Investigations of *In vitro* Digestibility of Proteins Bound to Food Colors

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Abstract: Colorants either synthetic or natural are commonly added to a variety of food systems to make them attractive and acceptable for the consumers. Our previous publications on Carmoisine, Allura Red, Sunset yellow and the present study showed that food colorants such as Erythrosine, Amaranth, Tartrazine, Quinoline yellow, Brilliant Blue bind with the proteins in variety of the food environments and the protein color complexes are digested by the proteolytic enzyme. The present study elaborates the active sites of the proteins involved in binding with various functional groups of food colors and on these sites modifies the tryptic digestibility of the different proteins. Colors show strong binding capacity with proteins which may block the active site for hydrolysis by the enzyme. The effect of the tryptic digestibility on color complexes of the protein such as the BSA a high molecular weight and nisin the low moleculer weight proteins are explored. The result shows the different color binding with protein have not similar effect on digestibility but in all cases digestibility decreases significantly as compared with blank.

Keywords: Food dyes, food proteins, protein digestibility

INTRODUCTION

Colors play a very prominent role in modulating the appearance and overall quality perception of the processed foods for selection of the product by the consumers. Colorants are also used to attract the attention of a particular age group of the consumers, especially in case of the children for indicating their choice of foods [1]. Food colorants have a long history of their use all over the world in raw and processed foods, although their use in food is still controversial in view of their adverse effects on health [2]. The European Union has allowed certain synthetic food dyes to be used in food processing [3] such as the Tartrazine (E102); Quinoline Yellow (E104); Sunset Yellow (E110); Carmoisine (E122); Amaranth (E123); Ponceau 4R (E124); Erythrosine (E127); Allura Red (E129); Patent Blue (E131); Indigo Carmine (E132); Brilliant Blue FCF (E133); Brown HT (E155) which have allowed by the Europe [4].

Synthetic food dyes are classified into the azo, triarylmethane, chinophthalon (quinoline yellow E 104), xanthene (erythrosine E 127), and indigo (E 132) [5]. Azo compounds are the most widely used as the synthetic food colorants [6]. Azo colors are characterized by at least one group N=N in the molecule with two or three aromatic rings.

Albumin is distributed in the plasma and in the extracellular fluids of skin, muscles and various other tissues in the body. According to Kragh Hansan BSA is composed of three homologous domains, these multi domain protein folded into three domains each of which is built of three loops on the basis of probe displacement method [7], there lie at least three relatively high specific binding sites in BSA molecules. Albumin is also responsible for the maintenance of blood pH [8]. The most extensively studied among the proteins is the serum albumin since past 50 years and about thirty five years ago its tertiary structure was determined by X-ray crystallography [9]. Its secondary structure comprises 67% Helix (six turns) consisting 17 disulfide bridges [10]. Several study had been done on the binding of drugs to plasma and tissue protein is an important factor affecting their distribution and rate of metabolism. Pharmacological effect is closely related to the free concentration of drug at its site of action. Acidic drugs commonly bind to plasma albumin [11]. These studies showed that the protein binds ligands selectively and covalently: few ligands and molecules bind, with a high heat of reaction. In fact, the binding of any substance is likely to affect the activity of the protein, either enhancing it [12] with potential medical inhibiting or significance inhibiting it [13]. Azo pigments are a structurally diverse and widely distributed group of synthetic compounds that include environmental pollutants. Baurm discovered poncean S (PS) and its homologs are used as food additives [14] as cosmetic pigments and biological stains, usually very effectively. Poncean S can permeate into the blood via skin absorption or enter in to the gastrointestinal tract via food intake. Azo reductases reduced azo compound, to

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produce aromatic amines, some of which are known carcinogens [15].

Color-protein interaction plays a very important role in food chain system, particularly in food processing. Natural colors are sensitive to heat, pH and pressure that's why synthetic dyes used to compensate the color loss [16]. In commercial food production, proteins are widely used as emulsifier due to presence of polar and nonpolar amino acids along their polypeptide backbone which provides simultaneous affininty to aquous (polar) and oil (nonpolar) phases. Protein molecules also provide a combination of electrostatic and stereo chemical interaction [17]. Colors are generally anionic in nature, that's why protein interacts with color strongly.

