Measuring pH and buffer capacity in fluids aspirated from the fasted upper gastrointestinal tract of healthy adults

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Abstract

Purpose: The design of biorelevant conditions for *in vitro* evaluation of orally administered drug products is contingent on obtaining accurate values for physiologically relevant parameters such as pH, buffer capacity and bile salt concentrations in upper gastrointestinal fluids.

Methods: The impact of sample handling on the measurement of pH and buffer capacity of aspirates from the upper gastrointestinal tract was evaluated, with a focus on centrifugation and freeze-thaw cycling as factors that can influence results. Since bicarbonate is a key buffer system in the fasted state and is used to represent conditions in the upper intestine *in vitro*, variations on sample handling were also investigated for bicarbonate-based buffers prepared in the laboratory.

Results: Centrifugation and freezing significantly increase pH and decrease buffer capacity in samples obtained by aspiration from the upper gastrointestinal tract in the fasted state and in bicarbonate buffers prepared *in vitro*. Comparison of data suggested that the buffer system in the small intestine does not derive exclusively from bicarbonates.

Conclusions: Measurement of both pH and buffer capacity immediately after aspiration are strongly recommended as "best practice" and should be adopted as the standard procedure for measuring pH and buffer capacity in aspirates from the gastrointestinal tract. Only data obtained in this way provide a valid basis for setting the physiological parameters in physiologically based pharmacokinetic models.

Keywords

Buffer capacity, stomach, small intestine, bicarbonates, pH, human intestinal fluid

1. Introduction

The design of biorelevant conditions for the *in vitro* evaluation of orally administered drug products is contingent on obtaining accurate values for physiologically relevant parameters such as pH, buffer capacity and bile salt concentrations. For this purpose, samples are often aspirated from the upper gastrointestinal (GI) tract. As values that have been reported for these parameters differ substantially among studies reported in the literature, the question arises as to whether the results may be influenced by the methodology used to collect and process the samples. If so, the aspiration study design needs to be harmonized to "best practices" in order to assure that meaningful and comparable results are reported. While Fuchs and Dressman(1) have discussed in general how various aspects of sampling can affect results (e.g. pooling of aspirates, location of aspiration etc.), in this study we focus specifically on the question of how sample handling procedures can influence pH and buffer capacity measurements in aspirates collected in the upper GI tract.

The buffer capacity of GI fluids, i.e. their resistance to change in pH, can be important to the *in vivo* dissolution of ionizable active pharmaceutical ingredients (APIs) and the release of APIs from pharmaceutical products with pH-dependent release mechanisms.(2–4) The buffer capacity of GI fluids is determined by the physiological pH-regulating agents that are present in the region of interest as well as any food and drink that is ingested by the patient. Further, the impact of the transfer of gastric contents to the small intestine and the contribution of various protein-based pancreatic secretions to the buffer capacity of the fluids in the upper intestine should be taken into consideration.

The intragastric pH in fasted, healthy adults is mainly regulated by the concentration of hydrochloric acid. Using perfusion techniques, hydrogen ion concentrations have been measured to range from 56 to 160 mM.(5–8) There is also a potential contribution of pepsin, lipase, or other protein-based components to

the buffer capacity of bulk gastric contents. Under conditions of reduced gastric acid secretion there may also be some contribution from bicarbonate ions. Bicarbonate concentrations in the acid-suppressed stomach using the carbon dioxide partial pressure and pH (pCO_2/pH) method (which is based on the Henderson-Hasselbalch equation and in which the total concentration of bicarbonates is considered to be the sum of carbon dioxide and free bicarbonates) have been reported to range from 1 to 20mM.(9,10)

In the fasted small intestine, on the other hand, the pH is considered to be mainly controlled by bicarbonates, which are secreted by the pancreas and the enterocytes and are also present in the bile.(11–14) Using the pCO_2/pH method, the bicarbonate concentration in the upper intestine (duodenum and jejunum) of fasted adults has been measured to range between 2 and 20 mM, and the influx of hydrochloric acid into the upper small intestine has been shown to result in a significant increase in bicarbonate secretion rates.(6,15–18) Here it should be mentioned that pCO_2/pH measurements have been criticized as sometimes leading to an underestimation of bicarbonate concentration,(19) in which case values at the upper end of the reported ranges for the stomach and upper intestine are likely to be closer to the true intraluminal values.

