**[JSCS 8197] (Current day date 19/06/2019)**

Dear Editor and Reviewer,

The authors would like to thank you for the time and efforts that improved the manuscript. We have revised our manuscript taking into account all your recommendations. We hope that the manuscript is now improved and acceptable for publication.

Sincerely Yours,

Authors

THE LIST OF SUGGESTED AMENDMENTS

**Referee(s)' Comments to Author:**

In the submitted manuscript, the authors compared "the quality" of spray- and freeze-dried isolated bovine/porcine hemoglobin preparations after two years of storage at room temperature with or without the addition of 30% maltose. Using various physic-chemical methods they convincingly showed the overall stabilizing effects of maltose on hemoglobin native structure and redox state, point to its use in formulation of long-term stable solid-state hemoglobin(s).

The overall impression of this study is very positive. It is very well written, with sufficient details and references to literature. I would like to ask the authors a few questions and suggestions to further improve the text of the paper.

**Reviewer’s comment No. 1:** The final maltose concentration in the hemoglobin preparations was 30% (w/w) (line 107). No information was provided on why the authors chose this particular concentration of sugar. Please very briefly discuss this important point!

**Response to Reviewer’s comment No. 1:** We thank the reviewer for the indicated omission of this important point. Although literature data provide that commonly used concentration of sugar as protein stabilizers is about 10-20% (w/w) (Chung et al. 1994, Labrude et al. 1989, Chang et al. 2005), the recent study shown that even higher investigated concentrations of sugars (up to 43.6% (w/v)) still have measurable effect on structure and stability of the protein, and more importantly shift the mechanism of protein stabilization from preferential exclusion (preferential hydration) to the neutral solvation (partial penetration of sugar into the hydration shell region) (Ajito et al. 2018).

Accordingly, brief discussion on the used maltose concentration in this study has been added at page 8, lines 230-237.

*References cited:*

J. Chung, S. Takeoka, H. Nishide, E. Tsuchida, *Polymers Adv. Technol.* 5 (1994) 385

(https://doi.org/ 10.1002/pat.1994.220050704)

P. Labrude, M. Rasolomanana, C. Vigneron, C. Thirion, B. Chaillot, *J. Pharm. Sci.* 78 (1989) 223 (https://doi.org/10.1002/jps.2600780311)

L. Chang, D. Shepherd, J. Sun, D. Ouellett, K.L. Grant, X.C. Tang, M.J. Pikal, *J. Pharm. Sci.* 94 (2005) 1427 (https://doi.org/10.1002/jps.20363)

S. Ajito, H. Iwase, S.I. Takata, M. Hirai, *J. Phys. Chem. B*. 122 (2018) 8685 (https://pubs.acs.org/doi/10.1021/acs.jpcb.8b06572)

**Reviewer comment No. 2.** It is more correct to write that vacuum aspiration removes the plasma and buffy coat (not only leucocytes). Please replace (line 96)!

**Response to Reviewer’s comment No. 2:** We agree that it is more correct to write that vacuum aspiration removes the plasma and buffy coat (not only leucocytes). This amendment has been done in agreement to the reviewer’s suggestion, and the sentence has been changed (page 3, line 97)

**Reviewer comment No. 3**.The first use of abbreviations pHb and bHb in the text (line 136) was without referring their meaning. Please introduce these symbols in a suitable place in the text.

**Response to Reviewer’s comment No. 3:** We apologize for the omission of abbreviations introduction at the first place of the appearance. According to the reviewer’s suggestion the abbreviations have been introduced at page 2, line 81.

**Reviewer comment No. 4:** The authors gave an explanation for (practically negligible) impurities in hemoglobin samples after isoelectric focusing analysis (band at pI 5.85),

but not after SDS-PAGE (bands of 30 and 70 kDa). Please either analogously supplements the text (ca. line 185) or (as an important point) remove presented assumption.

**Response to Reviewer’s comment No. 4:** At this moment we cannot precisely identify the 70 kDa protein and according to the reviewer’s comment we removed the presented assumption i.e. the sentence “*Noticed protein band at pI 5.85 with apparent molecular weight 30 kDa probably belongs to carbonic anhydrase*” (page 6, line 188-189) from the revised version of the manuscript. The reference No 28 was also removed from the manuscript.

