Dear Editor,

Thank You very much on reviewers responses for our manuscript entitled “Pro-oxidant Activity of β-Carotene and Lutein inside Multilamellar Liposomes Estimated by TBA-MDA Test“.

With thanks to reviewers for the given effort, please find below the responses to the reviewers:

**Reviewer G:**

1. Why UV-C irradiation was applied as it is well known that this part of UV spectra should be generally absorbed by stratosphere. On the other side, in the view of potential use for cosmetic sunscreens, it is of interest to use UV-A irradiation for elaboration of antioxidant/pro-oxidant activity of investigated Crts.

**Answer**: We have used UV-C and UV-B irradiation in order to get answer on UV-irradiation on some shorter time scales. We have noticed that the answer on UV-A irradiation is similar but after much longer irradiation time. Having in mind thermostability of carotenoids, we’ve wanted to avoid such long irradiations because of possibility of samples overheating.

1. Also, it is really well known that ß-carotene(s) are possible pro-oxidants and for that reason they are not welcomed in the cosmetic products in last few years. If this is a case, they are generally used encapsulated in microspheres, nanocapsules/nanoparticles, more than in a form of liposomes. It is clear that liposomes here served as a model of biomembranes but some outcomes were expected, although it is stressed that mechanism of interaction was investigated. Please, explain the rationale of the approach.

**Answer**: Yes, the liposomes are used as a very good biomembranes model which is convenient for investigations of mutual interactions of incorporated carotenoids as well as their interactions with phospholipid molecules, in order to discover interactions inside photosynthetic apparatus which is also embedded inside phospholipid bilayer of biomembranes. But, in our opinion there are still very much cosmetic formulations on the market based on β-carotene incorporated inside liposomes, so we wanted to give a small contribution in draw attention to this serious problem, especially when it comes to sunscreen products for children. In the same time, we tried to find potential differences in answer to UV-irradiation between carotenes (β-carotene) and xanthophylls (lutein), and potential use of less harmful xanthophylls in this kind of formulations.

1. If the main contribution of the study is estimation of usefulness of adjusted TBA-MDA test, please point at this issue, which may be suffer from small number of evaluated samples.

**Answer**: we’ve used TBA-MDA test in this study on two carotenoids in five concentrations. In our previous investigations we’ve used four carotenoids (β-carotene, lycopene, lutein and neoxanthin - Cvetković and Marković, Radiation Physics and Chemistry 77 (2008) 34–41), as well as two selected flavonoids, quercetin and rutin (Cvetković *et al*. J. Serb. Chem. Soc. 76(7) 973–985 (2011)). One step forward in this work is made because the liposomes have been used as the models, so the effect of molecular organization is present. The main contribution in our TBA-MDA test applications is in the initiation step. Namely, the initiation of lipid peroxidation in our work is performed by UV-irradiation, so initiators are avoided which cause significantly less complex reaction mixture since potential interactions of initiators and antioxidants are not possible (lines 302-306, Discussion part).

1. Also, there is an impression that number of cited references is overshoot.

**Answer**: Four older references are excluded, but in the same time two more recent references are added on Rev. I request.

**Reviewer I:**

INTRODUCTION

* Cited references are not up-to-date!!! The newest one is from 2011. Please add few of recent studies on the topic.

**Answer**:

Two more references are added (Ref.11&12). There are several references from 2012 and 2013, and one from 2016, now. In the same time four older references are excluded.

* Page 1, lines 31-32: For sentence “Beside their antioxidant activity, there are growing evidences for pro-oxidant activity of Crts in recent years” adequate references should be given.

**Answer**: we’ve decided to delete sentence “Beside their antioxidant activity, there are growing evidences for pro-oxidant activity of Crts in recent years”, and start the next one with „However, the exact mechanism of Crts anti- or pro-oxidant activity is still not fully understood although a lot of authors …” because all references in this paragraph are related to anti- or pro-oxidant activity of Crts.

* Page 2 Line 42-43 without adequate reference the sentence “The most commonly used liposomes are multilamellar vesicle (MLV), small unilamellar vesicle (SUV) and large unilamellar vesicle (LUV)" has no sense. Please edit it like: Depending on the number of bilayers, liposomes could be classified as uni- and multi-lamellar…Unilamellar ones are further classified by size as SUV, LUV, and GUV… Add adequate reference(s).

**Answer**: the sentence “The most commonly used liposomes are multilamellar vesicle (MLV), small unilamellar vesicle (SUV) and large unilamellar vesicle (LUV)" are edited in: „Depending on the number of bilayers liposomes could be classified as unilamellar, multilamellar and multivesicular. Unilamellar ones are further classified by size as small unilamellar vesicles (SUV), large unilamellar vesicle (LUV) and giant unilamellar vesicles (GUV)“. Also, a reference is added after this sentence (Ref. 24).

MATERIALS AND METHODS

* Page 3 Line 67-85. Given procedure for liposome preparation is very complicated and confusing. Please explain it. Is it some modified thin film hydration method already described by other authors? Examples from literature describe modification of thin film method by addition of sonication step or vortexing step or freeze-thawing step, but you have applied all of the mentioned? It is hard to believe that after described procedure you still have MLV???

