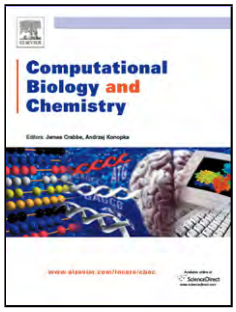


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High amounts of artificial food colorants present in infants' diets.

Children behavioral problems related with ACs point to attention disorders.

Comparison of HSA binding affinity to ACs and their natural industrial equivalents.

H-bonding is stronger for the five ACs studied than their natural equivalents.

# Study on the interaction of artificial and natural food colorants with human serum albumin: a computational point of view

Diego Masone*[1], Céline Chanforan[2]

**Abstract**

Due to the high amount of artificial food colorants present in infants' diets, their adverse effects have been of major concern among the literature. Artificial food colorants have been suggested to affect children's behavior, being hyperactivity the most common disorder. In this study we compare binding affinities of a group of artificial colorants (Sunset yellow, Quinoline yellow, Carmoisine, Allura red and Tartrazine) and their natural industrial equivalents (Carminic Acid, Curcumin, Peonidin-3-glucoside, Cyanidin-3-glucoside) to Human Serum Albumin (HSA) by a docking approach and further refinement through atomistic molecular dynamics simulations. Due to the protein-ligand conformational interface complexity, we used collective variable driven molecular dynamics to refine docking predictions and to score them according to a hydrogen-bond criterion. With this protocol, we were able to rank ligand affinities to HSA and to compare between the studied natural and artificial food additives. Our results show that the five artificial colorants studied bind better to HSA than their equivalent natural options, in terms of their H-bonding network, supporting the hypothesis of their potential risk to human health.

*Keywords:* Food colorants, Human Serum Albumin, Docking, Molecular Dynamics

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## 1. Introduction

In food manufacturing the addition of color is almost ubiquitous to improve consumers' perception of the processed products. Indeed, the addition of food colors allows insuring a standardized shade of food products. Today, natural colors are largely used as food ingredients in Europe and in the US, whereas in other regions, such as South America, artificial colors (ACs) are still considered the main solution for food coloring purposes (Hallagan et al., 1995; Wissgott and Bortlik, 1996), (for more details please see Global New Products Database 2014, www.gnpd.com). Although processed food might has powered human evolution (Kim, 2013), current levels of ACs in the acceptable daily intake are of major concern. Not surprisingly, the amount of ACs certified by the Food and Drug Administration of the United States has increased from 12 mg/capita/day in 1950 to 68 mg/capita/day in 2012 (Stevens et al., 2013a). Moreover, industrialized food designed to be consumed by infants may contain the highest concentrations of food colorants, either natural or artificial (Hofer and Jenewein, 1997). Consequently, the effects of these substances on human health have been extensively questioned in the literature (Abbey et al., 2014; Bolel et al., 2012; Stevens et al., 2013a,b, 2011; Arnold et al., 2012; Weiss, 2011). In particular, several studies on children behavioral problems related with ACs emphasize attention problems, hyperactivity, irritability, sleep disorders and aggressiveness, (Stevens et al., 2013a; McCann et al., 2007; Bateman et al., 2004).

In figure 1 ligand molecules are shown. Tartrazine is an artificial synthesized acid azo dye which is water soluble and yellow in solution (Li et al., 2014). Recent studies on rats pointed out that tartrazine and carmoisine (also known as azorubine) alter biochemical markers in vital organs such as liver and kidney, not only at high doses but also at lowest ones (Amin et al., 2010). While other studies demonstrated the adverse effects of tartrazine in learning and memory functions (Gao et al., 2011), as well as on the male reproductive function (Mehedi et al., 2009).

Figure 1

Allura red is a water-soluble, monoazo class of synthetic food pigment, with extraordinary stability in many manufactured food products such as candy coating, ice creams, drinks and confectionery (Wang et al., 2014). Allura red's side effects have been reported experimentally (Chung, 2000) and include DNA damage in male mice (Tsuda et al., 2001). Quinoline yellow is a synthetic colorant with possible genotoxic characteristics, as suggested from two different cellular model systems, human lymphocytes in vitro and Vicia faba root-tip meristems, in vivo (Macioszek and Kononowicz, 2004). While sunset yellow has shown reproductive and neurobehavioral effects (Tanaka, 1996) as well as hyperactivity (Ward, 1997).

