Responses to the EDITOR and reviewer

We appreciate the comments and advices from the editor and reviewer and would like to thank all of them for taking the time to study our paper. In the following, we will reply to their comments point-by-point.

**Reviewer E:**

ADDITIONAL COMMENTS and REPORT

This manuscript investigates physicochemical characteristics of beta-casein/resveratrol complex by employing UV and fluorescence spectroscopy. Furthermore, isomerization of resveratrol as well as its radical scavenging activity were measured and discussed. Therefore, the present manuscript can provide valuable information in creation of functional food. Applied methodology is suitable. Manuscript is well written but some parts need clarification.

The following specific comments were added to improve the manuscript:

*1. In Fig.4 radical scavenging activity of both protein and resveratrol are presented and discussed at p11-12. The authors stated that “scavenging capacity of b-casein increased in sigmoidal fashion”, but without any particular literature citation or further experiments to support this statement. However, by simple introspection of Fig.4 it is more likely that scavenging is rather parabolic (as often the case in high concentrations of antioxidants: e.g. Food Science and Human Wellness, Volume 2, Issue 2, June2013, Pages 68–74) than sigmoidal process. The authors should provide suitable literature support or further experiments if they want to prove that scavenging is sigmoidal process.*

In the original manuscript, sigmoidal fashion was used because a sigmoidal fit was used to fit the data. Thank the reviewer for his/her suggestion! As suggested, the word “sigmoidal fashion” has been changed into “parabolic fashion” and the reference in *Food Science and Human Wellness* has been cited on line 211 p12 in the revised manuscript.

*2. Minor comments:*

*(1). Please reformulate: p3 lines 38-40*

The sentence “Entrapped bioactive nutrients generally interact with the carrier protein, except that they may remain dissolved in a liquid phase entrapped within an emulsified carrier.” has been changed into “Entrapped bioactive nutrients generally interact with carrier proteins, except that they may remain dissolved in the inner liquid phase of protein-stabilized emulsions.” on lines 38-40 p3 in the revised manuscript.

*(2). p5 line 85: measured at absorbance…*

As requested, the text “measured as absorbance” has been changed into “measured at absorbance” on line 87 p5 in the revised manuscript.

*(3). p6 line 110: Blended = mixed*

As suggested, the word “blended” has been changed into “mixed” on line 113 p6 in the revised manuscript.

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**Reviewer F:**

*1. A native english speaker should edit the manuscript for some minor corrections (articles etc)*

As requested, English grammar has been checked carefully throughout the manuscript under the help of a native English speaker.

*2. page 3, line 54 - instead of binding number it is more appropriate to use mol per mol (mol/mol)*

As suggested, the text “binding numbers from 1.16 to 2.05” has been changed into “with 1.16 ~ 2.05 moles of vitamin D3 bound per mole of *β*-casein” on line 54 p3 in the revised manuscript.

*3. page 5, line 90- instead of as required give the concentration ratios*

Different concentrations of resveratrol and *β*-casein were used for different measurements (*e*.*g*., intrinsic fluorescence of 10 μM β-casein measured in the absence and presence of 2, 5, 10 and 20 μM resveratrol, fluorescence emission spectra of 20 μM resveratrol recorded in the absence and presence of 5, 10, 20 and 50 μM *β*-casein), so the authors prefer to keep the word “as required” in the manuscript. The concentrations of each material have been given in the part of different measurements.

*4. Why did the authors use 275 nm as the excitation wavelength for protein Trp emission instead of 295nm?*

*β*-casein contains one tryptophan residue and four tyrosine residues. When exciting at 295 nm only Trp residues are excited, while at 275 nm both Trp and Tyr residues are excited. Absorption spectrum of resveratrol has a broad peak between 300 and 320 nm as shown in Fig. 1 in the manuscript, 275 nm was used as the excitation wavelength to minimize the inner-filter effect that refers to the absorption of light at the excitation or emission wavelength by components present in solution. The text “The excitation wavelength at 275 nm was used to minimize the inner-filter effect.” has been added on lines 162-163 p9 in the revised manuscript.

*5. What is the concentration of beta caseine and folic acid (page 6, lines 103and 104)?*

The concentrations of *β*-casein and folic acid were 1μM and 10μM, respectively. The text “Fluorescence intensities of folic acid at the emission maximum (λmax≈ 455 nm) were recorded in the absence and presence of 1 μM resveratrol and/or *β*-casein using an excitation wavelength of 348 nm.” has been change into “Fluorescence intensities of 10 μM folic acid at the emission maximum (λmax ≈ 455 nm) were recorded in the absence and presence of 1 μM resveratrol and/or 1 μM *β*-casein using an excitation wavelength of 348 nm.” on lines 104-107 p6 in the revised manuscript.

*6. Does beta-caseine form complex with resveratrol? The existance of complex should be detected with technique other than by recording fluorescence spectra. Ultrafiltration of beta-caseine/resveratrol complex upon mixing by using a membrane with cut off of 10000 kDa followed by determination ofresveratrol concentration in filtrate could be useful*

Fluorescence spectroscopy has been widely used to study the interaction of *β*-casein with bioactive molecules (J. Phys. Chem. B, 2013, 117, 1287-1295; Journal of Food Engineering 2015, 167, 217-225). In this manuscript, intrinsic fluorescence of *β*-casein and fluorescence of resveratrol were used to analyze the formation of *β*-casein and resveratrol. Addition of resveratrol caused a decrease in the intensity and a red shift of λmax of intrinsic fluorescence of *β*-casein. *β*-Casein transferred resveratrol from the aqueous solution to a more hydrophobic environment. These results indicate that *β*-casein did form complexes with resveratrol.