Previous studies have shown that synthetic food colors form stable complexes with edible proteins, (also inedible .unpublished work) for uniform color distribution in all common food system [18-20].

MATERIALS AND METHODS

Materials

The bovine serum albumin (BSA) was purchased from Merck (E. Merck, 64271 Darmstadt, Germany) while the Nisaplin (nisin) was procured from Suzhou Hengliang Imp, Exp, (China). The source of trypsin was pancreas and that of protease was fungal type XIII (*Aspergillus saitoi*). Erythrosine, Ponceau 4R, Amaranth, Tartrazine, Quinoline yellow, Brilliant Blue FCF were supplied from National Foods (Pvt) Ltd. as a gift. The other chemicals used were of analytical grade. All the solutions were prepared in doubled distilled deionized (DDD) water.

Method

Standard solutions of BSA and nisin proteins were prepared using the amount of 10 mg/ml of 0.1M phosphate buffer, pH 7. Stock solution of food dyes such as erythrosine, amaranth, tartrazine, quinoline yellow and brilliant blue FCF were prepared as 0.1 mg/ml seprately. Equal volumes of each protein and color solution were mixed in 7 test tubes separetely and incubated at 37°C for two hours. The incubated mixtures of color bound proteins were digested separately by trypsin (at the enzyme concentration of 1 mg/50 mg of substrate) for various periods of time [21]. After completion the different time periods, the reactions were terminated by adding 1 ml of 10% TCA the respective enzyme and to precipitate the undigested proteins were precipitated as described above. The extent of proteolytic activity of the

Table 1: *In vitro* Digetibility of BSA Protein(Control) and BSA-Dye Complexes

Time (min.)	Controlled	Erytrocine	Amaranth	Tartrazine	Quinoline yellow	Brilliant Blue FCF
10	1.11±0.07	0.58±0.07	0.61±0.03	1.02±0.08	0.58±0.05	1.51±0.21
20	1.24±0.04	0.8±0.04	0.61±0.08	1.14±0.05	0.64±0.02	1.51±0.19
30	1.56±0.05	0.98±0.05	0.96±0.08	1.21±0.02	0.72±0.08	1.51±0.16
40	2.20±0.23	1.06±0.02	1.22±0.05	1.27±0.01	0.81±0.13	1.58±0.15
50	2.24±0.18	1.25±0.01	1.37±0.05	1.32±0.08	0.86±0.21	1.58±0.13
60	2.46±0.12	1.39±0.01	1.47±0.13	1.39±0.05	1.07±0.08	1.65±0.08

± standard deviation (n=5); In the column P<0.01

Table 2: *In vitro* Digetibility of Nisin Protein (Control) and Nisin-Dye Complexes

Time (min.)	Controlled	Erythrocine	Amaranth	Tartrazine	Quinoline yelow	Brilliant Blue FCF
10	1.01±0.08	0.54±0.13	0.89±0.05	1.72±0.13	1.28±0.21	1.56±0.176
20	1.24±0.05	0.68±0.13	0.98±0.02	1.74±0.21	1.39±0.08	1.63±0.11
30	1.34±0.01	0.73±0.21	1.53±0.15	1.75±0.07	1.40±0.01	1.84±0.21
40	1.56±0.13	0.98±0.15	1.57±0.21	1.83±0.04	1.42±0.08	1.92±0.09
50	1.65±0.13	1.18±0.21	1.57±0.16	1.83±0.05	1.48±0.05	2.22±0.16
60	2.01±0.21	1.37±0.16	1.57±0.15	1.85±0.02	1.68±0.13	2.84±0.14

± standard deviation (n=5); In the column P<0.01

supernatant was measured spectrophotometrically at 280 nm. The results are shown in Tables **1** and **2**.