In some food applications, such as confectionery and beverages, yellow ACs quinoline or tartrazine may be matched by their natural equivalent: curcumin (diferuloylmethane) (Hallagan et al., 1995; Aggarwal et al., 2007). This pigment is extracted from the rhizome of curcuma longa, and may also be used as natural flavor in food products. However, due to its light sensitivity and pH dependency, yellow ACs might be preferred by the food industry (Batista et al., 2006). Another yellow orange AC is sunset yellow which is matched in food applications by a natural pigment extracted from the cochineal insect (dactylopius coccus): carminic acid, (Koren, 1994). Allura red and carmoisine exhibit red and pinkish shades, respectively, when applied in foods. Anthocyanins are generally used as a natural solution for the replacement of these ACs. Natural colors based on anthocyanins consist of fruits or vegetables extracts, and their shade is pH dependent. These pigments, also used as ingredients in the pharmaceutical industry, contain antioxidants and have shown positive health effects (Tsuda, 2012; He and Giusti, 2010; Sarni-Manchado and Cheynier, 2006).

Aware of possible children's health issues related to the consumption of ACs, the European Parliament published a list of artificial food colors allowed to be used in processed food but requiring a warning label on food packaging (see table 1). This modification of the European regulation is aligned with the evolution of European and North American customers perception over the last years; indeed many customers are looking for food products with "all natural"

| Food Colorant | E number in EU | Natural equivalent | E number in EU |
|---|---|---|---|
| Sunset yellow | E 110 | Carminic Acid | E 120 |
| Quinoline yellow | E 104 | Curcumin | E 100 |
| Carmoisine | E 122 | Peonidin-3-glucoside | E 163 |
| Allura red | E 129 | Cyanidin-3-glucoside | E 163 |
| Tartrazine | E 102 | Curcumin | E 100 |

Table 1: Food colorants to be used in processed food allowed by the Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. For artificial ones the labeling shall mention: `may have an adverse effect on activity and attention in children.`

claims on packaging (Bartels and Onwezen, 2014; Tully and Winer, 2014; Hsu and Chen, 2014).

During the last decade atomistic simulations have been able to contribute in the understanding of critical problems in biophysics and computational chemistry, such as protein folding, protein-protein and protein-ligand docking (Piana and Laio, 2007; Pietrucci et al., 2009; Berteotti et al., 2009). These advances stand on the shoulders of newly developed methods that explore free energy landscapes more efficiently, together with better and faster computer processors (Van Der Spoel et al., 2005). Remarkably, collective variable driven molecular dynamics (MD) have shown to suitably sample complex conformational changes in biomolecules by accelerating rare events not observable by classical MD (Fiorin et al., 2013). In particular, restrained MD simulations are able to surpass intrinsic limitations of the physical models, resulting in a more efficient statistical sampling (Fiorin et al., 2013; Laio and Parrinello, 2002; Laio and Gervasio, 2008; Kumar et al., 1996). Besides, considering that large computer power is not available to all, the problem of rapidly obtaining realistic dynamic information on proteins, remains an issue of principal interest (Cossins et al., 2012).

In this study we analyze by computational means how 5 artificial food colorants and their 4 natural equivalents interact with Human Serum Albumin

(HSA), the most abundant protein in plasma which contributes to about 80% of the blood osmotic pressure (He and Carter, 1992; Peters, 1995). Together with alpha-1-acid glycoprotein, HSA is responsible for transporting drugs, steroids, bilirubin, thyroid hormones, fatty and colic acids (Zunszain et al., 2003; Peters, 1995; Honoré, 1990), as it contains two structurally selective binding sites (Jisha et al., 2006). The binding of such artificial and possibly toxic compounds to HSA is of physiological relevance, since binding to HSA can be the way to control these substances concentrations as well as their side effects (Pan et al., 2011). As Basu and Kumar recently pointed out, the available free concentration for toxic action can be regulated by high binding to serum proteins (Basu and Kumar, 2014). The binding between ACs and HSA could then affect the absorption, distribution, metabolism and toxicity of these compounds and may alter the functions and structure of the receptor protein (Wang et al., 2014).