The buffer capacity of the GI contents can be estimated by aspirating the luminal contents from the region of interest and titrating the sample with a strong acid or base. Handling of aspirates and any storage prior to titration appear to have an impact on the measured value, since both sample handling techniques and reported values for pH and buffer capacity vary widely among studies. It has been reported that the pH of samples aspirated from the upper intestine drift to higher values when the samples remain on the bench at room temperature.(20) The authors attributed the drift to the transformation of bicarbonates to carbon dioxide.(20) Moreover, Litou et al. demonstrated that subjecting the samples to a freeze-thaw cycle significantly reduces the measured buffer capacity values in both the stomach and in the upper intestine.(21)

To resolve the various issues described above and thus assist in achieving a standardized methodology for sample handling of GI aspirates, this study had three specific objectives: First, to investigate the impact of sample handling on values of pH and buffer capacity measured in gastric and intestinal aspirates. In several studies reported in the literature, the pH and buffer capacity values were determined after centrifugation of the aspirated samples and/or after subjecting the aspirates to a freeze-thaw cycle (22–26), while in others these measurements were made immediately after obtaining the sample (20,21). Second, to compare the impact of freeze-thaw cycling and centrifugation on the pH and buffer capacity of bicarbonate solutions prepared in the laboratory with the impact of these sample handling procedures on aspirated samples. Third, to evaluate the impact of drug administration on buffer capacity via locally and/or systemically mediated mechanisms, based on literature data.(21,23)

2. Methods

2.1 Data from published human intubation studies that were considered in the present work

For each clinical study published to date, the protocol as well as the aspirate collection and handling procedures prior to the *ex vivo* measurements are summarized in Table 1. In this work, data from the studies of Litou et al.,(21) Pedersen et al.,(24) Kalantzi et al.(20) and Persson et al.(26) were used to evaluate the impact of a freeze-thaw cycle on the buffer capacity values in gastric aspirates and in aspirates from the upper small intestine. In all these studies, the adult volunteers were healthy, had fasted overnight prior to the study day and had received no treatment prior to the aspirations. In each of these studies the buffer capacity was measured with NaOH in gastric aspirates and with HCl in aspirates from the upper small intestine. In the study by Kalantzi et al.(20) 10 mg/mL PEG 4000 was used as a non-absorbable marker. Data from the studies of Kalantzi et al. and Litou et al. were reported as individual values.(20,21) Data from the study of Pedersen et al.(24) were reported as mean (SD) values, resulting from six measurements in one pooled sample of gastric contents aspirated from three healthy volunteers, whereas data from the study of Persson et al.(26) were reported as a single value corresponding to one pooled sample of intestinal fluids aspirated from six healthy volunteers.

Individual data from the study of Litou et al.(21) were used to evaluate the impact of a freeze-thaw cycle on the estimated buffer capacity values in the stomach after treatment with famotidine to elevate the gastric pH. In that study the adult volunteers were healthy, had fasted overnight prior to the study day, and had received a treatment with famotidine prior to aspiration. In this case, the buffer capacity in the stomach was estimated immediately upon aspiration by titrating with NaOH and additionally after one freeze-thaw cycle by titrating with NaOH and by titrating with HCl (i.e. in both directions).

