak intensity occurs.

To choose the appropriate dye concentrations for all the measurements throughout the study, the fluorescence intensity of dye solutions from 0μM to 100μM are measured in order to find a linear range on the peak intensity – dye concentration plot and thus choose the dye concentration for the following studies.

Absorbance measurements are conducted at room temperature to better correct the fluorescence intensity results and therefore adjust the dye concentration in the samples to make it constant. Absorbance spectra are collected from 200-800 nm at a scan rate of 300nm/min for all samples. Blanks are subtracted from each spectra.

The fluorescence emission intensity is measured by titrating the macromolecule solution into the dye solutions (with a constant dye concentration). The starting volume

of dye concentration in the quartz cuvette is 1.5mL. The starting macromolecule concentration is 0, while the ending concentration is 0.5%. Concentrations for macromolecules: 0, 0.03%, 0.10%, 0.167%, 0.233%, 0.30%, 0.367%, 0.433%, 0.50%. Also, the dye stock solution will also be added in the sample each time for 4.08μL (the first addition is 2.04μL) to correct the dilution effect of adding macromolecule solution, according to concentration calculations and absorbance measurements. Each time the macromolecule solution is added, the sample will be stirred inside the instrument with a stir bar for 1 minute and kept still for 1 minute before measurement. Emission spectra are collected for all samples. Blanks (adding macromolecule solutions to distilled water without the presence of dye solution) are subtracted from the corresponding sample spectra. Temperature is kept constant at 20°C by a temperature controller connected to the spectrophotometer. All samples are run in triplicate.

Anisotropy values of the same sets of dye – macromolecule solutions are also collected by measuring the single-point anisotropy of the samples. Excitation / emission wavelengths for allura red are 540nm / 585nm, sunset yellow 480nm / 565nm, and fast green 580nm / 650nm respectively. Temperature is kept constant at 20°C by a temperature controller connected to the spectrophotometer. All samples are run in triplicate. To achieve more accurate results, polarizer alignment is usually conducted with amylopectin solution (light scatterer), FITC (fluorescein isothiocyanate) in ethanol solution (with a standard anisotropy value of ~0.02) and FITC in glycerol solution (with a standard anisotropy value of ~0.35).

Ionic strength study is conducted by measuring the fluorescence emission intensity when titrating sodium chloride solution into a constant concentration of dye – macromolecule solution, or titrating the macromolecule solution into a constant concentration of dye – sodium chloride solution. Also, calculated amount of the dye stock solution will also be added in the sample each time to correct the dilution effect of adding macromolecule solution. Blanks are subtracted from the corresponding sample spectra. Temperature is kept constant at 20°C by a temperature controller connected to the spectrophotometer. All samples are run in triplicate.

Temperature study is carried out by measuring the fluorescence emission intensity of a constant concentration of dye – macromolecule solution with varying temperatures. Temperature is set to 20°C, 25°C, 31°C, and 37°C by a temperature controller connected to the spectrophotometer. Before each measurement, samples are kept still in the spectrophotometer for 3 minutes after the temperature reaches the desired level. Blanks are subtracted from the corresponding sample spectra. All samples are run in triplicate.

2.5 Data Analysis

All data in the Horiba Scientific software (Fluor Essence) can be directly exported to Origin 9.0 software to be further analyzed. Blanks are subtracted from each corresponding sample data. Then the peak location (peak intensity and emission wavelength) of each spectra are identified using Microsoft Excel. Normalized maximum

intensity is calculated by dividing the peak emission intensity of a sample to the peak emission intensity of the starting dye solution without macromolecule solutions added. Then plot the normalized maximum intensity as y-axis and macromolecule concentration as x-axis. For anisotropy study, plot the anisotropy value as y-axis and macromolecule concentration as x-axis. For ionic strength study, plot the normalized maximum intensity value as y-axis and salt concentration or macromolecule concentration as x-axis. For temperature study, plot the normalized maximum intensity value as y-axis and temperature as x-axis. All plotting are done by Microsoft Excel.

According to material specifications, the average molecular weight for the following macromolecules are used in the calculations of thermodynamic parameters in chapter 3. Methyl cellulose: 40000 g/mol; BSA: 66000 g/mol; whey protein: 5000 g/mol and gelatin: 50000 g/mol.

3. RESULTS AND DISCUSSION

3.1 Choosing the appropriate dye concentration

To choose appropriate dye concentrations for this study, fluorescence emission intensity of water solutions of allura red, sunset yellow and fast green are measured in different concentrations (Figures 3.1.1, 3.1.2, 3.1.3). The intensity of all dyes exhibit an increase in low concentrations, then reach a maximum point, and then decrease in higher concentrations. The concentrations for maximum intensity are allura red (~60μM, ~$1.4*10^5$cps), sunset yellow (~40μM, ~$5*10^4$cps) and fast green (~15μM, ~$2.5*10^5$cps). The differences in intensity values and concentrations for maximum intensity indicate that with the same concentration, fast green is the most sensitive fluorescent probe in water solutions while sunset yellow is the least sensitive. The decrease of fluorescence intensity can be explained as inner filter effect. At high concentrations, the dye molecules near the light source absorb more light, thus blocking the light pathway and at the end of the cuvette there is less light than at the front. Therefore, the intensity-dye concentration experiment is essential for choosing the appropriate dye concentration to use for the entire study. The chosen dye concentration must fall into the linear range where the fluorescence emission intensity can be considered as proportional to concentration. According to the results, 20μM is chosen for allura red and sunset yellow solution, and 5μM is chosen for fast green solution for the entire study.

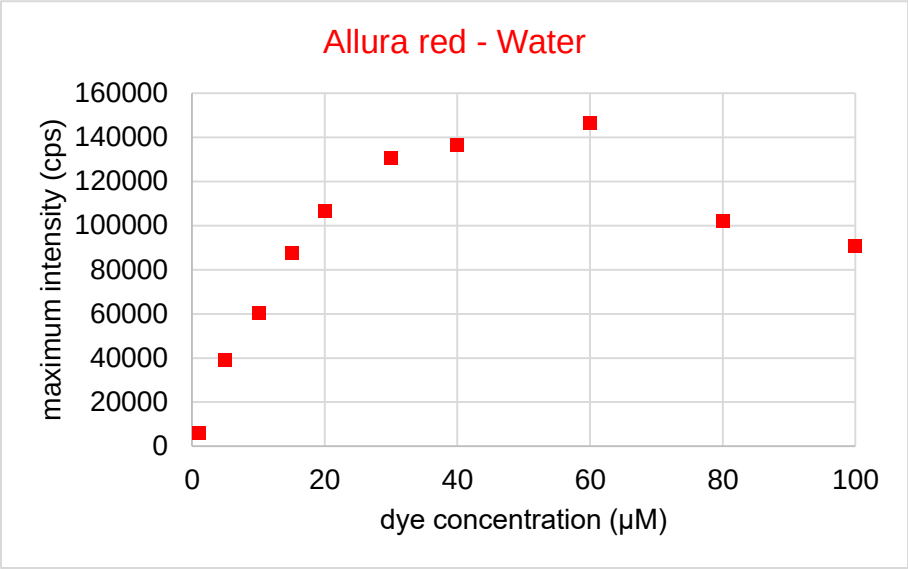


Figure 3.1.1 Fluorescence intensity of allura red in water

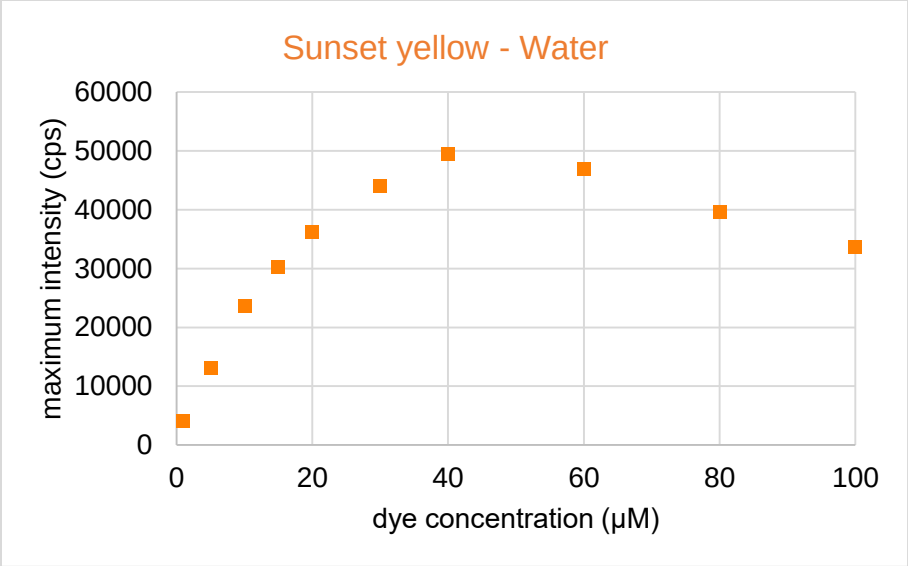


Figure 3.1.2 Fluorescence intensity of allura red in water

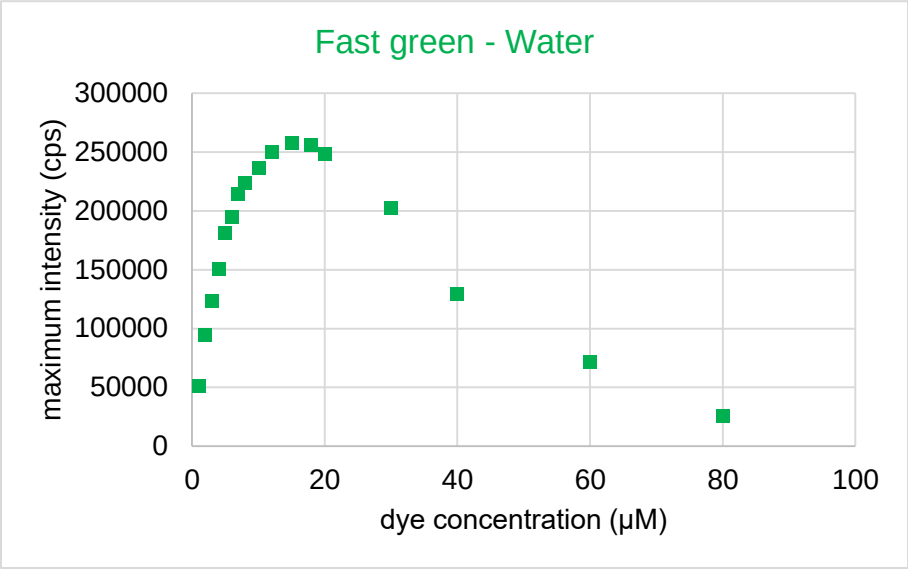


Figure 3.1.3 Fluorescence intensity of fast green in water

3.2 Dye – Carbohydrates fluorescence intensity study

In order to compare the fluorescence sensitivity of allura red, sunset yellow and fast green in different food carbohydrates and thus identify any possible molecular interactions, the fluorescence emission intensity of three dyes are measured by the addition of the carbohydrate solutions.

