Dear Reviewers,

We have received your report on our manuscript entitled “ **Application of RP-HPLC method for determination of p*K*a 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 blue color (since in the first round review were in yellow).

**Reviewer’s comment:** The manuscript should be condensed and published as a short  
communication.

**Answer:** The manuscript was condensed in a short communication form according to the reviewer`s suggestion and guidelines to the authors.

**Reviewer’s comment:** The title of the manuscript/short communication should be  
reflective of the content. For example: A modified HPLC method for...".

**Answer:** It was clarified that the used method was modified, so the title has been changed into: **A modified RP-HPLC method for determination of p*K*a values of synthesized β-hydroxy-β-arylalkanoic acids.”** The same thing was clarified along the text.

**Reviewer’s comment:** The focus is on the  replacement of citrate based buffers with phosphate and acetate buffers. Are these bufferes compendial?

**Answer:** The buffers that have been used to replace four original (citrate) buffers were phosphate and acetic buffers. These buffers were also used in the original method, just in this method modification, their amounts were adjusted to ensure the same pH values as replaced ones had.

**Reviewer’s comment:** Discussion on pKa, gastric/intestinal pH and extent of  
absorption/bioavailability should be omitted, as the information provided by  
the authors is inaccurate (i.e. ibuprofen is equally well absorbed under  
fasted and fed conditions, an as such stomach pH has no impact on ibuprofen  
bioavailability.

**Answer:** The discussion on pKa, gastric/intestinal pH and extent of absorption/bioavailability was omitted.

All major changes considering manuscript condensation are listed below.

1. Abstract was shortened:

* Sentence: „Ibuprofen was used for two reasons: p*K*a value for ibuprofen obtained with this method is already reported (so repeatability of method can be confirmed), and ibuprofen contains the same phenylpropanoic moiety as analyzed acids” was deleted
* Sentence: “swpH values were measured after mixing methanol and appropriate buffer” was deleted.
* Sentence: Difference in the extent of ionization between synthesized compounds and ibuprofen in stomach is insignificant. was deleted according to the suggestion of the Reviewer
* It was stressed out that the used method is modified (Line 10).
* Two sentences: „Predicted pKa values for this type of compounds in MarvinSketch 5.11.5. program showed poor correlation with experimental results. Prediction of pKa values using ACD/I-Labs is imprecise due to the fact that pKa values are given as wide range“

were condensed into one: „Predicted p*K*a values for this type of compounds in MarvinSketch 5.11.5. program were in a poor correlation with experimental results, while in ACD/I-Labs p*K*a values were calculated as a wide range”.

2. Introduction was condensed

* Paragraph: „Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used for decades in the treatment of fever, pain and inflammation. Their mechanism of action is inhibition of enzyme cyclooxygenase (COX) which catalyzes the production of inflammatory prostaglandins. This class of drugs contains many compounds which may be divided into groups based on different aspects. One of the most important divisions of NSAIDs is on non-selective inhibitors of both COX-1 and COX-2 (conventional NSAIDs) and on selective inhibitors of COX-2.1 Historical development of NSAIDs went from non-selective towards selective drugs and was guided by the objective to avoid gastric side effect. This side effect occurs as a consequence of inhibition of COX-1 isoform which is considered to be physiological isoform participating in the production of gastric mucus. Gastric side effects can be mild like gastric irritation, but also very serious like bleeding and ulceration.2-5 Withdrawal of some selective inhibitors-coxibes due to their cardiovascular side effect put the search for new selective NSAIDs into the focus again.“

Was changed into: „Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used for decades to treat fever, pain and inflammation. The search for new NSAIDs is still a challenge because of gastric side effects of non-selective and cardiovascular side effects of selective NSAIDs.”

* The sentence: „The most important information that can be calculated from p*K*a value is the percent of compound that is ionized at specific pH“ was deleted.
* Paragraphs: „The most important information that can be calculated from p*K*a value is the percent of compound that is ionized at specific pH. p*K*a affects compound`s solubility and permeability through biological membranes. Ionized form of a compound is better dissolved in water, but weakly passes through biological membranes, while the reverse applies for the molecular (non-ionized) form. It should be stressed out that the diffusion of drugs through membranes is a very complex process that depends on many factors, and p*K*a is only one of them.

Besides on absorption, p*K*a has a great impact on binding process of a drug to a receptor. There is a difference between interactions that can be achieved throughout nonionized form of drug (hydrophilic, hydrophobic interactions) and throughout ionized form (salt bridges, dipole-dipole interactions).9

Knowledge on p*K*a value is very important for the choice of optimal conditions in drug analysis, especially for optimization of chromatographic separations of ionizable compounds.

Because all mentioned above, it is clear that it is very desirable to determine pKa values at the early stage of screening of any newly synthesized potentially biologically active compound.”

Were condensed into: „Aqueous dissociation constant (*K*a) is a physicochemical parameter with a great impact on biopharmaceutical parameters of each drug candidate. Negative logarithm of aqueous dissociation constant (p*K*a) affects compound`s solubility, permeability through biological membranes, receptor binding process and a choice of optimal conditions in drug analysis.

Determination of p*K*a values at the early stage of screening of any newly synthesized potentially biologically active compound is highly desirable.”

* Sentence: „Two often used programs for estimating pKa values are MarvinSketch 5.11.5.10 and ACD/I-Labs was transformed into: Two frequently used programs for estimating p*K*a values are MarvinSketch 5.11.5. and ACD/I-Labs.
* Sentence: “This makes experimental data valuable noting that experimental procedure must be carefully selected and precisely monitored” was deleted
* Sentence „ Because synthesized compounds are week acids sparingly soluble in water, having the same phenylpropanoic moiety as ibuprofen, the modified chromatographic method reported by Oumada et al. was used.27 was changed into “Because synthesized compounds are week acids sparingly soluble in water, having the same phenylpropanoic moiety as ibuprofen, the modified chromatographic method reported by Oumada et al. was used.27 in order to clarify that the used method is modified.
* According to the text condensation some references were deleted, and three new ones were added.

3. Experimental section was condensed

* Table I was condensed, names and molecular weights of tested compounds were omitted considering that they are not necessary.
* Paragraphs regarding used solvents, solid materials and procedure were technically condensed, so they occupy less space.
* Sentences „ values of the aqueous buffers were measured. After mixing the aqueous buffer with methanol,  values of the prepared mobile phases were also measured” were condensed into one:”  values of the aqueous buffers were measured, as well as  values of the prepared mobile phases.”

4. Conclusion was shortened

* Sentences: „pKa of ibuprofen was also determined in order to confirm repeatability of the used method. Potentiometric system was calibrated with aqueous buffers, but in calculations are used values of mobile phases because it is already shown that this gives more accurate results.“ were deleted.