Data from the study of Hens et al.(23) were used to evaluate the impact of administration of ibuprofen (a weak acid) prior to initiation of aspiration on the pH and buffer capacity in the stomach and upper small intestine. In that study aspirates collected from another study by the same group were used(27). In the Koeningsknecht et al. study(27) the healthy adult volunteers fasted overnight prior to the study day and received 800 mg ibuprofen prior to aspiration. 25 mg of phenol red were used as a non-absorbable marker. Buffer capacity was measured with NaOH in aspirates collected from the stomach and with HCl in aspirates collected from the upper intestine, after centrifuging at 21000 g for 5 min and then freezing the samples at -80 °C. At an undisclosed time-point during the sample handling and the buffer capacity measurement, pure mineral oil was added to the sample. In the Hens et al. study the mean buffer capacity and pH values were reported at each aspiration time. Relevant data from this study were digitalized from the published figures using WebPlotDigitizer (v. 4.0, Texas, USA).

Bergström et al. reported a median value for jejunal buffer capacity, but provided no information about either the protocol of the clinical study or of the sample handling procedures.(28) Perez de la Cruz Moreno et al. did not clarify whether the titrations were performed with NaOH or HCl.(22) Fadda et al. did not clarify which samples were measured immediately upon aspiration and which after freezing and thawing.(25) Therefore, data from those three studies could not be used in the present analysis.

Pairwise statistical comparisons were performed in all cases using parametric or distribution-free tests, depending on the results of the normality and equal variance tests, using SigmaPlot 11.0 (Systat Software Inc. Chicago, IL, USA) and setting the Type I error at 0.05.

2.2 Impact of centrifugation on the pH of aspirates from the fasted upper small intestine

In a further study (29), eight successive aspirates were collected from the upper intestine of a fasted volunteer between 5 and 70 minutes after administration of 30 mg dipyridamole as an aqueous solution. The aspirates were centrifuged at 37 °C for 10 min and 12560 g immediately after aspiration, and the pH after centrifugation was compared with the pH measured immediately upon aspiration. On a separate occasion, eight successive aspirates were collected over 5-70 minutes from the same volunteer after administration of 90 mg dipyridamole as an aqueous solution. These samples were placed in centrifuge vials, which were immediately sealed and then centrifuged at 37 °C for 10 min and 12560 g: here, too, the pH values after centrifugation were compared with the pH measured immediately upon aspiration. The comparative data are presented in this work for the first time. The differences between the pH values before and after centrifugation were evaluated using either the paired t-test or Wilcoxon Signed-Rank test, depending on the results of normality and equal variances testing, with the Type I error set at 0.05. The statistical analysis of the data was performed using the SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA).

2.3 Titration methodologies for determining buffer capacity

In all studies identified in the literature the buffer capacity of samples aspirated from the stomach was determined by titration with NaOH. In the case of samples aspirated from the upper small intestine, most published buffer capacity values were determined by titration with hydrochloric acid. It should be noted that the contents of the upper intestine are more resistant to a decrease in pH value when a strong acid is added than to an increase in pH when an equivalent molar amount of a strong base is added.(21,26)

2.4 In vitro experiments with bicarbonate solutions

Bicarbonate buffers of 10, 20, 30, 50 and 100 mM were prepared using the appropriate amount of sodium hydrogen carbonate (Alfa Aesar, LOT: Z07C065, ThermoFisher GmBH, Kandel, Germany) and adjusting the final pH of the buffer to 6.5 with HCl with the aid of a pH electrode (pHenomenal®, VWR Int. Leuven, Belgium). Buffer capacity measurements were performed by dropwise addition of HCl after various storage conditions and sample handling procedures, as follows:

a) immediately upon buffer preparation,

b) after freezing the sample in a sealed vial (-20 ^{o}C, 10 d),

c) after centrifuging (20 °C, 21000 g, 5 min) and freezing the sample in a sealed vial (-20 °C, 10 d), and

d) after leaving the sample in a sealed vial on the bench for 4 or for 24 h.