**Answer**: Yes, this procedure is modified thin film hydration method. Using this phospholipids mixture we had to additionally modify the usual method. We have used the freeze-thaw primarily because of Crts incorporation improving. Comparing to liposomes prepared from pure phospholipids, we’ve concluded (according to fractionate centrifugation method and SEM micrographs) that we need much more energy (vortexing and sonication in the bath) to reduce the size of liposomes obtained from PL90 mixture of phospholipids. After longer use of this phospholipids mixture (more than 10 years) we firmly believe that we can obtain unilamellar liposomes from PL90 mixture only after extrusion in an extruder (we usually use AVESTIN, 100 nm pore diameter filter).

* No scientific research without statistical analysis, please add statistical analysis section at the end of materials and methods, directly before the results section, indicated to the program used in data analysis, data expression, limit of significance.

**Answer**: The statistical analysis section is added at the end of Experimental section.

RESULTS

* Page 5, lines 135-136. Are SEM micrographs of empty liposomes provided only? Left and right micrograph just differ in magnification, aren’t they? Please provide the SEM micrograph of Crts encapsulated liposomes. From given SEM micrographs obtained liposome formulation seems very polydisperse, please report the average size of formed liposomes as well?

**Answer**: Yes, SEM micrographs of empty liposomes have been only provided. The very similar SEM micrographs of β-carotene and lutein encapsulated liposomes are now provided. The estimated average size of formed liposomes is reported at the end of Fig. 2 capture.

* Page 5. Line 139-142. Move these lines from Fig. 2  into M&M section

**Answer**: These lines are moved into M&M section, on the end of “*SEM microscopy*“ part.

* Results of encapsulation efficiency for any of used Crts have not been reported. Please provide these data.

**Answer**: It is assumed that the encapsulation efficiency of used Crts in the reported concentration is 100% (please see the last Answer).

* Fig. 3 and 4. Only FTIR spectra of empty and liposomes with lutein and β-caroten in 0.007% conc are presented? Explain why? Was the resolution of 2 cm-1 enough to use applied FTIR analysis as quantitative method?  Or you have used it only as qualitative method  to explain the interaction of lutein with lipid bilayer?

**Answer**: The samples are chosen as an illustration of this approach in indirect proving of Crts incorporation. Crts incorporation inside liposomes is very difficult for demonstration. Unfortunately, it is always limited on indirect methods. We believe that this Crts concentration is illustrative for interpretation of possible interactions inside lipid bilayer of formed liposomes and proving of their incorporation and orientation. In this context, we have used FTIR as qualitative method trying to explain interactions of Crts with phospholipid molecules in liposomes bilayer, but, also to emphasize the differences between β-carotene and lutein in this regard.

* Page 7. Line 160-161. The sentence “Carotenoids are incorporated in concentrations of 0.005, 160 0.0075, 0.02, 0.07 and 0.5 mol% relative to lipids” has to be removed since it is already given in M&M section.

**Answer**: The sentence “Carotenoids are incorporated in concentrations of 0.005, 160 0.0075, 0.02, 0.07 and 0.5 mol% relative to lipids” is removed from this part.

DISCUSSION

* Interactions of Crts with phospholipids by FTIR spectroscopy is good explained, including the presumed model of interaction given in Fig 7. However, discussion on TBA-MDA test with Crts encapsulated liposomes is poor. Much more is given report on literature. Also, my main concern is related to lack of data regarding Crts encapsulation efficiency (EE). Namely, authors did not specify if they performed separation of un-encapsulated Crts from Crts encapsulated liposomes? Only in the case the EE was 100%, authors can speculate on real behavior of Crts encapsulated in liposomes after irradiation. In all other cases of EE (much more probable than 100%), the tested samples were mixture of free, un-encapsulated, unprotected Crts (more prone to oxidation) and encapsulated Crts (protected from oxidation). In view of all this, conclusions are maybe overstressed, and the title of the manuscript should be changed to something like:  Investigation into the potential chemical mechanism of Crts pro-oxidant activity inside liposomes under UV- irradiation, for example.

**Answer**:

It is assumed that the encapsulation efficiency of used Crts in the reported concentration is 100% basing on UV-Vis spectra of Crts measured directly inside liposomes using specially constructed optics on Olis Aminco DW2 spectrometer which is specialized for measured of spectra in mitochondria, tissue suspensions and another samples with large scattering (Figure S1). Having in mind that Crts form aggregate in contact with water, which is resulted in significant changes in their absorption spectra (loss of fine structure as well as large red or blue shift depending of formed aggregate type), we’ve concluded that Crts are not in contact with water in our liposomes dispersion, i.e. that they are completely encapsulated inside lipid bilayer of formed liposomes and that their chromophores are surrounded by hydrophobic fatty acids chains.



Figure S1. UV-Vis absorption spectra of used Crts inside PL90 liposomes measured on Olis Aminco DW2 spectrometer.

(These spectra can be provided as Supplementary material).

One sentence is added in the Discussion part before *TBA-MDA test*, colored in yellow.

We are also very grateful for new title preposition, so the title is changed in: “Investigation into the potential chemical mechanism of carotenoids pro-oxidant activity inside liposomes under UV- irradiation“.

* Page 11, lines 300-302 The sentence “The liposomes were obtained by thin film method and Crts are incorporated by mixing method at various concentrations (0.005, 0.0075, 0.02, 0.07 and 0.5 mol%)” is already given in M&M. Please remove it.

**Answer**: The sentence “The liposomes were obtained by thin film method and Crts are incorporated by mixing method at various concentrations (0.005, 0.0075, 0.02, 0.07 and 0.5 mol%)” is removed from this part.