Figure 2

## 2. Computational methods

### *2.1. Docking*

In order to locate the protein binding site of each one of the 9 ligands (4 natural colorants and 5 artificial ones, since curcumin is used to replace both tartrazine and quinoline yellow in the food industries) we performed a docking analysis using the freely available PatchDock web server (Schneidman-Duhovny et al., 2005). This large-scale docking web-tool allows for structure prediction and scoring of protein/small-molecule-complexes through both, geometric fit and atomic desolvation energy. The crystal structure of the Human Serum Albumin (HSA) is available in the PDB (www.pdb.org) with PDB ID 1AO6 (Sugio et al., 1999). Ligand molecules were downloaded from PubChem (Bolton et al., 2008) (tartrazine: CID 164825, curcumin: SID 12626, carmoisine: CID 22717453, Cyanidin-3-glucoside: CID 25244576, sunset yellow: CID 6093232, allura red: CID 6093299, quinoline yellow: CID 6731, peonidin-3-glucoside: SID

14288, carminic acid: SID 87565853) and their 3D structures were optimized with the academic version of Maestro 9.7 (SchrödingerLLC) before docking. Docking analysis provided a set of 100 candidates ranked by Patchdock's scoring function for each of the protein-ligand systems.

Docking simulations located the protein's pocket near HSA's subdomain IIA (see figure 2), a principal binding site together with IIIA. IIA binding site has been characterized as both electrostatic and hydrophobic (Sudlow et al., 1975, 1976) which has been also observed experimentally (He and Carter, 1992).

### *2.2. Docking refinement*

Initial configurations of HSA-ligand were exported to Gromacs package in order to use docking results as a starting point for dynamics. For each protein-ligand system only the top1 docking candidate was selected, according to PatchDock's scoring function. Each ligand was parametrized for Gromacs using the PRODGR web server (Schüttelkopf and van Aalten, 2004) and the Gromos45a3 force field. Systems were solvated in SPC-water (Berendsen et al., 1981) minimized and equilibrated with Gromacs-4.6.3 (Hess et al., 2008). Docking refinement simulations were run under the NPT ensemble for 20ns using the V-rescale thermostat (Bussi et al., 2007) and Berendsen's barostat (Berendsen et al., 1984) at 298K.

In order to account for major conformational changes in the receptor protein while the ligand binds, we slowly simulated the entering path of each molecule using restrained MD with a reaction coordinate $D$ (see eq. 1). This collective variable pulls the ligand into the pocket binding site of HSA, according to each docking prediction. Once the ligand is in the pocket we continued to sample by restraining the value of $D$=0, meaning that the ligand is forced to stay in the binding site. The collective variable defined $D$ measured the three-dimensional distance between the ligand center of mass $L_{com}$ and the geometric center of the binding site $P_{center}$, (see eq. 1):

$$\vec{D}(x,y,z) = \vec{L}_{com} - \vec{P}_{center} \tag{1}$$

This -in place- simulations allowed for further docking refinement and evaluation of hydrogen bond formation. To do so, we imposed a second collective variable defined $H$ (see eq. 2) to efficiently bias dynamics. Accordingly, collective variable $H$ counts the number of hydrogen bonds between a group donors and acceptors to drive the system into the formation of hydrogen bonds. User defined values where set to $r_0 = 2.5$, n = 6 and m = 12, where $i$ counts over the group of donors (ligand) and $j$ over the group of acceptors (protein). The switching function form of $H$ ensures it to be differentiable.

$$H = \sum_{ij} \frac{1 - (\frac{d_{ij}}{r_0})^n}{1 - (\frac{d_{ij}}{r_0})^m} \tag{2}$$

To perform restrained MD simulations we patched Gromacs-4.6.3 with Plumed 2.0.1 (Tribello et al., 2014), an open source library for free energy calculations, in order to implement the reaction coordinates to drive each ligand along a binding path into the protein's pocket and to bias the system into the formation of protein-ligand hydrogen bonds. We used a time step of 2 fs, all bond-lengths were constrained using the sixth-order `LINCS` algorithm (Hess et al., 1997), the Particle-Mesh-Ewald (PME) method was used for the long-range electrostatics (Essmann et al., 1995) with a Fourier grid spacing of 0.16 nm and all cut-offs set to 1 nm.

Protein and ligand figures were created using the academic version of Maestro 9.7 Molecular Modeling Environment (SchrödingerLLC). Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) has been extensively used for visualizing MD trajectories and docking results.