Frozen samples were allowed to thaw on the bench at room temperature for about 1 h before measuring the pH and buffer capacity. Experiments were performed at least in triplicate. The statistical evaluation of differences was performed with one-way ANOVA or the Kruskal-Wallis test, depending on the results of normality and equal variance testing, and post hoc comparisons were carried out using the Tukey test (SigmaPlot 11.0, Systat Software Inc., Chicago, IL, USA). The Type I error was set at 0.05 in all cases.

3. Results

3.1 Impact of sample handling on pH and buffer capacity of aspirates from the upper gastrointestinal tract of healthy adult volunteers in the fasted state

3.1.2 Gastric aspirates

For aspirates collected from the fasted healthy adult stomach,(20,21,24) measurements immediately after aspiration, or after one freeze-thaw cycle indicate that the pH is not significantly different (pH 1.73, n=60 (20,21) vs. pH 1.92, n=16 (21,24), Mann-Whitney, p=0.078). There appears to be a relation between the buffer capacity and gastric pH (Figure 1). As can be observed from the insert graphs in Figure 1, there is a linear correlation between the measured buffer capacity value and the hydrogen ion concentration (calculated according to the measured pH value), independent of whether the measurement was performed immediately after aspiration (R^2 = 0.85) or after one freeze-thaw cycle (R^2 = 0.82). The outlying datum for buffer capacity after one freeze-thaw cycle (Figure 1B) from the study of Pedersen et al.(24) could be related to the fact that no water was administered in that study prior to aspiration, unlike in the studies by Kalantzi et al.(20) and Litou et al.,(21) in which 240 mL of water had been administered prior to aspiration (see Table 1).

The median buffer capacity of gastric fluids measured immediately upon aspiration (17.4 mmol/L/ΔpH, n=60 (20,21) was far higher than after one freeze-thaw cycle (6.6 mmol/L/ΔpH, n=16 (21,24); Mann-Whitney, p=0.007).

Data measured in aspirates collected from the stomach after pretreatment with famotidine indicated that one freeze-thaw cycle did not affect the pH significantly (paired t-test, p=0.301). The absence of a clear relationship between pH and buffer capacity in this case can be attributed to the lack of HCl and the presence of other components in the gastric aspirates (Figure 2).(30–32)

The mean buffer capacity of gastric fluids after treatment with famotidine was significantly higher when measured immediately after aspiration (0.62 mmol/L/ΔpH), than after one freeze-thaw cycle (0.21 mmol/L/ΔpH; paired t-test, n=16, p<0.001).

3.1.2 Intestinal aspirates

When vials containing aspirates from the fasted upper small intestine were not sealed prior to centrifugation, the centrifugation procedure (37 °C, 12560 g, 10 min) increased the median (range) pH values significantly from 6.11 (2.67-6.74) to 6.70 (2.67-7.29) (p=0.008).(29) When the vials were sealed prior to centrifugation, the effect was reduced, with the pH rising from 5.84 (4.38-7.03) to 5.89 (4.38-7.48), but still statistically significant (p=0.031).(29)

pH values of aspirates from upper intestine were not significantly affected by one freeze-thaw cycle (6.35, n=47 vs 6.86, n=18, Mann-Whitney, p=0.168)(20,21,26). The median buffer capacity measured immediately upon aspiration (7.0 mmol/L/ΔpH, n=45(20,21)) was significantly higher than that after one freeze-thaw cycle (4.8 mmol/L/ΔpH, n=17(21,26)) (Mann-Whitney, p=0.019) (Figure 3).