Statistical analysis

The statistical analysis was carried out using software Minitab version 13.1. The statistical mean of five replicates for each analysis was calculated and is shown in Figures **2** to **7**. The linear regression analysis was carried out for the digestibility of the proteins and the color bound proteins. The regression analysis showed that the digestibility was linearly related to the time interval of the exposition to the enzyme since the R^2 values calculated were in the range of 0.90–0.99 in most of the cases. The statistical significance of the difference between analytical samples was also estimated (Significance level was set at 95%) in Table **3**.

RESULTS

The results of the present study show the protein binding with food colors and enzyme activity on its digestibility. In modern life style the consumption of safe food colors and their binding with body proteins is highly impacted, particularly for the control of adverse effect of the synthetic colors .In the case of BSA the digestibility pattern increase with all colors sample at different time of interval. The tryptic digestibility of erytrocine bind BSA and nisin complexes compared to unbound protein (Figure **3**) clearly demonstrate that digestibility decrease significantly. Similarly in the case of Amaranth link BSA digestibility decrease but in case of nisin bound Amararnth show approximately equal digestibility as control (Figure **4**). Tartrazine bind BSA digestibility found same on 20 min. interval but after that color bind BSA digestibility decrease significantly but in case of nisin there is no change of digestibility as compare to control (Figure **5**). BSA binds with Quinone yellow as shown in Figure **6** decrease the digestion because it acts as a partial agonist, as far as the nisin slightly increase the digestibility as compared with control. Quinone yellow in the case of BSA restrict the proteolysis may be the active sites for trypsin action blocked. Brilliant blue bind BSA found decrease in digestibility as compare to control but nisin color conjugates digestibility decrease by the increase of

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10          20          30          40          50          60
MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA FSQYLQQCPF

70          80          90          100         110         120
DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK VASLRETYGD MADCCEKQEP

130         140         150         160         170         180
ERNECFLSHK DDSPDLPKLK PDPNTLCDEF KADEKKFWGK YLYEIARRHP YFYAPELLYY

190         200         210         220         230         240
ANKYNGVFQE CCQAEDKGAC LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA

250         260         270         280         290         300
RLSQKFPKAE FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE

310         320         330         340         350         360
CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL GSFLYEYSRR

370         380         390         400         410         420
HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL KHLVDEPQNL IKQNCDQFEK

430         440         450         460         470         480
LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS RSLGKVGTRC CTKPESERMP CTEDYLSLIL

490         500         510         520         530         540
NRLCVLHEKT PVSEKVTKCC TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP

550         560         570         580         590         600
DTEKQIKKQT ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV

STQTALA
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Figure 1: The amino acid sequence of BSA is obtained from Swiss-Prot with accession number P02769. The two amino acids around the cleavage sites found for trypsin digestion are shown in underline and possible dye binding amino acids shown in bold.

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10          20          30          40          50
MSTKDFNLDL VSVSKKDSGA SPRITSISLC TPGCKTGALM GCNMKTATCH CSIHVSK
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Figure 2: The amino acid sequence of nisin-A is obtained from Swiss-Prot with accession number P13068. The two amino acids around the cleavage sites found for trypsin digestion are shown in underline possible dye binding amino acids shown in bold.

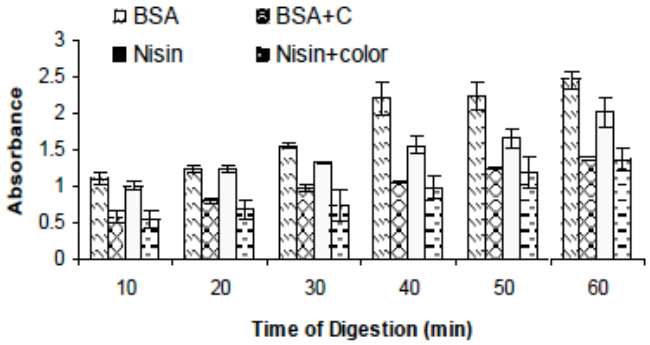


Figure 3: Tryptic digestibility of BSA and erythrocine-bound BSA (BSA-C), nisin and erythrocine bound nisin (NISIN-C).