### *2.3. Docking scoring*

To score docking refined poses for each protein-ligand system the formation of hydrogen bonds was evaluated along the refinement MD trajectories, as defined by collective variable $H$. The value of hydrogen bonds' spatial and directional properties has shown to be of major interest in analyzing docking conformations (Meyer et al., 1996). Hydrogen bonds are a key contributor to

the high specificity of macromolecule interactions, having distributions in good agreement with energy landscapes obtained through electronic structure calculations (Morozov et al., 2004). As previously demonstrated, hydrogen bond refinement has proven to consistently bring initial models closer to natives structures (Masone and Grosdidier, 2014; Masone et al., 2012). Several available softwares currently allow for hydrogen bond optimizations following a combination of local geometry restraint and a conformational search (Bhattacharya and Cheng, 2013) and reorienting hydroxyl and thiol groups, water molecules, amide groups of ASN and GLN residues, together with the imidazole ring in histidines (Madhavi Sastry et al., 2013). Hence, we ranked our protein-ligand poses in terms of the amount of hydrogen bonds formed during MD refinement trajectories under the action of collective variables $D$ and $H$. Finally, we compared the docking affinity of all the systems under study.

## 3. Results and discussion

Modeling protein-ligand interactions can be considered a two-step problem of increasing complexity: (a) pose generation and (b) pose refinement and scoring. Normally, the first step is performed under an approximated but very efficient rigid body docking algorithm, usually leaving full flexibility for the second step. Due to time and speed limitations, most algorithms do not use all-atom force fields to score docking poses, but instead, rely on soft scoring functions to correct for steric clashes and for the lack of optimized hydrogen bond networks. The scoring energy function then analyzes the conformation of the protein-ligand pose and returns a numeric representation of the total energy. Although having little meaning alone, this number can be used relatively to get an idea of how stable the different docking conformations are. As a consequence, computational studies of protein-ligand interactions from an energetic point of view are also important to comprehend their essential principles and thus to improve modeling.

From docking calculations binding site's relevant residues were identified in

agreement with previous studies: between subdomains IIA and IIIA in (Artali et al., 2005) and binding site 1 in (Deeb et al., 2010), (see figure 3). Selected residues for reaction coordinate definition include: hydrophobic Trp214, Leu219, Phe223, Leu234, Leu238, Leu260, Ile264, Ile290, Ala291, hydrophylic Arg222, Arg257 and His242 (He and Carter, 1992), free Cys34 and reactive residues Lys195, Lys199 and Tyr411 located at the entrance of the IIA/IIIA binding sites (Yvon et al., 1990). The center of mass defined by these residues is $P_{center}$, dynamically calculated during simulations and used by the reaction coordinate to constantly measure distance $D$ (see eq. 1). Given that protein-ligand interactions are not only driven by hydrogen bonding but also by other force contributions such as hydrophobic, van der Waals and electrostatics, figure 3 depicts in detail the molecular interactions between each ligand and the surrounding residues in HSA's binding pocket. At the docking stage, most hydrophobic ligands are carminic acid, peonidin-3-glucoside, cyanidin-3-glucoside and tartrazine. Curcumin and tartrazine are the ones with more positively charged electrostatic interactions and allura red is the one showing more negatively charged electrostatic interactions. Allura red, carmoisine and sunset yellow present the larger amount of polar interactions.

Figure 3

Figure 4 shows the scoring results for all ligands studied in terms of the amount of protein-ligand H-bonds, as measured by variable H, here used as scoring function along 20ns trajectories. It is observed how in all cases, the binding affinity (in terms of the H-bonding network) of the AC is systematically higher than its natural equivalent in terms of shade. This result suggests that these artificial molecules might easily reach other targets in the body as they are transported by HSA. Such is the case of tartrazine, classified as DNA binder, toxic to human lymphocytes and a contributor to primary biliary cirrhosis (Carocho et al., 2014).

Figure 4

The combination of docking prediction methods with molecular dynamics simulations allows for efficient docking refinement through exhaustive exploration of the protein potential energy landscape. As shown by Król and collaborators, accounting for full flexibility during relaxation tends to increase the amount of recovered native contacts among sets of docking poses (Król et al., 2007a,b). Alonso and collaborators have shown how molecular dynamics simulations have been able to accurately, although expensively, refine a few selected candidates from a previous fast docking stage used to sample high complexity configurational spaces (Alonso et al., 2006). Yet, for most biological systems, the complete atomistic description in long time scales is still beyond classical molecular dynamics, as a result of the femtosecond time step needed for energy conservation. Then, it becomes critical to reduce the number of degrees of freedom into a few parameters which can be biased to enhance dynamics in a controlled manner. However, the major limitation of collective variable based dynamics is the reduced amount of reaction coordinates that can be implemented to avoid excessive computational costs. Besides, the initial selection of the reaction coordinates might introduce erroneous biases on how events of interest happen (Abrams and Vanden-Eijnden, 2010), meaning that, choosing a correct set of collective variables remains an unsolved issue.