3.2 The impact of ibuprofen together with centrifugation/freezing of samples on the pH and buffer capacity in aspirates from the upper gastrointestinal tract of healthy adult volunteers in the fasted state

3.2.1 Gastric aspirates

Administration of 800 mg ibuprofen significantly elevated the gastric pH value; median pH values were 1.73 (n=60)(20,21) measured immediately upon aspiration without prior drug administration compared with pH 2.63 (n=13)(23) measured immediately upon aspiration after administration of ibuprofen (Mann-Whitney, p<0.001). However, the buffer capacity of the gastric contents measured after administration of ibuprofen and after centrifuging/ freezing the samples was not significantly affected. In aspirates that were obtained from volunteers who had not received ibuprofen and which underwent a freeze-thaw cycle prior to measurement the median value was 6.6 mmol/L/ΔpH (n=16) (21,24), whereas in aspirates that were

obtained from another set of volunteers who had received ibuprofen and which had undergone both centrifugation and a freeze-thaw cycle, the median value was 4.7 mmol/L/ΔpH (n=13) (23) (p=0.283, Mann-Whitney) (Figure 4A).

3.2.2 Intestinal aspirates

The pH values measured immediately upon aspiration in samples collected from the upper small intestine after administration of 800 mg ibuprofen (median 5.51, n=26 (23)) were significantly lower than those measured immediately upon aspiration with no prior drug administration (median 6.35, n=47 (20,21)) (p=0.002, Mann-Whitney). The observation is in line with data reported by Hoffman et al. (33) who measured the intestinal pH with a Heidelberg capsule in eight healthy volunteers. In that study, values reported after administration of an ibuprofen suspension at various infusion rates were lower than average population data. However, the exact region of the upper small intestine at which the pH was measured was not confirmed in this study.(33)

Given that the pH values in aspirates collected from the upper small intestine are significantly lowered by prior administration of ibuprofen and/or centrifuging of aspirates, the buffer capacity values are also expected to be affected. Indeed, the buffer capacity measured in aspirates under ibuprofen administration combined with centrifugation/freezing sample handling (median 1.0 mmol/L/ΔpH, n=26 (23)) was significantly lower than in other studies in which no ibuprofen was administered and the samples were frozen without having been centrifuged (median 4.72 mmol/L/ΔpH, n=16(21,26)) ($p<0.001$, Mann-Whitney) (Figure 4B).

3.3 The impact of handling and storage on buffer capacity of bicarbonate solutions prepared in the laboratory

Data with respect to the impact of sample handling procedures and storage conditions on the buffer capacity of solutions of bicarbonate prepared in the laboratory at concentrations of 10 mM to 100 mM are presented in Tables 2 and 3.

At a 10 mM concentration of bicarbonate, subjecting the samples to centrifugation followed by one freeze–thaw cycle increased the pH (Kruskall-Wallis, $p=0.004$) and decreased the buffer capacity (one-way ANOVA, $p=0.021$) significantly. By contrast, subjecting the samples to just the freeze–thaw cycle (without centrifugation) did not affect either the pH or the buffer capacity significantly (Table 2). Keeping the sample on the bench for 4 h or 24 h (Table 3) led to a statistically significant increase in pH with an attendant decrease in buffer capacity (one-way ANOVA, $p<0.001$ for both parameters, all pairwise comparisons were significantly different for both parameters).

At a bicarbonate concentration of 30 mM buffer, centrifuging and/or freezing the sample significantly increased the pH and decreased the buffer capacity (one-way ANOVA, $p<0.001$, for both parameters). Keeping the sample on the bench for 24 h significantly increased the pH (one-way ANOVA, $p<0.001$, all pairwise comparisons) and decreased the buffer capacity (one-way ANOVA, $p=0.012$).

At a very high bicarbonate concentration of 100 mM buffer, centrifuging and/or freezing the sample did not affect the pH or the buffer capacity (one-way ANOVA, $p=0.197$). While keeping the sample on the bench for 24 h significantly increased the pH (6.50 vs. 7.04, (Kruskal – Wallis, $p=0.004$), the buffer capacity was not significantly altered (one-way ANOVA, $p=0.123$).