3.2.1 Allura red – carbohydrates

Figure 3.2.1.1 shows the fluorescence emission spectrum of allura red in varying concentrations of methyl cellulose (MC) and carboxymethyl cellulose (CMC). The

fluorescence intensity of allura red gradually increases with the addition of MC, while it does not increase with the addition of CMC.

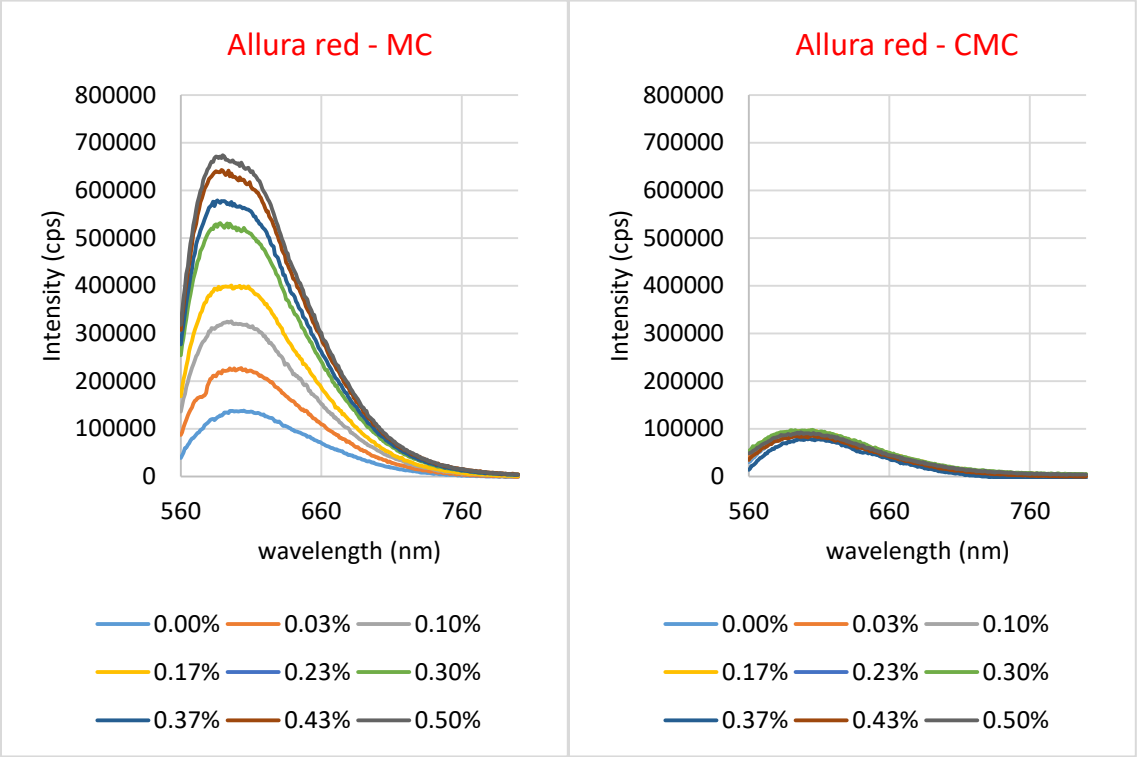


Figure 3.2.1.1 Fluorescence spectra of allura red in MC and CMC solutions as a function of added carbohydrate (wt. %)

Previous researches have indicated that both MC and CMC, as viscosity modulating hydrocolloids, can cause lower fluorescence intensity of dye probe compared to glycerol or sucrose solutions with the same bulk viscosity, because of more free water and less molecular crowding around the dye probe (Kashi, 2015). However, according to Figure 3.2.1.1, the fluorescence intensity of allura red is showing a dramatic increase with the presence of MC rather than CMC. Therefore there should be certain molecular

interactions between allura red and MC molecules. MC and CMC are both polymer compounds derived from cellulose. They differ in molecular structure by the substitute groups, which are methoxy groups and carboxymethyl groups, respectively. Allura red molecule is able to have hydrophobic interactions with MC while it doesn't do with CMC, thus leading to the increase of fluorescence sensitivity of allura red. Another reason that CMC and allura red do not have molecular interactions is that carboxymethyl group is negatively charged, so does allura red.

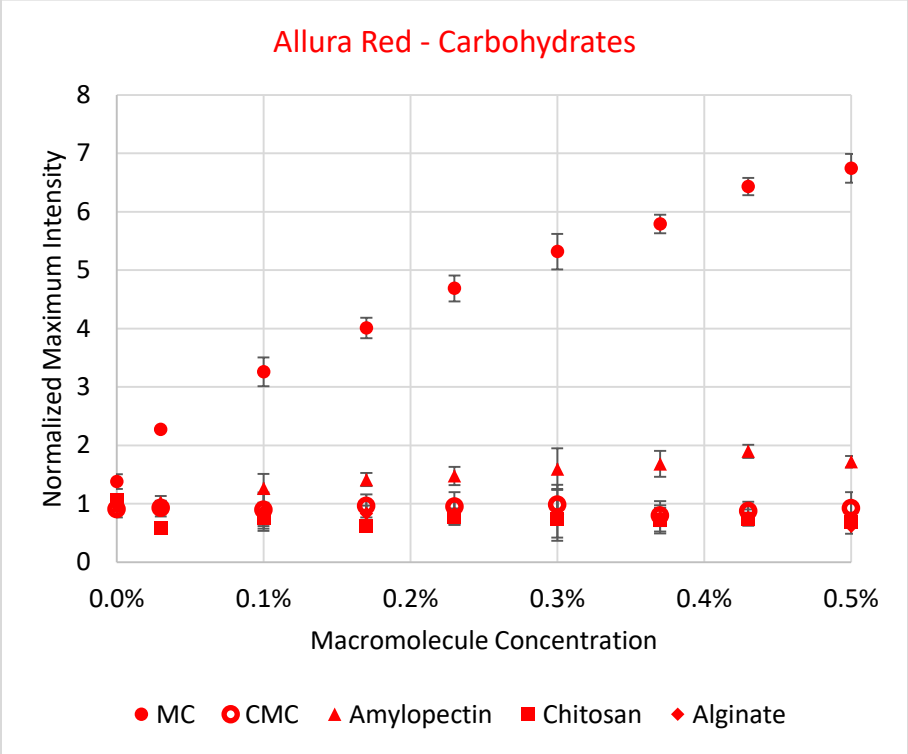


Figure 3.2.1.2 Fluorescence intensity of allura red in carbohydrates

The normalized maximum intensity of allura red in all five carbohydrates are compared in Figure 3.2.1.2. The intensity of allura red in MC increases by 7 times from

zero to 0.5% MC, increases by 1 time in amylopectin, and does not obviously change with the addition of CMC, chitosan or alginate. The behavior of allura red in MC is attributed to the molecular interactions between the two molecules. For amylopectin, the increase and a larger variation in results indicate the impact of light scattering, majorly caused by the turbidity of amylopectin solution. Chitosan, which is positively charged, is not binding with the negatively charged allura red either. This may due to the low pH level which causes the conformational change of allura red molecules. Therefore only the interactions between allura red and MC should be further studied.

To determine the binding constant and stoichiometry of the binding interaction between allura red and methyl cellulose, the modified Stern–Volmer equation below can be used.

$$\log \frac{F-F_0}{F} = \log K_a + n \log[Q]$$

Where F_0 is the initial fluorescence intensity without addition of macromolecule solution, F is the intensity with the addition of macromolecule, K_a stands for the binding constant (L/mol), n is number of binding sites, which represents the stoichiometry of the complex (dye molecule : marcomolecule), and [Q] is the macromolecule concentration (mol/L). So the binding constant can be calculated by plotting the double-logarithm regression curve of fluorescence emission intensity data.

The Gibbs free energy change of the binding interactions can be calculated by the following equation:

$$\Delta G = -RT \ln K_a$$

Where K_a is the binding constant (L/mol), R is the universal gas constant 8.3145 J/(mol K), T is the temperature (K), and ΔG means the Gibbs free energy change (kJ/mol).

Table 3.2.1 Binding constants and Gibbs free energy change for allura red and MC (r^2 stands for the correlation coefficient for the linear fit.)

	T (K)	K_a (L/mol)	r^2	n	ΔG (kJ/mol)
MC	293	76.63±0.02	0.997	0.58±0.01	-10.57±0.01

Table 3.2.1 indicates that at 20°C the interactions between allura red and MC are spontaneous because ΔG<0. Hydrophobic interactions should serve as the major molecular interaction between allura red and methyl cellulose.

3.2.2 Sunset yellow – carbohydrates

The normalized maximum intensity of sunset yellow in all five carbohydrates are compared in Figure 3.2.2.1. With the addition of MC, CMC, chitosan and alginate, the fluorescence intensity of sunset yellow does not obviously change. However, in the study of allura red with MC, the intensity increases dramatically, while for sunset yellow, which has a similar molecular structure with allura red, the addition of MC does not affect its fluorescence sensitivity. This result also verifies the explanation that there are specific

molecular interactions between MC and allura red that leads to the change of fluorescence behavior.

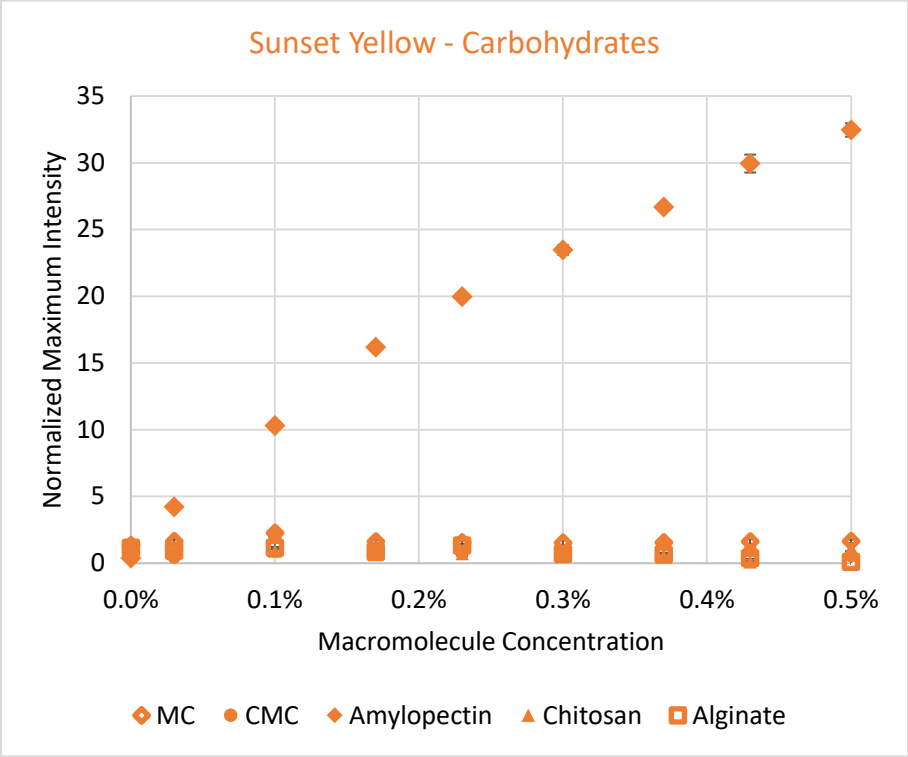


Figure 3.2.2.1 Fluorescence intensity of sunset yellow in carbohydrates

According to the molecular structure of allura red and sunset yellow, there are two more methyl groups on the allura red molecule than sunset yellow, The methyl groups are increasing the hydrophobicity of the allura red molecule and thus make it possible for allura red to have hydrophobic interactions with MC. Another possible explanation is that the binding of MC and allura red restricts the rotation of the allura red molecule but not affects the rotation of sunset yellow molecule.