**Reviewer comment No. 5:** If I understood properly, storage conditions were controlled temperature and protection from moisture (desiccators). What about light (Ref. 41; line

340)? I think it should be stated (Experimental section) if hemoglobin preparations were kept on ambient light or in the dark.

**Response to Reviewer’s comment No. 5:** We thank the reviewer for the indicated omission of the storage conditions details. According to the reviewer’s suggestion this is added in Experimental section, page 4, lines 124, 132.

**Reviewer comment No. 6.** What the exact temperature was during two years storage of examined dried forms of hemoglobin? 20 ± 5 °C (lines 22 or 123) or 25 °C (line 420).

Please clarify (I suppose the first one)!

**Response to Reviewer’s comment No. 6:** The exact temperature during two years storage of examined dried forms of hemoglobin was 20 ± 5 °C, as stated at the first appearance in the text (lines 22 or 124 and 132), not 25 °C as stated by mistake in the line 420. This typo was corrected at page 16, line 420.

**Reviewer comment No. 7:** I would suggest α2β2 instead of 2α2β for the labeling of hemoglobin tetramers (lines 194 and 299).

**Response to Reviewer’s comment No. 7:** We agree with the reviewer’s suggestion, and accordingly in revised manuscript we have changed the labelling of hemoglobin tetramers as α2β2 instead of 2α2β (lines 193 and 305).

**Reviewer comment No. 8**: Please add on room temperature at the end of Figure 2. (legend) text (line 243).

**Response to Reviewer’s comment No. 8:** According to the reviewer’s suggestion this is added in the legend of Figure 2, line 249.

**TECHNICAL EDITOR**

**Technical Editor’s comment No. 1:** IMPORTANT NOTE FROM TECHNICAL EDITOR:

SOME RESULTS SHOULD BE GIVEN AS SUPPLEMENTARY MATERIAL!

**Response to Technical Editor’s comment No. 1:** Since it has not been specified which results should be presented as Supplementary Material, we agreed to move Table 1 into Supplementary Material.

**Minor changes added by authors**:

Besides the text added according to the reviewers’ suggestions, RED font colour was also used for the text added with reason of additional polishing, and which it is not substantial to the manuscript.

Throughout the manuscript the typo “*30 % (w/w)*” has been changed to “*30 % (w/v)*”

References No 28 has been removed in the revised manuscript. In accordance with this amendment the list of the references has been modified.

page 1, line 17: The sentence “*In order to obtain stable solid-state formulation of this protein, we have investigated the effect of 30 % (w/w) maltose addition on isolated bovine and porcine hemoglobin during freeze- and spray- drying, and on obtained powders storage (without maltose: Hb; with maltose: HbM) at room temperature.*” has been rewritten to “*In order to obtain stable solid-state formulation of this protein, we have investigated the effect of maltose (30 % (w/v)) addition on isolated bovine and porcine hemoglobin during freeze- and spray- drying, and on obtained powders storage (without maltose: Hb; with maltose: HbM) at room temperature.*”

page 2, line 39: “are” and “huge” have been replaced with “is” and “large”, respectively

page 2, line 40: “but” has been replaced with “and”

page 2, line 48: “it is possible to calculate” has been replaced with “we calculated”

page 2, line 49: parentheses have been moved

page 3, line 88: “obtained” has been added

page 4, line 120: “Europe” has been removed

page 5, line 148: “by diluting the samples with PBS” has been added

page 6, line 181: “two weak protein bands” has been added

page 6, line 185: “The” has been replaced with “A”

page 11, line 306: “of” has been added

page 11, line 308: “the” has been added

page 12, line 318: “(20±5 ºC)” has been added

page 12, line 319: “and light” has been added

page 15, line 373: “especially when high concentration of sugars (>20%) is used.” has been added

page 16, line 389: “being” has been replaced with “having been”

page 16, line 390: “and light” has been added

page 16, line 392: “prevented formation” has been replaced with “demonstrated the absence of”

Page 17: Abstract in Serbian has been extensively modified