Overall, it was observed that keeping the sample on the bench for 4 h leads to a significant increase in the pH and to a significant decrease in the buffer capacity at bicarbonate concentrations up to 30mM. Likewise, freezing and/or centrifuging the sample affects the pH and buffer capacity significantly at

concentrations of up to 30 mM (i.e. within the physiological range of bicarbonate concentrations that have been observed). The observed differences are greater when the sample has been both centrifuged and frozen than when it is simply frozen before storage. The results are in general agreement with the study of Leijssen et al., in which the "loss of label" (i.e. decrease in concentration) of bicarbonate solutions was investigated *in vitro*. The authors reported that different stirring rates (when the bicarbonate solution was placed in a beaker) resulted in a loss of label up to 58% in one hour and that that the percentage loss could be reduced by increasing the bicarbonate buffer concentration from 1 to 10 mM.(34)

In summary, at bicarbonate concentrations in the physiological range of values observed in the fasted state in the small intestine, both the pH and buffer capacity become very sensitive to the sample handling procedure, so it is imperative to ensure that the sample handling procedure is closely controlled.

4. Discussion

A general comment on the studies with aspirates from the stomach and upper intestine is that the sample handling, use of marker compounds and pretreatment with drugs all vary from study to study. Although this is to be expected to some extent because of the different aims of the studies, it impedes a straightforward comparison of the results. At least for the purposes of determining inter-subject variability in parameters like pH and buffer capacity (and other relevant upper GI parameters such as bile salt concentrations), it would be extremely helpful to have a harmonized protocol.

4.1 pH in aspirates

It can be concluded that the pH of the samples aspirated from the fasted stomach and upper small intestine is not significantly affected by a single freeze-thaw cycle (section 3.1). By contrast, centrifugation of intestinal aspirates upon collection increases the pH of the sample (section 3.1). It has been reported that the pH of samples aspirated from the upper small intestine drifted to higher values when the samples were kept on the bench at room temperature. The authors attributed the drift to the transformation of bicarbonates to carbon dioxide.(20) Taken together, these observations suggest that different sample handling procedures can have an effect on the measured pH values.

4.2 Buffer capacity in aspirates

The data presented here show that the buffer capacity of samples aspirated from the either the fasted stomach or the fasted small intestine is lowered significantly by subjecting the sample to a freeze-thaw cycle (section 3.1). Further, comparing studies in which ibuprofen was administered and the samples were centrifuged before freezing with studies in which no drug was administered and samples were frozen without having been centrifuged (section 3.2), it appears that centrifugation also leads to a decrease in buffer capacity. Thus, it is evident that the accuracy of the buffer capacity measurements of fluids aspirated from the upper GI tract is compromised when they are not performed immediately upon

aspiration. Since centrifugation or leaving the sample on the laboratory bench for several hours both affect the pH, these sample handling procedures are expected to have a knock-on effect on the accuracy of the measurement of the buffer capacity as well.

Similar concerns with respect to the effects of sample handling on pH and buffer capacity have been made for other body fluids. For example, Gittings et al. performed pH and buffer capacity measurements in human saliva collected from healthy volunteers, immediately upon collection and after storing the samples at -80°C, respectively.(35) The authors recognized that bicarbonate buffer is a dynamic system and opined that in saliva samples carbon dioxide may be lost from the system.

4.3 In vitro testing

Comparison of the *in vivo* and *in vitro* observations provides experimental evidence for non-exclusivity of bicarbonates in the regulation of pH in the fasted upper small intestine as well as in the fasted stomach at elevated pH. The results from the *in vitro* experiments indicated that both the buffer capacity and the pH of bicarbonate solutions up to 30 mM are affected by subjecting the samples to a freeze-thaw cycle. Since subjecting the samples to a freeze-thaw cycle does not significantly affect the pH of aspirates from the upper small intestine or from the stomach when the subjects are pretreated with famotidine (section 3.1), the question of whether bicarbonate is the sole contributor to the buffer system in the upper gastrointestinal tract arises. It appears that in these aspirates, species other than bicarbonates e.g. enzymes and/or mucin glycoproteins, may play an important role in regulating the intraluminal pH. This possibility is also supported by recent data concerning the importance of bicarbonates in biorelevant media simulating the conditions in the stomach under elevated gastric pH conditions and in the upper small intestine in the fasted state.(21,36)