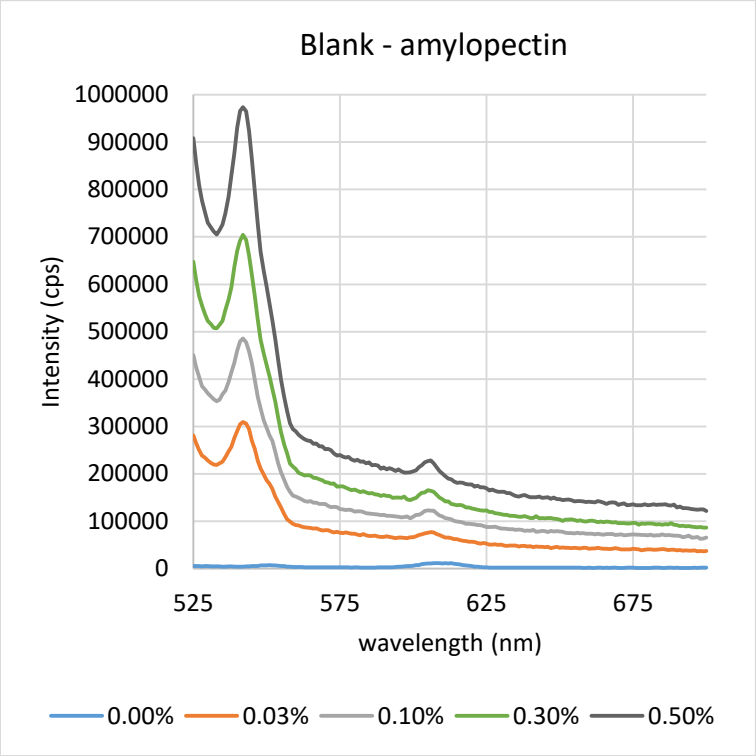


Figure 3.2.2.2 Fluorescence intensity of blank amylopectin solutions

For sunset yellow in amylopectin solutions, there is a dramatic increase of fluorescence intensity by about 33 times in 0.5% gelatinized amylopectin solution. However, this result can be explained by the fluorescence emission spectrum of amylopectin solution blank samples (Figure 3.2.2.2). The high intensity levels around 540nm indicate the impact of light scattering of the amylopectin sample. Compared to sunset yellow, the light scattering of amylopectin is not much effective in allura red, and there are two reasons. One is that the excitation and emission wavelengths of the two probes are different (for allura red, excitation wavelength is 540nm and emission wavelength is 585nm; for sunset yellow, 480nm and 565nm respectively), where the

wavelengths of sunset yellow are closer to the excitation wavelength of the light source. The other is that the initial maximum intensity of the two probes are also different. The maximum intensity of allura red is around $1.5*10^5$cps, while for sunset yellow is around $5*10^4$cps. So sunset yellow is a weaker fluorescent probe than allura red, and the minor turbidity of the sample solution can cause a greater influence on the fluorescence behavior of sunset yellow. Therefore the increase of fluorescence intensity in Figure 3.2.2 cannot reflect the binding between amylopectin and sunset yellow. So the molecular interactions between sunset yellow and the carbohydrates used in this study cannot be identified.

3.2.3 Fast green – carbohydrates

The normalized maximum intensity of fast green in all five carbohydrates are compared in Figure 3.2.3. There is no increase in fluorescence intensity of fast green in MC, CMC and alginate solutions, suggesting no molecular interactions between fast green and these three carbohydrates. A three time increase of intensity is observed for fast green in amylopectin solution, which can also be explained by the light scattering effect caused by the turbidity of the gelatinized amylopectin solution. In addition, there is also a slight intensity increase of fast green in chitosan solution. T

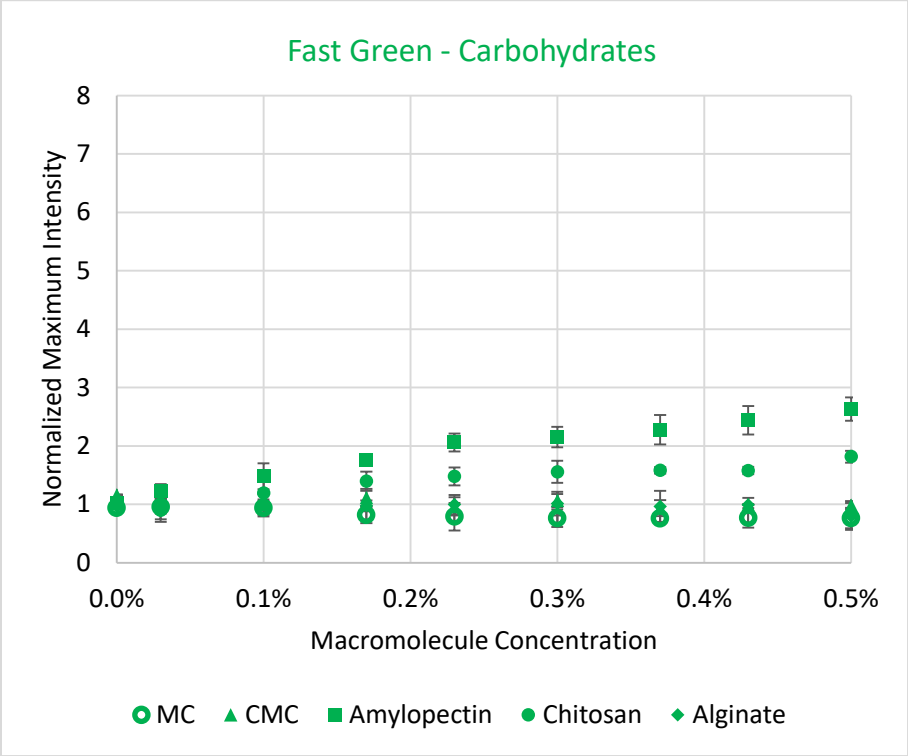


Figure 3.2.3 Fluorescence intensity of fast green in carbohydrates

his result should be caused by the increasing acidity of the sample solution (chitosan is prepared in 0.1M acetic acid solution) that changes the charge of the fast green molecule. Therefore fast green is not shown to have any specific molecular interactions with all five carbohydrate molecules tested.

3.3 Dye – Proteins fluorescence intensity study

In order to compare the fluorescence sensitivity of allura red, sunset yellow and fast green in different food proteins and thus identify any possible molecular interactions, the

fluorescence emission intensity of three dyes are measured by the addition of the protein solutions.

3.3.1 Allura red – water-soluble proteins

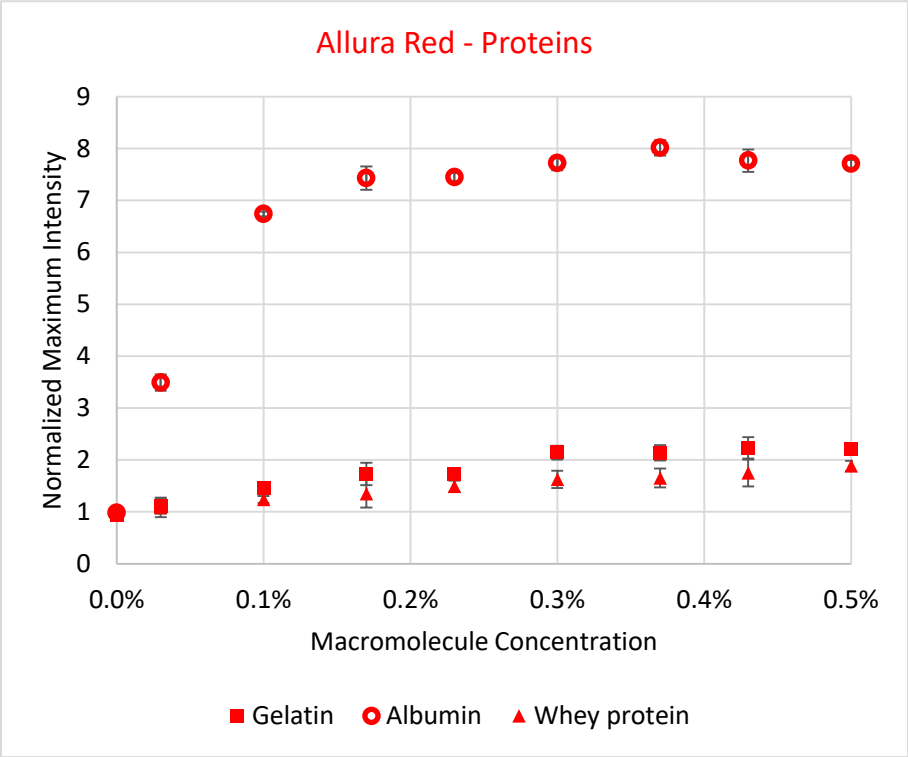


Figure 3.3.1 Fluorescence intensity of allura red in water-soluble proteins

The normalized maximum intensity of allura red in all three water soluble proteins are compared in Figure 3.3.1. A ~100% increase of maximum intensity of allura red is observed with the addition of gelatin and whey protein solutions, suggesting molecular interactions between the allura red molecule and the protein molecules. The intensity of

allura red increases to about 7 times of the initial intensity with the addition of only 0.1% bovine serum albumin (BSA) solution, and the intensity value tends to become constant with further addition of albumin. This result not only verifies the previous researches that allura red is binding with BSA complexes, but also suggests that there are more binding sites between allura red with albumin than allura red with other proteins such as whey and gelatin. The rotation of allura red as an azo dye is restricted by its electrostatic binding or hydrogen binding with protein molecules, thus enhancing its fluorescence sensitivity. By the addition of ~0.4% albumin, the binding sites are saturated for all allura red molecules so that its fluorescence activity will not be influenced by the concentration of albumin. Therefore it can be concluded that allura red, though having molecular interactions with all three proteins, has the strongest binding with BSA, leading to its highest fluorescence emission intensity.

Table 3.3.1 Binding constants and Gibbs free energy change for allura red and water-soluble proteins (r^2 stands for the correlation coefficient for the linear fit.)