As for ultrafiltration, we did perform the experiment with an Ultra-4mL device (10 kDa MWCO, Millipore, Bedford, MA) at 7,500 g for 30 min. Fig. 1 shows absorption spectra of resveratrol before and after ultrafiltration. Only 11% and 23% of resveratrol solutions passed through the membrane respectively at 10 and 20 μM, indicating that a large amount of resveratrol absorbed to the membrane, possibility due to its amphiphilic nature. Therefore, the complexation between *β*-casein and resveratrol was not analyzed using ultrafiltration method.



Fig. 1 Absorption spectra of 10 (black), 20 (blue) μM resveratrol before (solid) and after (dash) ultrafiltration.

*7. If the complex is formed, what is its stability during 24 hours?*

In solution, equilibrium exists between *β*-casein and *β*-casein-resveratrol complexes and between resveratrol and *β*-casein-resveratrol complexes, since the driving force between *β*-casein and resveratrol are non-covalent interactions (J. Phys. Chem. B, 2013, 117, 1287-1295). Resveratrol is basically stable within 24 hours (Biomacromolecules, 2008, 9, 50-56). The scavenging capacity of *β*-casein/resveratrol complexes remained stable after storage for 200 hours (Fig. 4 in the revised manuscript). Accordingly, it can also speculate that *β*-casein/resveratrol complexes are stable. As shown in Fig. 2, resveratrol-induced change in the fluorescence of *β*-casein and *β*-casein-caused change in the fluorescence of resveratrol were stable after storage 24 hours, indicating that *β*-casein/resveratrol complexes were stable during 24 hours. The text “At 20 μM resveratrol, the λmax around 338 nm and its intensity was about 88% that of pure protein.” has been changed into “At 20 μM resveratrol, the λmax around 338 nm and its intensity was about 88% that of pure protein, which kept invariable after 24 hours (data not shown).” on lines 171-172 p9 in the revised manuscript. The text “At 10 μM, the *β*-casein-caused change in the fluorescence of resveratrol were stable after 24 hours (data not shown).” has also been added on lines 189-190 p10 in the revised manuscript



Fig. 2 (A) Fluorescence emission spectra of 10 μM *β*-casein in the absence and presence of 20 μM resveratrol after storage of 0 and 24 hours, (B) fluorescence emission spectra of 20 μM resveratrol in the absence and presence of 10 μM *β*-casein after storage of 0 and 24 hours.

*8. Does the percentage of ethanol (75%) in which the resveratrol was dissolved influence the native conformation of beta-casein, especially when protein concentrations were kept low (page 6, line101)?*

In the revised manuscript, the text “Stock solution of resveratrol (also 200 μM) was prepared freshly by dissolving in 75% ethanol and then diluting in phosphate buffer.” has been changed into “Stock solution of resveratrol was prepared freshly by dissolving in 75% ethanol at 2 mM and then diluting to 200 μM in phosphate buffer.” on lines 90-92 p5. When resveratrol was used at the highest concentration (20 μM), the final content of ethanol was 0.75% in the samples. In our previous study, it was found the ethanol content less than 7% had on influence on native conformation of *β*-lactoglobulin, a globular protein in milk whey (Biomacromolecules, 2008, 9, 50-56). Accordingly, 0.75% ethanol should have no impact on the structure of *β*-casein. Since the large amount of proline residues disrupt the formation of *α*-helical and *β*-sheet (Songklanakarin J. Sci. Technol, 2005, 27, 201-212), *β*-casein is classifiedas intrinsically unstructured protein (J. Phys. Chem. B, 2013, 117, 1287-1295). Native conformation of *β*-casein was thus not study in the presence of tiny ethanol in the study.

*9. Could one expect these kinds of interactions between resveratrol and beta-caseine isolated from milk (native protein)?*

Beta-casein used in the manuscript is obtained from bovine milk. Far-UV CD spectrum of *β*-casein has a negative peak around 195 nm (Fig. 3), indicating a random coil structure. It belongs to the family of unstructured protein due to unique unfolded structure under native conditions (J. Phys. Chem. B, 2013, 117, 1287-1295). Therefore, it can be speculated that these kinds of interactions could occur between resveratrol and *β*-casein in native state and isolated from milk.



Fig. 3 Far-UV CD spectrum of *β*-casein at 10 μM and 10mM phosphate buffer at pH 7.4.

*10. REPORT: Paper contains original and interesting information considering interactions between molecules that could prove to be useful in making functional food. The authors need to confirm the actual formation of complex and determine its stability.*

The formation of *β*-casein-resveratrol complexes was proved using both intrinsic fluorescence of *β*-casein and fluorescence of resveratrol, please see response to question 6. The stability of *β*-casein-resveratrol complexes has been checked after storage of 24 hours, please see response to question 7.

**EDITORIAL REQUEST:**

*1. Y-AXIS IN FIGURE 1 SHOULD NOT BE ITALIC (DELTA); REFORM*

As requested, Y-axis in Fig. 1 in the revised manuscript has been reformed according to the requirement of JSCS.

*2. EQUATIONS IN REQUIRED FORMAT.*

As requested, equations have been reformed with Arabic numbers consecutively in parenthesis at the end of the line.