Dear Editor,

On behalf of all the authors, I am sending the file containing the list of corrections of the manuscript *“*Application of RP-HPLC method for determination of p*K*a values of synthesized β-hydroxy-β-arylalkanoic acids" by Jelena Savić et al.

We appreciate the careful evaluation made by the reviewers and the suggestions provided to improve our manuscript. Their observations were followed and appropriate corrections were made.

Dear Reviewers,

We have received your report on our manuscript entitled *“Application of RP-HPLC method for determination of pKa values of synthesized β-hydroxy-β-arylalkanoic acids"*. Thank you for your valuable comments.

Below you will find our answers. Changes in the manuscript are highlighted with yellow color. Below each comment, our answer is given together with line numbers of the manuscript where the changes were made.

Reviewer 1

1. Reviewer`s Comment: The introductory part is too long and repetitions should be avoided.

Answer: The introductory part was shorted. Next sentences were deleted:

(*Line 43-45 from the first submission*) „The research field of NSAIDs was ruffled up by the fact that highly selective COX-2 inhibitors such as coxibes produce cardiovascular side effects which can have lethal outcome.”

(*Line 80-84 from the first submission*) „Literature proposes a HPLC method for determination of aqueous dissociation constants of some water insoluble NSAIDs like ibuprofen, flurbiprofen, ketoprofen, diclofenac in series of solvents made from different amounts of isopropyl alcohol and water.23 In this case p*K*a values that are obtained are extrapolated to pure water and that is the usual approach in many cases.”

Also, parts of some sentences were deleted or reformulated in order to avoid repetitions. All changes are highlighted in yellow.

1. Reviewer`s Comment: Information provided in the abstract/introductory part states that the initial parameter used for the calculations is obtained from the plots (i.e.

inflection points) presented in Figure 1. For the reviewer is almost impossible to derive the parameters from Figure 1 (a small size, 4 plots presented in one Figure). My suggestion is to expand the plots to allow transparency in the parameter estimate based on the inflection point.

Answer: Plots were expanded, and one figure is transformed into 4, so the transparency of the parameter determination from the inflection point is better.

1. Reviewer`s Comment: Be more focused on the link between the p*K*a values established using the proposed method and biological/pharmacological activity of the test compounds (according to the authors, Ref 25-27, the activity has been tested).

Answer: Correlation between p*K*a and biological activity is discussed in the following paragraph (Lines 222-230):

„On molecular level, acidic compounds with carboxylic group inhibit both COX-1 and COX-2 by maintaining one of the most important interactions-salt bridges with Arg120. It is desirable for these compounds to have low p*K*a values which will ensure their existence in ionized form at physiological pH (7,4). In inflamed tissue pH level is decreased compared to the physiological, so it can be concluded that it is ensured that examined β-hydroxy-β-arylalkanoic acids will be almost completely ionized at lower pH compared to the ibuprofen, which will promote salt bridges formation. At pH 5 in inflamed tissue 94.8-97.5% of synthetized compounds, while 84.3 % of ibuprofen will be ionized (calculated from equation 4).“

(Lines 248-250): “Lower pH values of synthetized compounds will promote inhibition of COX because of forming salt bridges with Arg120 with ionized form of compound in comparison to ibuprofen.”

Differences between determined p*K*a are very small so, comparison with ED50 values would be, in our opinion, inadequate.

Reviewer 2:

Thank you for pointing out serious remarks in the manuscript. We tried to remove some shortcomings according to your remarks.

1. Reviewer`s Comment: The manuscript entitled "Application of RP-HPLC method for determination of p*K*a values of synthesized β-hydroxy-β-arylalkanoic acids" has a very limited scientific contribution. There is nothing new in Analytical chemistry.

Answer: We agree that the method is already published, but the intention was to show that this method is applicable on our structures. It was estimated that it would be more efficient to choose adequate method from literature, than to seek for a new one.

1. Reviewer`s Comment: Lines 175-176: "The original procedure was modified in the way that four original mobile phases were replaced with three new ones."

Unsufficient scientific contribution!

Answer: The text which justifies the replacement is added (Lines 169-147): “Four replaced mobile phases in original method were consisted of citric acid, potassium dihydrogen citrate, potassium sodium hydrogen citrate and sodium citrate. Instead of them, different amounts of phosphate and acetic buffers are used. The mobile phase preparation is simplified by using fewer substances in buffers while appropriate pH range is achieved (3.126 to 8.679 (Table II))”.

1. Reviewer`s Comment: Standard softwares were used.

Answer: The main motive was exactly to examine applicability of commonly used softwares on synthesised acids having in mind that many laboratories use these softwares. It was emphasised in the manuscript (Line 64, 201).

1. Reviewer`s Comment: Authors reported some basic facts, almost common knowledge, more appropriate for undergraduate students.

Lines 211-215: "Dissociation constants have a great impact on absorption in gastrointestinal tract. Fraction or percentage of the absorbable and nonabsorbable forms of a weak acid can be calculated if the pH condition at the site of administration is known. The relationship between pH and pKa and extent of ionization for weak acids is given by Henderson-Hasselbalch equation (Equation 4)."

Answer: Thank you for noticing this fact. We deleted sentences refering basic knowledge, so paragraph is now changed into (Lines 207-209):

“Using Henderson-Hasselbalch equation (4) it can be calculated that at pH levels that are 2 units above p*K*a values 99% of compound will be ionized. In that case the absorption is expected to be poor”.