Protein	T (K)	K_a (L/mol)	r^2	n	ΔG (kJ/mol)
BSA	293	8453±2.15	0.932	2.45±0.14	-22.03±0.02
Whey protein	293	756±2.57	0.996	1.03±0.07	-16.15±0.01
Gelatin	293	941±3.40	0.998	1.17±0.08	-16.68±0.01

The binding constant K and binding sites n are calculated by the modified Stern–Volmer equation after plotting the double-logarithm regression curve of fluorescence emission intensity data. And the Gibbs free energy change can be calculated through the equations mentioned in Chapter 3.2.1.

According to Table 3.3.1, allura red is binding BSA, whey protein and gelatin spontaneously at 20°C because $\Delta G<0$. However, the nature of these binding cannot be concluded at this time because enthalpy change ΔH and entropy change ΔS values are not acquired until temperature studies. This will be discussed in chapter 3.6.

3.3.2 Sunset yellow – proteins

The normalized maximum intensity of sunset yellow in all three water soluble proteins are compared in Figure 3.3.2. There is no obvious change of fluorescence emission intensity of sunset yellow with the addition of albumin, whey protein or gelatin solutions, suggesting no binding interactions between sunset yellow and the protein complexes. Sunset yellow does not have a methyl group and methoxy group as compared to the molecular structure of allura red. Since allura red is interacting with all three proteins, it is indicated that the methyl group and methoxy group on the allura red molecule tend to bind with protein molecules. Therefore sunset yellow is not shown to have any molecular interactions with the carbohydrates and proteins studied above, and

plus its weak fluorescence intensity compared to allura red and fast green, it is not considered as a good fluorescent probe for detecting molecular interactions between food dyes and macromolecules.

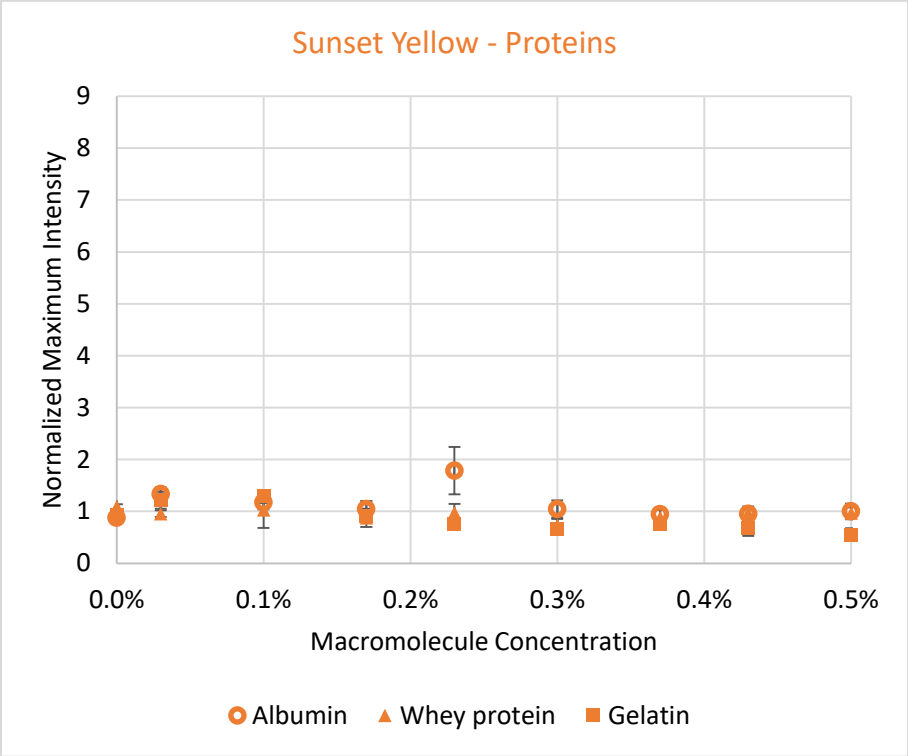


Figure 3.3.2 Fluorescence intensity of sunset yellow in water-soluble proteins

3.3.3 Fast green – proteins

The normalized maximum intensity of fast green in all three water soluble proteins are compared in Figure 3.3.3. An 8 time increase of maximum intensity of fast green is observed with the addition of gelatin, while a 10 time increase is seen by the addition of whey protein.

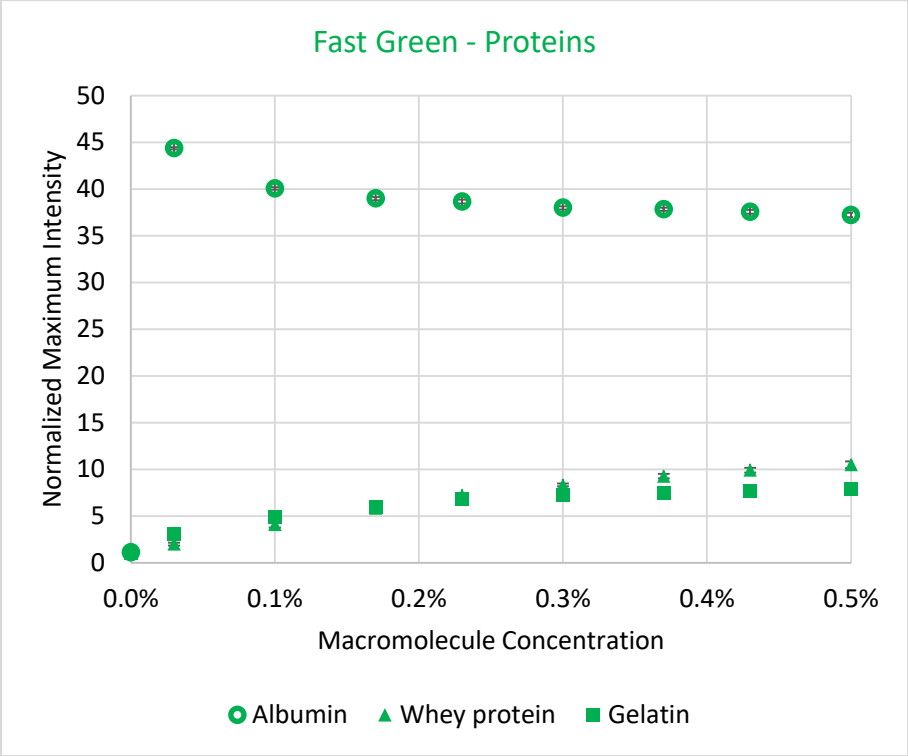


Figure 3.3.3 Fluorescence intensity of fast green in water-soluble proteins

These results suggest strong molecular interactions between the fast green molecule and the protein molecules. In addition, the intensity of fast green increases to about 45 times of the initial intensity with the addition of only 0.03% BSA solution, and the intensity value tends to decrease and then become constant with further addition of albumin. This finding verifies previous researches that fast green is capable of binding multiple protein complexes and also indicates that there are stronger binding sites between fast green with albumin than with whey and gelatin. By the addition of ~0.03% albumin, the binding sites are saturated for all fast green molecules so that its fluorescence activity will not be influenced by the concentration of albumin. A little

amount of maximum intensity decrease by the addition of more BSA solution suggest that minor quenching effect takes place when remaining BSA molecules partially disrupts the strict binding with fast green molecules, or there is redistribution of dye molecules when BSA increases, leading to fewer binding sites. Therefore fast green shows better fluorescence sensitivity than allura red and sunset yellow in interacting with proteins molecules, which makes it a highly potential probe in the quick detection of chemical properties in food systems.

The binding constant K and binding sites n are calculated by the modified Stern–Volmer equation after plotting the double-logarithm regression curve of fluorescence emission intensity data. And the Gibbs free energy change can be calculated through the equation mentioned in Chapter 3.2.1.

Table 3.3.3 Binding constants and Gibbs free energy change for fast green and water-soluble proteins (r^2 stands for the correlation coefficient for the linear fit.)

Protein	T (K)	K_a (L/mol)	r^2	n	ΔG (kJ/mol)
Whey protein	293	9563±4.51	0.994	3.43±0.22	-22.33±0.04
Gelatin	293	10741±6.12	0.995	3.22±0.29	-22.61±0.05

As shown in Table 3.3.3, the molecular interactions of fast green-whey protein and fast green-gelatin are both spontaneous reactions at 20°C because $\Delta G<0$. The K_a value of

fast green – BSA cannot be calculated due to the immediate saturation of binding sites at fairly low concentration of BSA. But it can be deduced that the reaction between fast green and BSA is also spontaneous ($\Delta G<0$). Further explanation of the rate of binding will be discussed in chapter 3.6 by calculating the enthalpy changes ΔH and entropy changes ΔS of the interactions.