Hence, the easier binding process of ACs to HSA, demonstrated here from a computational point of view, may be linked with the involvement of these molecules in health issues as largely reported in the literature (see introduction for references to each molecule).

## 4. Conclusions

This study provides an insight on protein-ligand interactions for 5 commonly used artificial food colorants and their natural equivalents in terms of shade. The protocol presented here identifies the protein-ligand binding site through a docking algorithm and refines the docking solutions by restrained molecular dynamics simulations. Two reaction coordinates are used to control the three

dimensional distance from the ligand to the receptor protein binding site and to bias the system into the formation of protein-ligand hydrogen bonds.

We have performed atomistic molecular dynamics to allow for the conformational space exploration that takes place when ligands bind to HSA. Refinement of docking solutions is then critical due to the intrinsic approximated nature of the docking algorithm. This kind of analysis is only possible with proper collective variables defined to bias sampling and efficiently accelerate an event (such as a ligand going inside a receptor protein and the formation of hydrogen bonds between a protein and a ligand), that otherwise would not be possible to observe within current computer simulations times.

Our results indicate that ACs bind to HSA creating a stronger H-bond network than their natural equivalents in terms of shade. Being HSA the most abundant protein in plasma (He and Carter, 1992; Peters, 1995) responsible for transporting a wide variety of molecules (Zunszain et al., 2003; Peters, 1995; Honoré, 1990) its interaction with artificial compounds is of major physiological importance (Pan et al., 2011) and may control the free concentration of these compounds (Basu and Kumar, 2014). Besides, the interaction of HSA with these ACs may as well interfere with HSA's functions (Wang et al., 2014).

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## 6. Declaration of conflicting interests

DM and CC declared no conflicting interests with respect to the research, authorship and publication of this article.

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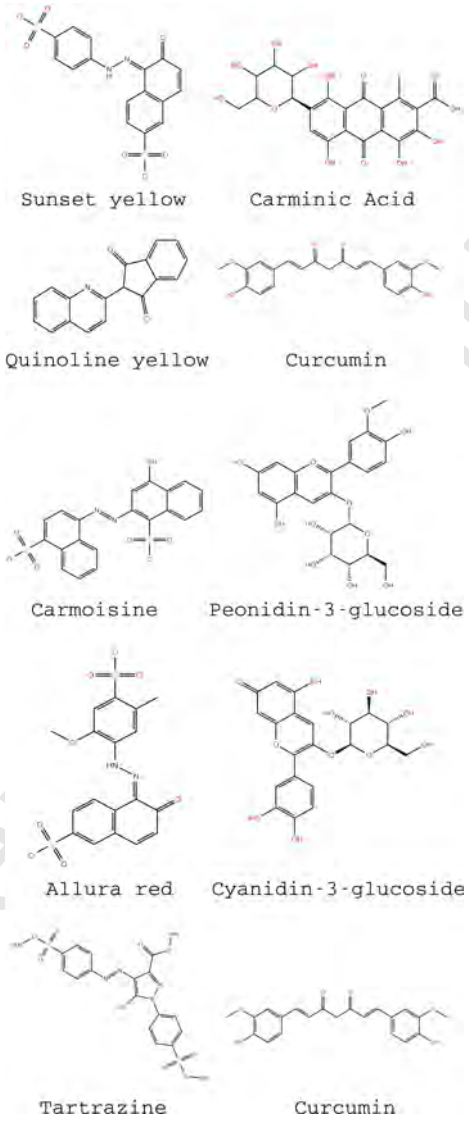


Figure 1: Selected artificial food colorants (left) with their respective natural equivalents (right).

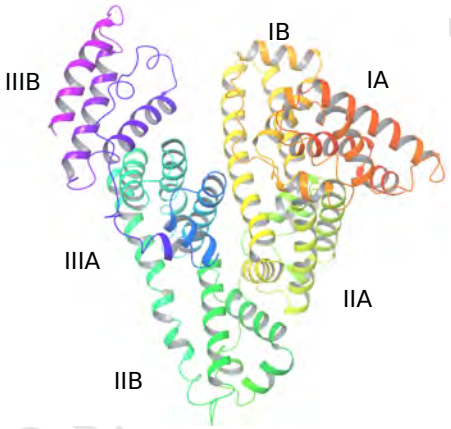


Figure 2: Crystal structure of Human Serum Albumin (HSA) protein. Available in the Protein Data Bank (www.pdb.org) with PDB ID 1AO6. Subdomain classification is both structural and functional having different ligand-binding properties.