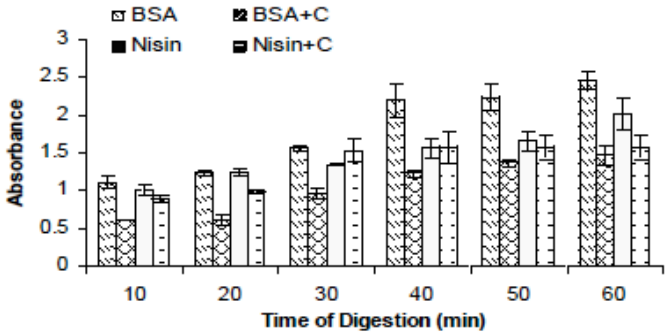


Figure 4: Tryptic digestibility of BSA and Amaranth-bound BSA (BSA-C), nisin and Amaranth bound nisin (NISIN-C).

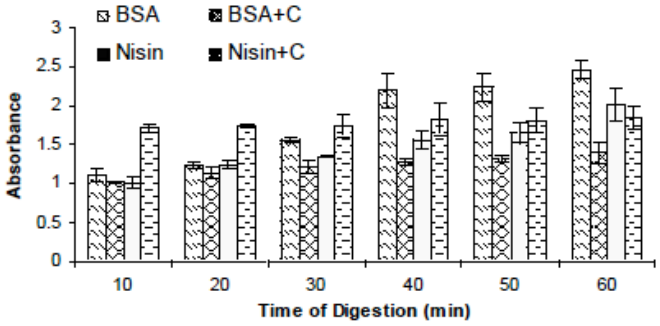


Figure 5: Tryptic digestibility of BSA and Tartrazine-bound BSA (BSA-C), nisin and Tartrazine bound nisin (NISIN-C).

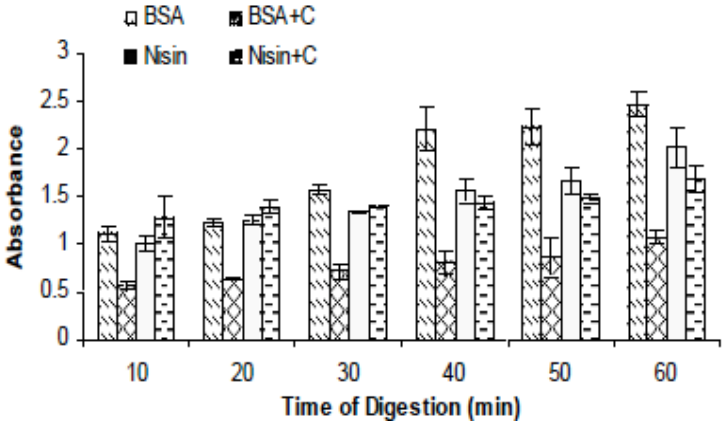


Figure 6: Tryptic digestibility of BSA and Quinoline yellow-bound BSA (BSA-C), nisin and Quinoline yellow bound nisin (NISIN-C).

time interval (Figure **7**). Linear regression analysis of the protein digestibility is strongly correlate with time and most of the cases color protein conjugates digestion decreases in comparison with blank as mentioned in the Table **3**.

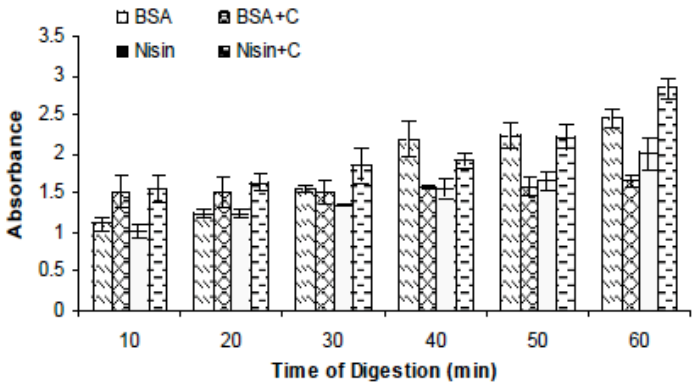


Figure 7: Tryptic digestibility of BSA and Brilliant Blue-bound BSA (BSA-C), nisin and Brilliant blue bound nisin (NISIN-C).