3.3.4 Dyes with gliadin and zein

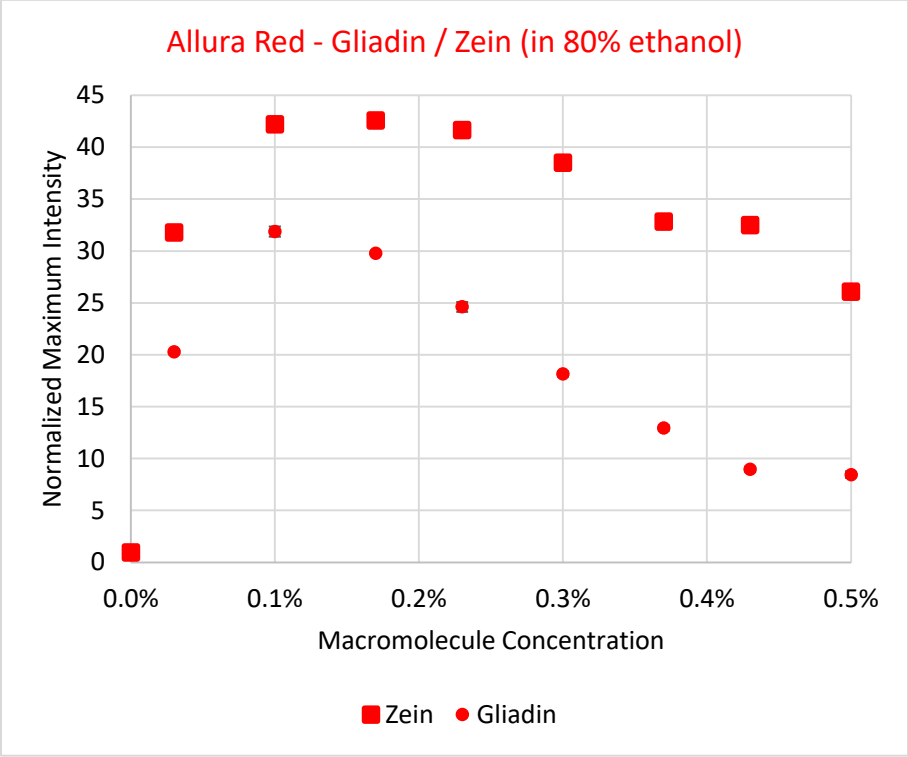


Figure 3.3.4.1 Fluorescence intensity of allura red in gliadin and zein

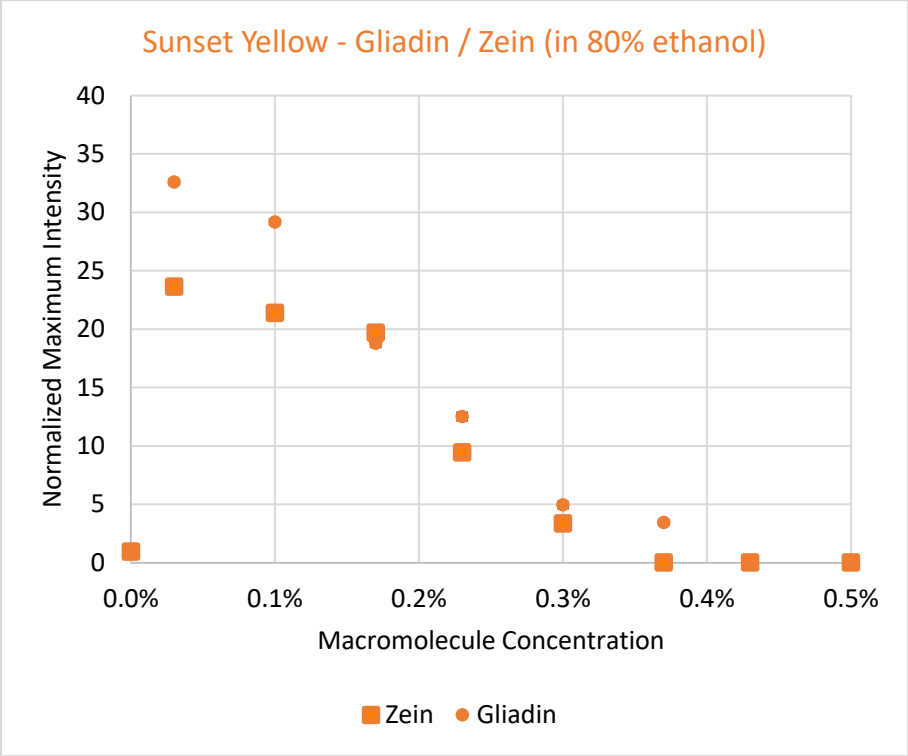


Figure 3.3.4.2 Fluorescence intensity of sunset yellow in gliadin and zein

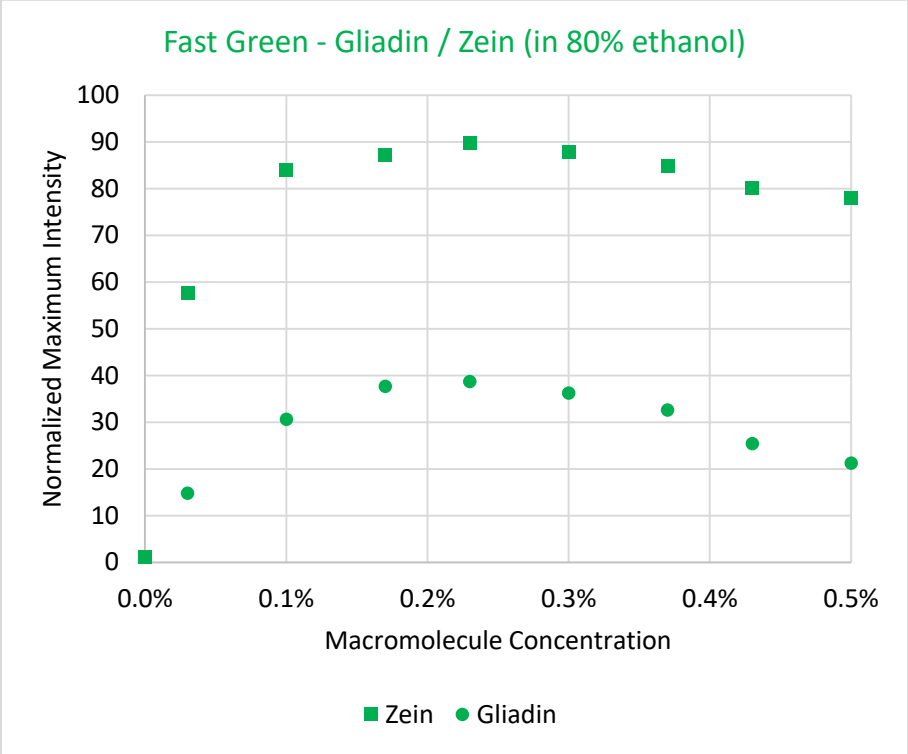


Figure 3.3.4.3 Fluorescence intensity of fast green in gliadin and zein

The normalized maximum intensity of three dyes in zein and gliadin are compared in Figure 3.3.4.1, 3.3.4.2 and 3.3.4.3. Zein and gliadin are both prepared in 80% ethanol-water solution to achieve complete dissolution. The same tendency of maximum intensity of all three dyes are observed with the addition of zein and gliadin, where the intensity increases dramatically at low concentrations and then dramatically decreases at higher concentrations. Molecular interactions between food dyes and zein or gliadin clearly exists, but cannot be identified through these results. Firstly, the intensity values are higher than the optimal range (10^5~10^6cps) of the instrument, which may cause inaccuracy of the measurement. Secondly, the intensity decrease may be attributed to the concentration quenching. Higher concentrations of zein/gliadin in ethanol may cause the dye molecules to self-aggregate and thus not absorbing and emitting enough light, leading to a decrease of fluorescence sensitivity. Third and most importantly, the existence of ethanol in the system may alter the structure of the protein and the dye molecules. Therefore it is difficult to explain the fluorescence behavior of food dyes in water-insoluble proteins. This experiment also shows that organic solvents may become an obstacle in studying the molecular interactions between food dyes and macromolecules.

3.4 Dye – Macromolecule anisotropy study

Anisotropy study is carried out to identify if the molecular interactions between food dyes and macromolecules are having impact on the rotational time of the dye molecule. The average anisotropy value of the food dyes is measured each time by the addition of macromolecule solutions.

3.4.1 Allura red anisotropy

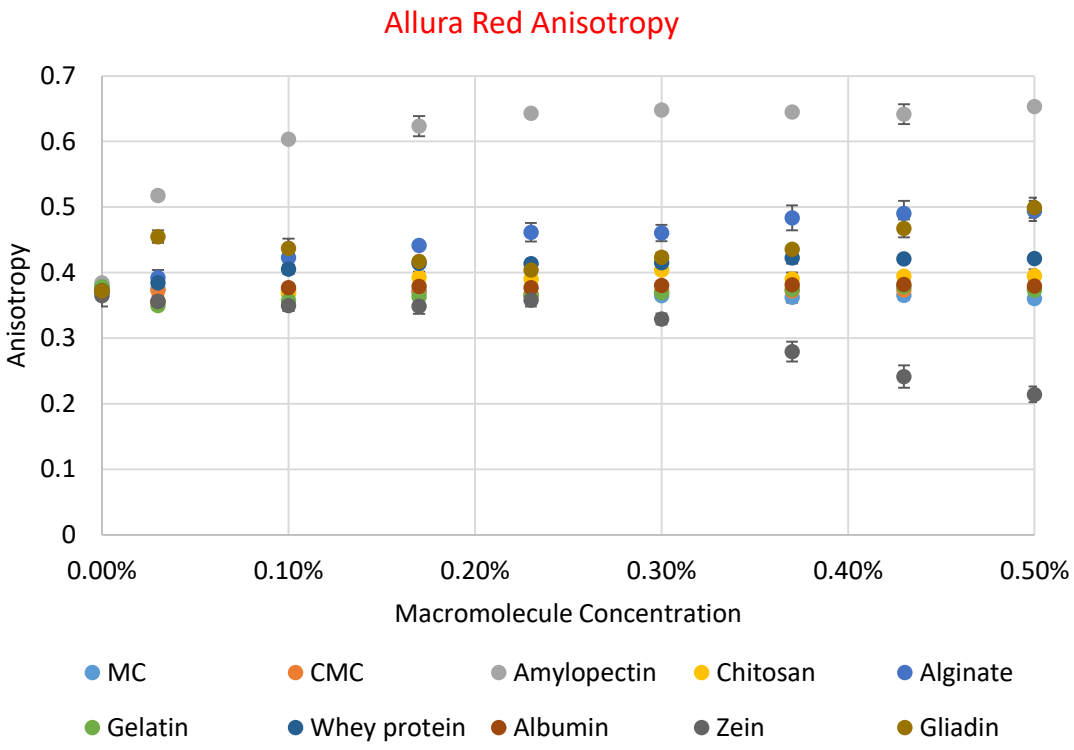


Figure 3.4.1 Anisotropy of allura red in macromolecule solutions

The anisotropy values of allura red in different concentrations of all macromolecule solutions are shown in Figure 3.4.1. Initial anisotropy value for 20μM allura red in water is ~0.38. This value is close to the theoretical maximum value 0.4, so it indicates that allura red molecule is immobile on the fluorescence lifetime in fluid water solution. The anisotropy of allura red increases with the addition of: amylopectin, MC, alginate; decreases with the addition of zein (in 80% ethanol); and does not change with the addition of other macromolecule solutions.

3.4.2 Sunset yellow anisotropy

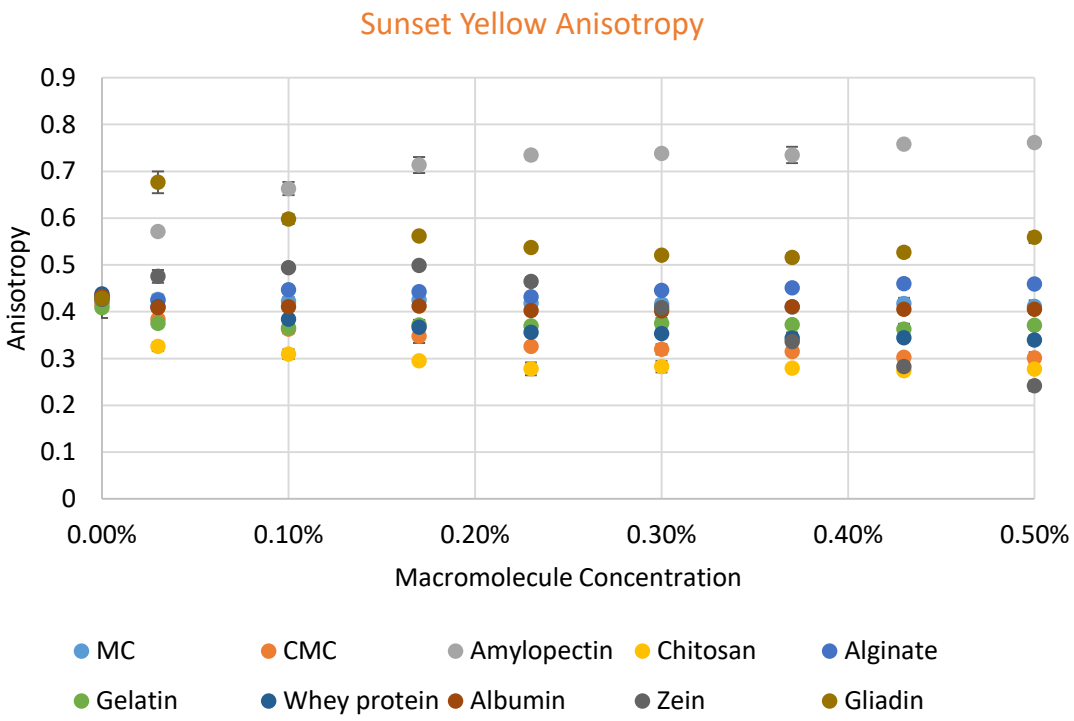


Figure 3.4.2 Anisotropy of sunset yellow in macromolecule solutions

The anisotropy values of sunset yellow in different concentrations of all macromolecule solutions are shown in Figure 3.4.2. Initial anisotropy value for 20μM sunset yellow in water is ~0.43. This is also a high anisotropy value, which exceeds the theoretical maximum of 0.40. Though alignment and measurements are both done in correct ways, measuring the anisotropy of sunset yellow molecules is not applicable. The anisotropy of sunset yellow increases with the addition of: amylopectin, MC, gliadin (in 80% ethanol); and decreases with the addition of zein (in 80% ethanol), chitosan, CMC.