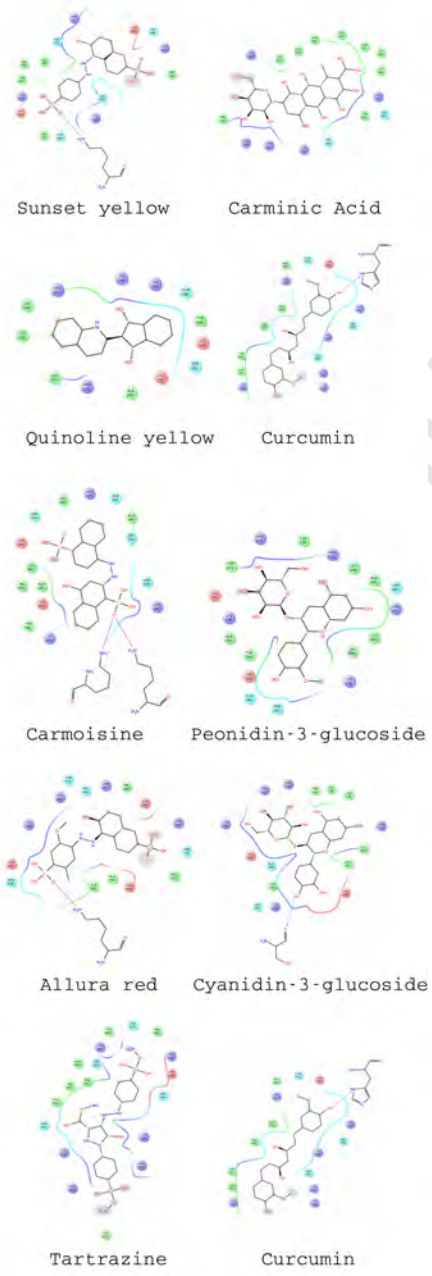


Figure 3: Ligand docking to HSA. Artificial food colorants (left) with their respective natural equivalents (right). HSA's residue names green highlighted depict hydrophobic interactions, blue indicates electrostatic positively charged, red electrostatic negatively charged and cyan polar interactions.

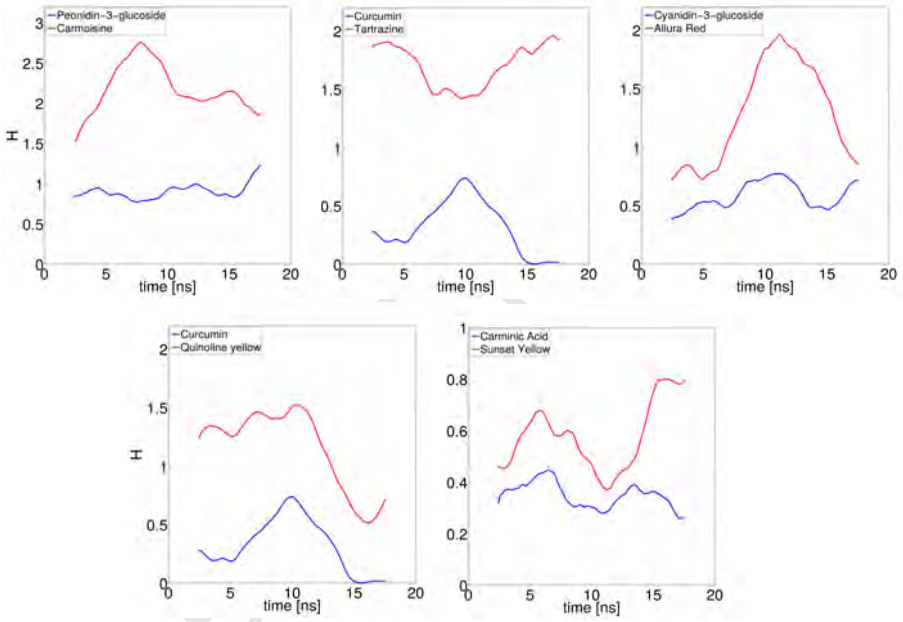


Figure 4: Comparison of H-bond formation during restrained molecular dynamics as measured by collective variable H.