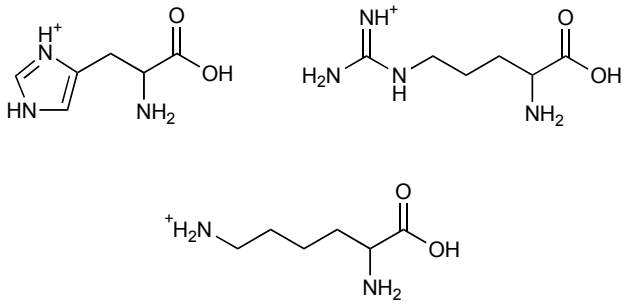
Table 3: Regression Analysis of the Digestibility of Protein with Respect to Time

Smaples	BSA Protein				Nisin Protein		
	Regression equation	R^2	SSE		Regression equation	R^2	SSE
Control	A=0.76+0.296T	0.942	0.0943		A=0.82+0.018T	0.969	0.0192
Erythrocine	A=0.46+0.016T	0.987	0.005		A=0.32+0.016T	0.972	0.014
Amaranth	A=0.36+0.019T	0.951	0.034		A=0.83+0.014T	0.737	0.138
Tartrazine	A=0.98+0.007T	0.977	0.002		A=1.68+0.002T	0.907	0.0014
Quinine Yellow	A=0.46+0.091T	0.946	0.008		A=1.21+0.006T	0.83	0.014
Brilliant Blue	A=1.46+0.003T	0.84	0.003		A=1.17+0.0235T	0.84	0.144

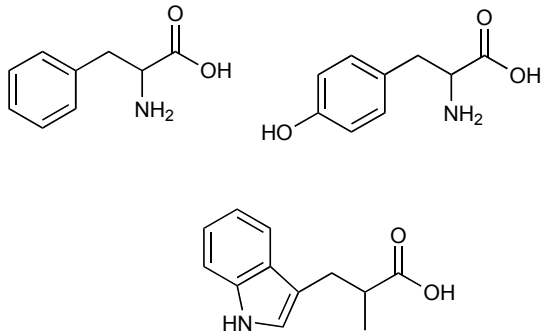
SSE= sum of square of error; Significance level was set at 95%.

DISCUSSION

Bovine serum albumin (BSA) is macromolecular protein. The molecular mass of BSA is 68 KD and its consisting 607 amino acid residues. It has a number of binding sites for protein staining ligand dye. All dyes use in this study has different color. It is expected that the dye molecule interacts strongly with positively charged lysine, histidine and arginine residues of BSA structure.

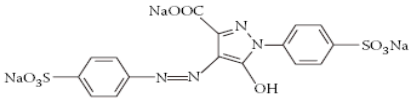
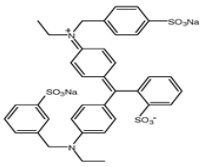
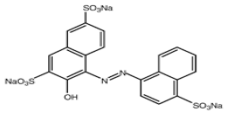
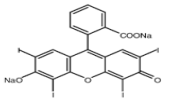
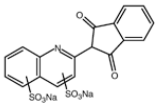


Another, weaker interaction between the dye and BSA molecule is thought to occur that involves the aromatic amino acids phenylalanine, tyrosine and tryptophan.



According to Sohl and Plittergerber who suggested that a spatial juxtaposition of a basic amino acid side chain and the side chain of an aromatic amino acid may be required for initial dye binding [22]. Dye molecule may also bond to dye molecule already bound to protein, creating a “Stacking” interaction between the phenyl rings of adjacent dye molecule. According to this interpretation of the binding events, the larger intrinsic binding constants also present ionic interaction between the positively charged basic amino acid side chains and negatively charged sulfonate groups on the dye molecule. The smaller intrinsic binding constants may represent a hydrophobic or Vander Waals interaction between phenyl rings of