3.4.3 Fast green anisotropy

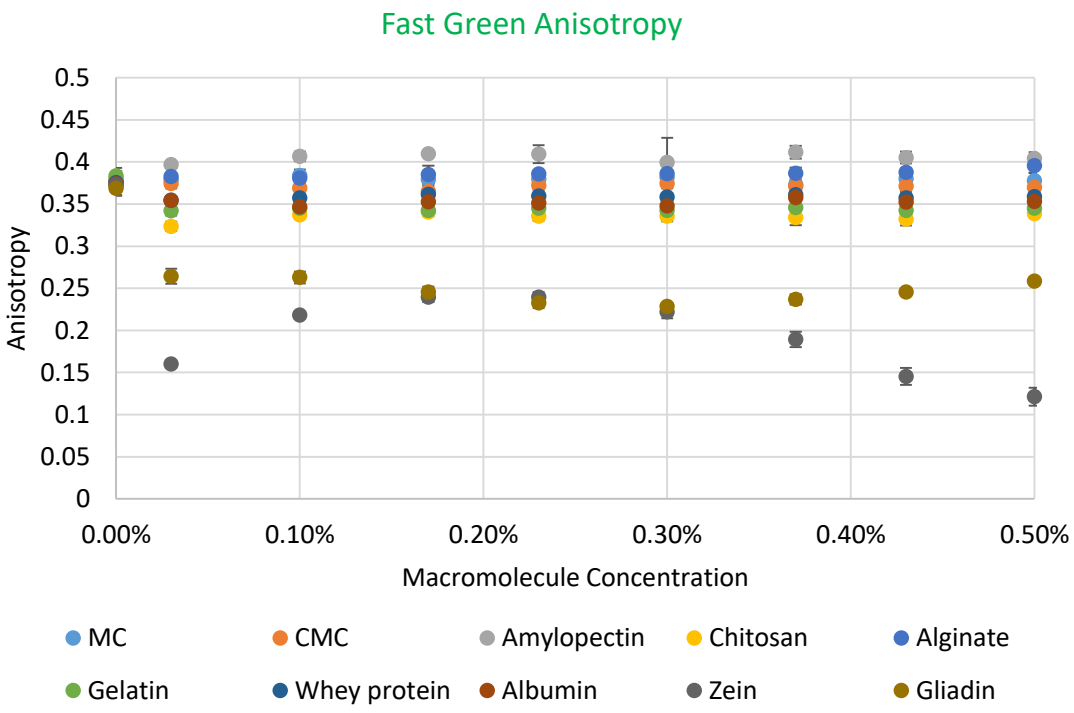


Figure 3.4.3 Anisotropy of fast green in macromolecule solutions

The anisotropy values of fast green in different concentrations of all macromolecule solutions are shown in Figure 3.4.3. Initial anisotropy value for 5μM fast green in water is ~0.37. This is a possible value but still too high for an ideal anisotropy measurement. The anisotropy of fast green decreases with the addition of zein, gliadin and does not change dramatically with the addition of other macromolecule solutions.

According to the equation of observed anisotropy, $r = \frac{r_0}{1+{}^{\tau}/_{\phi}}$ the molecular interactions between food dyes and macromolecules can be deduced from the rotational time constant (ϕ) if any binding occurs. Firstly, the fluorescence lifetime (τ) is also an important factor that affects anisotropy. When the rotational time constant changes, the lifetime of the dye probe still remains too short so that ${}^{\tau}/_{\phi}$ will not be affected at an obvious level, thus leading to no changes in observed anisotropy. That explains why the interacting combinations found in the fluorescence intensity study do not show changes on anisotropy values with the addition of macromolecules. Secondly, anisotropy decreases of the dyes with gliadin and zein verifies results from the intensity study that the molecular structure and fluorescence behavior can be affected by organic solvents such as ethanol. Thirdly, increases in anisotropy for all three dyes in amylopectin solution suggests that light scattering has caused the intensity difference of light on different directions of polarization. Extreme turbid solutions (such as high concentration un-gelatinized amylopectin) are often used for alignment of the instrument before anisotropy and polarization measurements. Fourthly, the anisotropy results are mostly

around or above the theoretical maximum value 0.4, which suggest that there are systematic error in the instrument and light scattering in the samples. So it can be concluded that anisotropy measurement does not serve as an appropriate method to discover the molecular interactions between synthetic food dyes and macromolecules because the factors are hard to control. Therefore, to further study the possible interactions, ionic strength and temperature should be two major factors taken into consideration.

3.5 Ionic strength study

Ionic strength study aims to decide whether electrostatic interactions are involved in the molecular interactions between food dyes and macromolecules. Ionic strength study is needed since electrostatic binding are vulnerable to increasing ionic strength, while hydrogen bonds and hydrophobic interactions are not. The fluorescence emission intensity of allura red and fast green are measured in selected macromolecules by the presence of sodium chloride solution.

3.5.1 Effect of constant salt solution

The fluorescence emission intensity of allura red is measured at various concentrations of BSA with and without the addition of 0.05M sodium chloride solution.

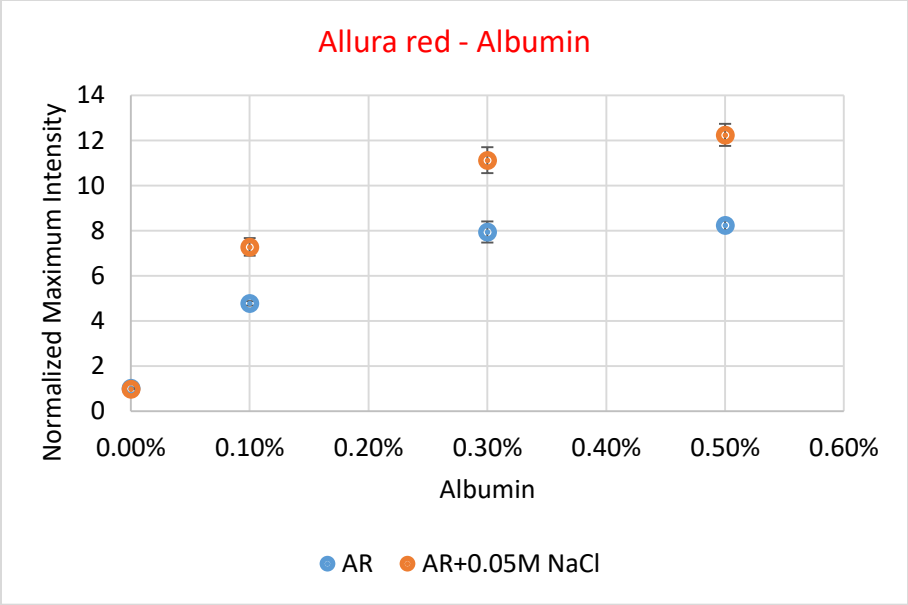


Figure 3.5.1.1 Effect of salt solution in allura red – albumin

Figure 3.5.1.1 shows that with the presence of 0.05M NaCl solution, the fluorescence maximum intensity of allura red increases up to 50% compared to samples without addition of salt. This indicates that ionic strength, at relatively low concentration, can enhance the fluorescence sensitivity of the allura red molecule. Previous research (Lelis, 2017) have suggested that hydrophobic interaction is the major molecular interaction between allura red and BSA. The hydrophobicity of allura red is caused by its three benzene rings as a heterocyclic compound, and it tends to bind the Sudlow's site I on the BSA complex. Therefore by the addition of low concentration salt solution, the hydrophobic interaction is insensitive to the increasing ionic strength, and may lead to restriction of the rotation of allura red molecules, thus causing the fluorescence sensitivity of allura red to increase.

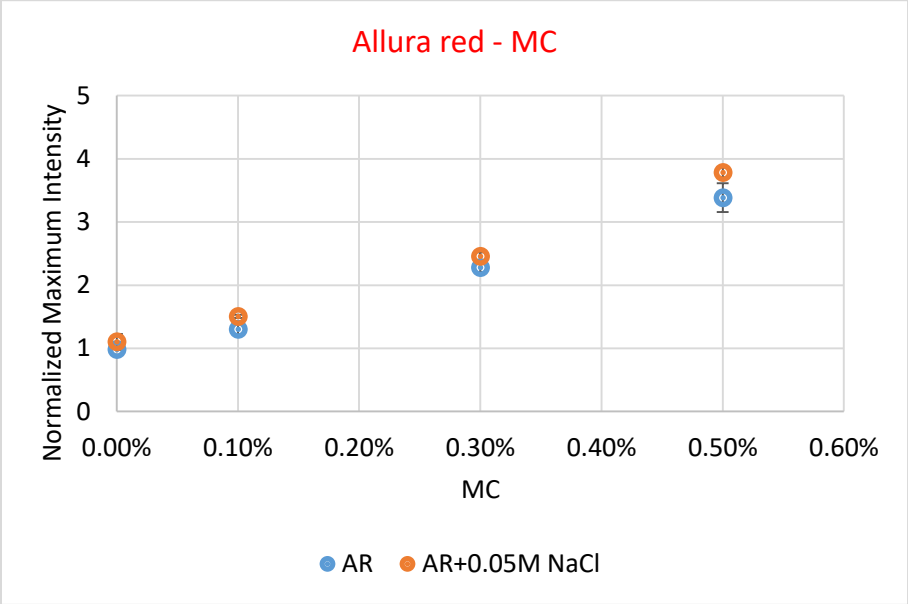


Figure 3.5.1.2 Effect of salt solution in allura red – MC

The fluorescence emission intensity of allura red is measured at various concentrations of methyl cellulose with and without the addition of 0.05M sodium chloride solution. Figure 3.5.1.2 indicates that with the presence of 0.05M NaCl solution, the fluorescence maximum intensity of allura red does not have an obvious increase or decrease. This result suggest that low ionic strength is not affecting the molecular interactions between allura red and methyl cellulose molecules. Different from allura red and BSA, the molecular interaction between allura red and methyl cellulose cannot be characterized as hydrophobic interaction. One possible explanation is that there is hydrophobic interactions between allura red and MC, thus there is no effect of low ionic

strength on the hydrogen bonds. On the other hand, the influence of local viscosity on the azo dye is also considered as a minor factor on the increase of its fluorescence sensitivity.

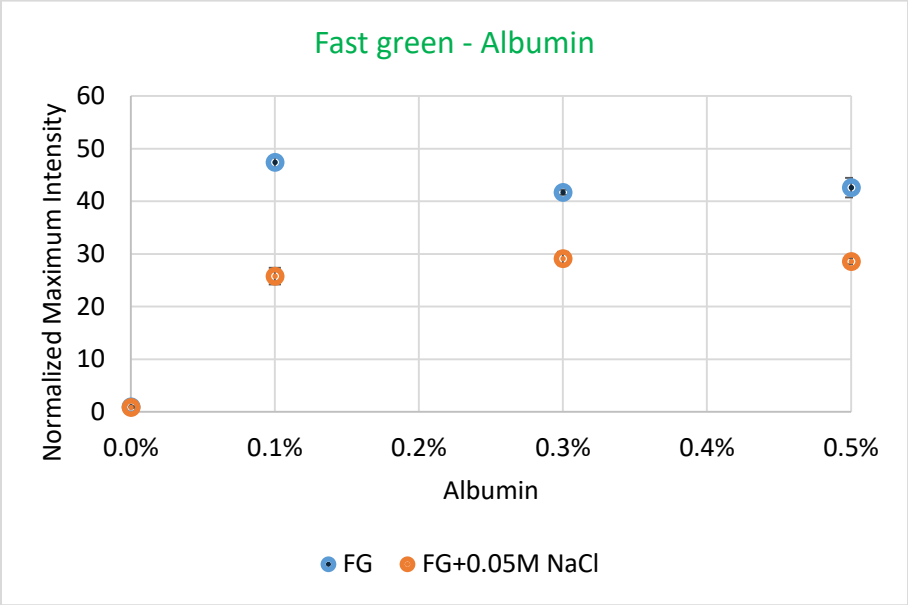


Figure 3.5.1.3 Effect of salt solution in fast green – albumin

The fluorescence emission intensity of fast green is measured at various concentrations of BSA with and without the addition of 0.05M sodium chloride solution. Figure 3.5.1.3 indicates that with the presence of 0.05M NaCl solution, the fluorescence maximum intensity of fast green is decreased by 30-50%, which means that ionic strength is strongly disrupting the molecular interactions between fast green and BSA, leading to the tendency of non-radiative decay for fast green molecules. Therefore the molecular interaction between fast green and BSA involves at least electrostatic interactions because electrostatic interactions are highly sensitive to ionic strength. The three sulfonate groups

on fast green, which are negatively charged, may have strong interactions with those amino acid side chains of BSA, which are positively charged. Also, another factor that causes the decrease of fluorescence intensity decrease is that fast green, having multiple hydrogen bond acceptor units on its molecular structure, can form strong hydrogen bonds with BSA complex. This also explains that why the fluorescence intensity is dramatically increased by increasing albumin concentration. The electrostatic interactions are vulnerable to ionic strength, so an intensity decrease is expected by the addition of salt solution.

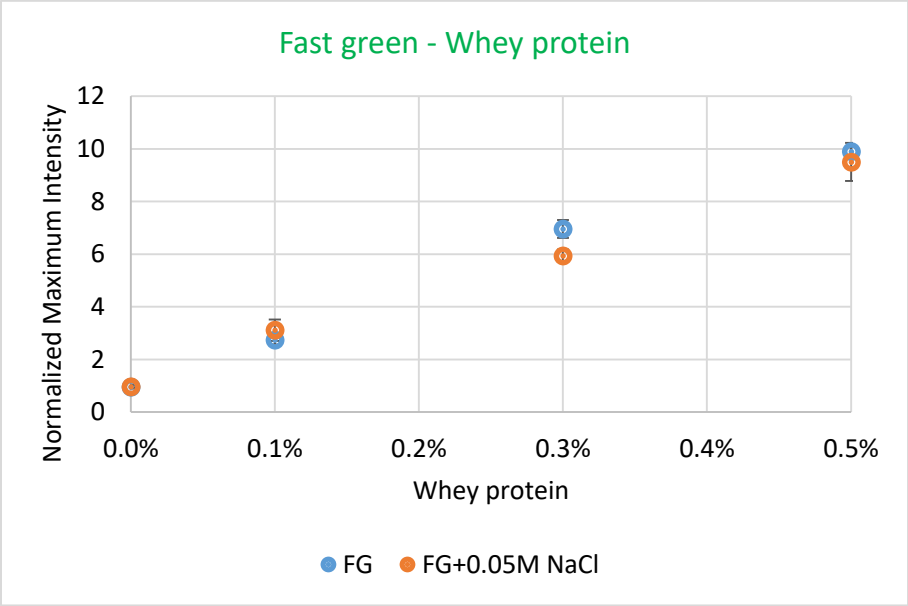


Figure 3.5.1.4 Effect of salt solution in fast green – whey protein

The fluorescence emission intensity of fast green is measured at various concentrations of whey protein with and without the addition of 0.05M sodium chloride

solution. Figure 3.5.1.4 indicates that with the presence of 0.05M NaCl solution, the fluorescence maximum intensity of fast green does not change obviously. This suggests that ionic strength, at low concentration, is not affecting the molecular interactions between fast green and whey protein molecules. In the fluorescence intensity – protein concentration study, it is suggested that the molecular interaction between fast green and whey protein is much weaker than that of fast green and albumin. Even though electrostatic interaction is still the major interaction for both situations, the binding sites of hydrogen bonds must be lesser in fast green – whey protein. Therefore when fast green binds to these proteins, there is a more obvious conformational change for the BSA complex than the whey protein complex. Binding sites of fast green on BSA are identified by previous molecular docking research (Wang, 2014) verifying the strong electrostatic interactions between fast green and BSA.

3.5.2 Effect of varying concentration of salt solution

The fluorescence emission intensity of the allura red is measured in 0.50% macromolecule solution with the presence of sodium chloride solutions at different concentrations (0, 0.05M, 0.25M, and 0.50M). From Figure 3.5.2.1, for allura red in methyl cellulose, maximum intensity gradually decreases with increasing salt concentration.

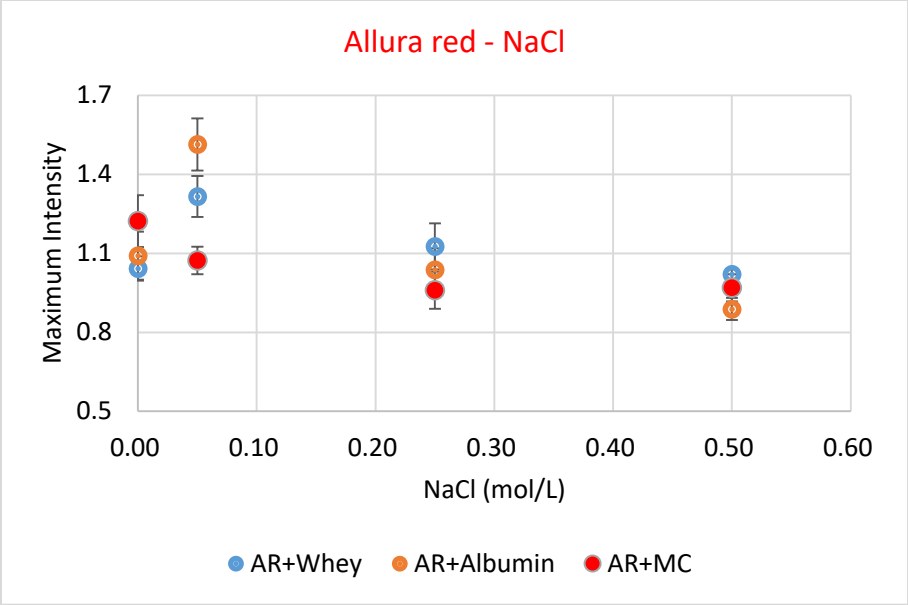


Figure 3.5.2.1 Effect of salt concentration in allura red – macromolecules

For allura red in both whey protein and albumin, the maximum intensity first increases with the addition of 0.05M NaCl, and then decreases with increasing salt concentration. All three dyes, shows a ~10% decrease of maximum intensity with the addition of 0.50M NaCl, compared to its initial maximum intensity without the presence of salt. This result verifies the ionic strength study for allura red that electrostatic interaction is the involved in the molecular interaction between allura red and methyl cellulose. Surprisingly, there is an increase of fluorescence intensity by the addition of 0.05M salt solution in BSA and whey protein. This suggests that besides from electrostatic interactions, other kinds of interactions may still play a role in the molecular interactions between allura red and proteins, which is insensitive to ionic strength. But

with further increase of salt concentration, the fluorescence intensity is still decreasing since the electrostatic interactions are being disrupted by the ionic strength.

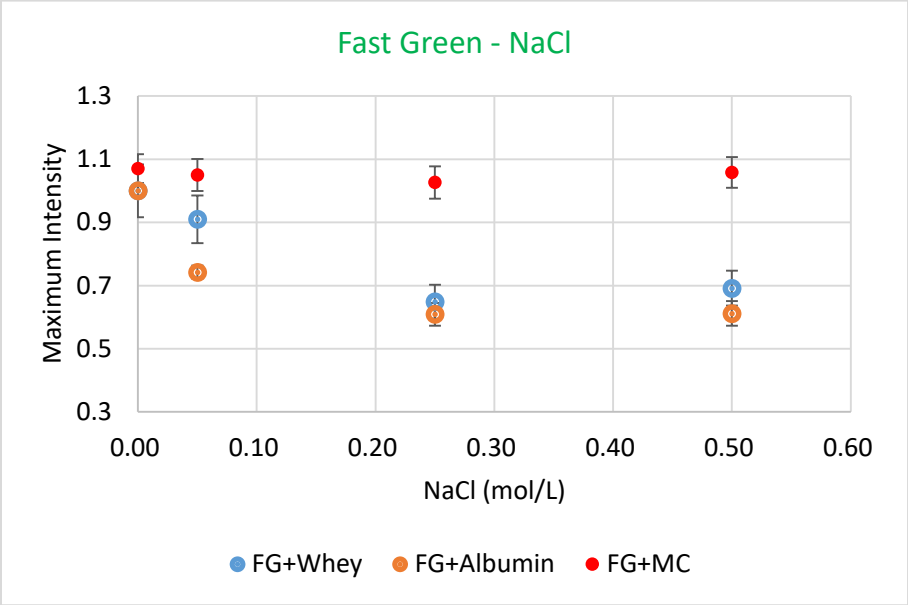


Figure 3.5.2.2 Effect of salt concentration in fast green – macromolecules

The fluorescence emission intensity of the fast green is measured in 0.50% macromolecule solution with the presence of sodium chloride solutions at different concentrations (0, 0.05M, 0.25M, and 0.50M). From Figure 3.5.2.2, for fast green in methyl cellulose, maximum intensity does not have an obvious change with increasing salt concentration, which verifies the fluorescence intensity – macromolecule concentration study that fast green in not interacting with methyl cellulose. For fast green in both whey protein and albumin, the maximum intensity decreases with increasing salt concentration, and shows a 30%-40% decrease of maximum intensity with the addition of

0.50M NaCl, compared to its initial maximum intensity without the presence of salt. This result verifies that electrostatic interaction is a contributor to the fast green – protein interaction which leads to the increase of its fluorescence sensitivity. At low salt concentrations the fluorescence intensity of fast green has a higher decrease in BSA (~30%) than in whey protein (~10%), suggesting that the electrostatic bonds between fast green and BSA are more susceptible to ionic strength than those between fast green and whey protein.