Physicochemical Properties of Food Colors and Structures Used in Current Study

Name of dye	Structure of Dye /Chemical name	Formula / Molecular Weight	Lamda max	Color	ADI (mg/Kg, bw)	solubility	Refrence
Tartrazine (E102, FD&C Yellow No. 5, CI Food Yellow 4)	 trisodium 5-hydroxy-1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-H-pyrazole-3-carboxylate	$C_{18}H_9NNa_2O_8S_2$ (principal component) / 477.38 mw	426 nm	light orange	0 to 7.5	water / sparingly soluble in ethanol	a & c
Brilliant Blue FCF (E133, FD&C Blue No. 1, CI Food Blue 2)	 disodium 3-[N-ethyl-N-[4-[[4-[N-ethyl-N-(3-sulfonatobenzyl)-amino]phenyl] (2-sulfonatophenyl)methylene]-2,5-cyclohexadiene-1-lidene]ammoniomethyl]benzenesulfonate	$C_{37}H_{34}N_2Na_2O_9S_3$ 792.84 mw	630 nm	reddish blue	0 to 10	water / slightly soluble in ethanol	a.b.c.d.e.
Amaranth (E123, CI Food Red 9)	 trisodium 3-hydroxy-4(4-sulfonato-1-naphtylazo)-2,7-naphthalenedisulfonate)	$C_{20}H_{11}N_2Na_3O_{10}S_3$ 604.47 mw	520 nm	reddish -brown	0 to 0.5	water / sparingly soluble in ethanol	a.b.c.d.e.
Erythrocine (E127, FD&C Red No. 3, CI Food Red 14)	 2-(2,4,5,7-tetraiodo-3-oxido-6-oxoxanthen-9-yl)benzoate monohydrate	$C_{20}H_6I_4Na_2O_5$ 879.86 mw	526 nm	red	0 to 0.1	water/ ethanol	a,b,c,e
Quinoline Yellow (E104, CI Food Yellow 13)	 disulfonates of 2-(2-quinodyl) indan-1,3-dione	$C_{18}H_9NNa_2O_8S2$ 477.38 mw	411 nm	yellow	0 to 10	water / sparingly soluble in ethanol	a.b.c.d.e.

[a] Commission Directive 95/45/EC; [b] U.S. Food & Drug Administration , 1999; [c] JECFA ; [d] Ref. [27]; [e] Food and Agriculture Organization , 2006

adjacent dye molecule. It may possible by the nonpolar regions of protein and hydrogen bonding interactions between the dye and the protein [23]. Generally serum albumin has a great binding affinity for small negatively charged hydrophobic molecule. BSA contains several hydrophobic sites, which can be expected to be involved in hydrophobic interaction with the aromatic regions of the dyes [24]. BSA has a high degree of helical content as a cylinder with an open channel and small molecules with suitable functional groups can occupy this channel [25]. Trypsin cleaves on the C-terminal side of the basic residues lysine and arginine. Trypsin digest partially both BSA (Figure **1**) and Nisin (Figure **2**) protein.

The tertiary structure of serum albumin consists of three domains. These three domains encompass residues 1-80, 187-372 and 379-570, respectively and include 96% of the 583 amino acid include [26].

The possible total number of amino acids may provide binding on BSA molecule is 156 out of 607 amino acid but in case of nisin 10 amino acids may bind with dye out of 57 amino acid. These amino acids

are not only the possible binding to the dye other bonding found in the protein molecule and tertiary structure is responsible for the dye binding and digestibility mechanism.

In conclusion, the present study demonstrates that synthetic dyes bind to both low and high molecular weight proteins. The tryptic digestibility of the colour-bound proteins has shown that not the drastic change in digestibility. In general digestibility decreased or in some cases slightly increases in comparison with control.

SUMMARY

Color is very important parameter for any food product to determine of its acceptability. In the food industry, food dyes dyes are used in colorless food products or in natural color containing foods to compensate for color lost. These colors not only use in confectionary and snacks but also used in baby foods and in medicine. These color required more studies regarding its safety and use. ADI values is set for all food dyes but it is required more studies related to toxicity and digestibility. Current studies gives an idea that *in vitro* digestibility of protein decreases by color interaction but not ceased.

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