3.6 Temperature study

Temperature study is carried out in order to further understand the binding between allura red, fast green and the water-soluble proteins. Thermodynamic parameters can be calculated through measurements at multiple temperatures. The fluorescence emission intensity of allura red and fast green are measured in 0.50% selected macromolecule solutions at the following temperatures: 20°C, 25°C, 31°C and 37°C. At these temperatures denaturation of protein or structure changes of dye molecules will not occur, therefore the binding properties can be better studied.

3.6.1 Effect of temperature on allura red – protein

Figure 3.6.1 shows the fluorescence emission intensity of allura red in 0.50% macromolecule solution with varying temperatures (20°C, 25°C, 31°C and 37°C). For both albumin and whey, the maximum intensity of allura red decreases significantly with increasing temperature.

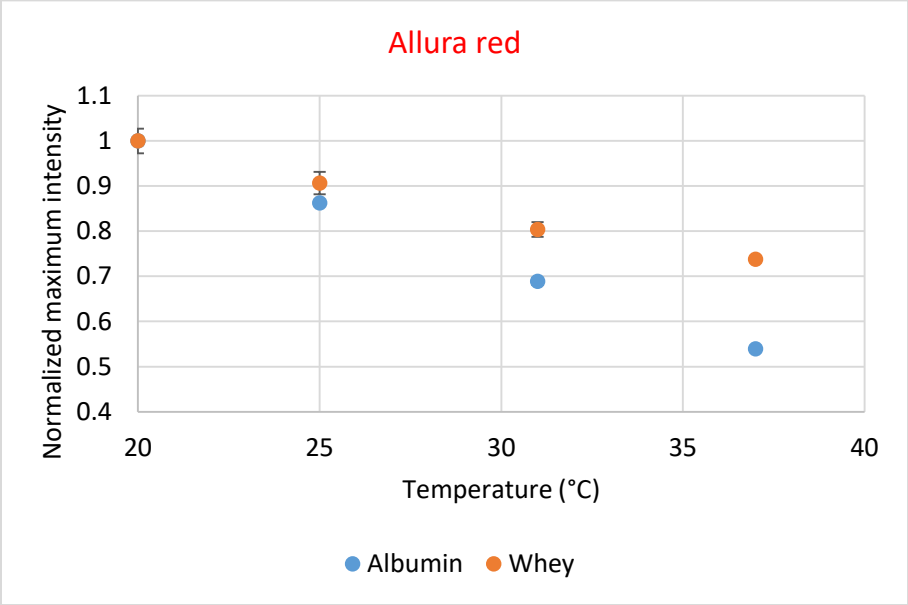


Figure 3.6.1 Effect of temperature in allura red – proteins

Compared to the initial maximum intensity at 20°C, the intensity of allura red at 37°C shows a ~25% decrease in whey protein, and a ~45% decrease in BSA. This verifies that the theory that hydrogen bonding and hydrophobic interactions are involved in molecular interactions between allura red and protein complexes, so these interactions will be disrupted by increasing temperature.

Enthalpy change and entropy change of the dye-protein interaction can be calculated according to van't Hoff equation:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

Where K_a stands for the binding constant (L/mol), R is the universal gas constant 8.3145 J/(mol K), T is the temperature (K), ΔH refers to enthalpy change (J/mol), and ΔS refers to entropy change (J/mol). Since the value change of binding sites n in the modified Stern–Volmer equation can be neglected within small temperature changes, the approximate values of ΔH and ΔS can be calculated by plotting the $\ln K_a$ – 1/T curve for linear regression.

Table 3.6.1 Enthalpy and entropy changes for allura red-protein interaction

(r^2 stands for the correlation coefficient for the linear fit.)

	ΔH (kJ/mol)	ΔS (kJ/mol)	r^2
BSA	-58.77±2.45	-0.12±0.01	0.980
Whey protein	-45.12±2.06	-0.08±0.01	0.992

From Table 3.6.1, for interactions between allura red and both BSA and whey protein, the negative ΔH values suggests that the major force of the molecular interactions is hydrogen bonding. ΔS values are both negative, indicating that hydrophobic interactions should not be a major contributor to the molecular interactions between allura red and

proteins. Therefore it can be concluded that from fluorescence intensity studies, allura red interacts with water-soluble proteins by forming hydrogen bonds.

3.6.2 Effect of temperature on fast green – protein

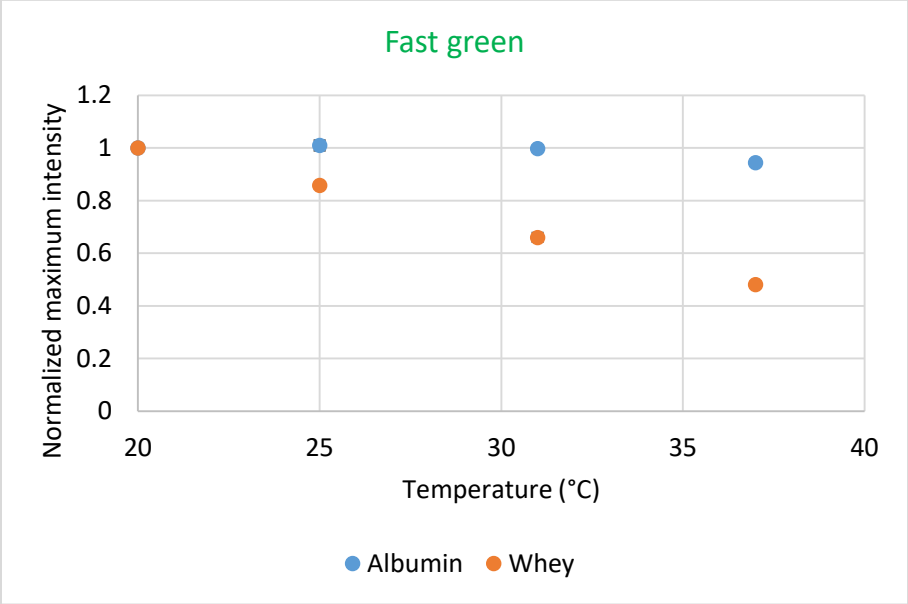


Figure 3.6.2 Effect of temperature in fast green – proteins

Figure 3.6.2 shows the fluorescence emission intensity of fast green in 0.50% macromolecule solution with varying temperatures (20°C, 25°C, 31°C and 37°C). For whey protein, the maximum intensity of fast green decreases significantly with increasing temperature. Compared to the initial maximum intensity at 20°C, the intensity of fast green at 37°C shows a 50% decrease in 0.5% whey protein. However, the intensity of fast green in BSA does not change obviously with increasing temperatures compared to the

initial maximum intensity. This result indicate that the molecular interactions between fast green and BSA are so strong that increasing temperature is not able to disrupt the specific binding sites.

Table 3.6.2 Enthalpy and entropy changes for fast green-protein interaction

(r^2 stands for the correlation coefficient for the linear fit.)

	ΔH (kJ/mol)	ΔS (kJ/mol)	r^2
BSA	-11.45±0.23	0.16±0.001	0.957
Whey protein	-24.07±0.45	-0.005±0.001	0.993

Table 3.6.2 shows the thermodynamic parameters for fast green-BSA interactions and fast green-whey protein interactions. For fast green and whey protein, the driving force of the interaction is probably hydrogen bonding because $\Delta H<0$, while the negative ΔS means hydrophobic interactions is not considered as a contributor to the interaction. However, for BSA and fast green, ΔH is negative while ΔS is positive, which means that probably both hydrogen bonding and hydrophobic interactions are playing important roles in the strong interaction between BSA and fast green. The contribution of hydrophobic interaction also explains the result that at higher temperatures the fluorescence intensity of fast green in BSA hardly decreases.

4. CONCLUSIONS

The fluorescence sensitivity of three synthetic food dyes (allura red, sunset yellow and fast green) in ten selected food macromolecule solutions (methyl cellulose, carboxymethyl cellulose, amylopectin, alginate and chitosan, bovine serum albumin, whey protein isolate, porcine gelatin, wheat gliadin and corn zein) are studied and compared by macromolecule concentration effect study, anisotropy measurements, ionic strength effect study and temperature study.

It is found that sunset yellow, as the weakest fluorescent probe among the three dyes, barely shows any fluorescence intensity increase in all macromolecule solutions, suggesting no obvious molecular interactions between sunset yellow and these carbohydrates and proteins.

The fluorescence intensity of fast green is significantly affected by the addition of all water-soluble proteins. Fast green exhibits a very strong interaction with BSA involving electrostatic interactions, hydrogen bonding and hydrophobic interactions, also it interacts with whey protein and gelatin due to hydrogen bonding and electrostatic interactions. On the other hand, fast green is not shown to have molecular interactions with any of the carbohydrates studied.

Allura red, which also has an increase in fluorescence intensity by the addition of all water-soluble proteins, shows less sensitivity to protein molecules than fast green. Major molecular interactions between allura red and BSA, whey protein and gelatin involve

hydrogen bonding. For carbohydrates, allura red only exhibits sensitivity to methyl cellulose, due to the effect of hydrophobic interactions.

These results provide insights into the molecular interactions between synthetic dyes and food macromolecules. It can be concluded that both allura red and fast green have the potential of being luminescent probes in monitoring food quality, especially considering their strong molecular interactions with water-soluble proteins. The fluorescent probes can possibly be used in the future for quick detection of certain food additives or chemical adulterants, and even microbial activity and shelf life of food products.

Further studies should focus on the fluorescence behavior of lipo-soluble dye molecules, or use synthetic food dyes to discover interactions between complicated food systems, such as protein-protein interactions, carbohydrate-protein interactions, effect of emulsifiers, etc. The findings may contribute to the development of novel fluorescence technology which may one day be massively applied in the food industry as a highly efficient analytical method.

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