Proteins are present both in gastric and intestinal fluids. Lindahl et al. reported, among others, concentrations of proteins in the fasted gastric fluids of 2.1 ± 1.2 mg/mL.(37) This value is in general agreement with the study of Litou et al., where concentrations of 0.27 ± 0.14, 0.53 ± 0.18 and 0.71 ± 0.35 mg/mL at 10, 20 and 35 min after administration of 240 mL of water, respectively, were reported.(21) With regard to the upper small intestine, Lindahl et al. reported protein concentrations in jejunal fluids of 1.8 ± 0.7 mg/mL.(37) Similar values were reported by Kalantzi et al. for the fasted duodenum (3.1 mg/mL),(20) Persson et al. (1 ± 0.1 mg/mL)(26) and Litou et al. (1.00 ± 0.37, 1.8 ± 1.2, 2.7 ± 1.7 and 3.7 ± 0.11 mg/mL at 5, 10, 30 and 50 min after administration of 240 mL of water).(21) Since the freeze-thawing process can denature or destabilize proteins,(38) it is important to measure their contributions to buffer capacity by titrating immediately after collection of the aspirate. From the observations in this study as well as the literature data on other physiological fluids (39–44), it seems that bicarbonates may not be the only contributors to the buffer system of the luminal fluids in the upper gastrointestinal tract and that proteins likely have an important role.

4.4. Effects of drug administration on pH and buffer capacity

Some authors have administered a drug prior to aspirating samples from the upper GI tract and it is quite clear that the administration of some drugs prior to the initiation of aspirations can have an effect on the measured pH and/or buffer capacity of the luminal aspirates.

A case in point is famotidine, a histamine 2 receptor antagonist, which like proton pump inhibitors is often used to elevate the gastric pH. In the Litou et al. study(21) it was shown that a 40 mg dose of famotidine (20 mg famotidine 14 h and 2 h prior to aspirations) elevates the gastric pH to values of pH 7 or more. Under these conditions the buffer capacity is reduced to a very low value (mean 0.62 mmol/L/ΔpH) due to the suppression of gastric acid secretion combined with the intake of a glass of water prior to aspiration. Interestingly, even at these extremely low buffer capacities, subjecting the sample to a freeze-thaw cycle prior to measurement resulted in a further decrease of the buffer capacity (section 3.1).

Hens et al. reported that the buffer capacity decreased after administration of 800 mg ibuprofen.(23) This can be partly explained by the decrease in pH when ibuprofen dissolves in the intestinal lumen to a value far lower than the pKa of the bicarbonate buffer system, thus weakening the buffer capacity of the bicarbonate. However, the pharmacological effect of ibuprofen should be also taken into consideration when interpreting its effects on pH and buffer capacity in the gastrointestinal tract. It has been suggested that bicarbonate secretion from the duodenal mucosa is regulated through cephalic-vagal stimulation, non-humoral mediators activated by the presence of acid in the stomach, as well as locally produced prostaglandins of the E-type (PGEs), which stimulate the bicarbonate secretion in the proximal and distal duodenum and are released by the presence of acid in the intestinal fluids.(12,13,45–48) The suppression of proximal and distal duodenal bicarbonate secretion after administration of an non-steroidal anti-inflammatory drug (NSAID) has been investigated in healthy subjects (50 mg of indomethacin orally administered 13 h and 1h prior to the study, or 50 mg of indomethacin rectally administered at identical time intervals, n=10).(49) In that study, the authors concluded that administration of NSAIDs could cause duodenal mucosal bicarbonate injury at least partly by decreasing mucosal prostaglandin generation.(49) It seems, therefore, that the decrease in luminal pH and buffer capacity induced by ibuprofen is mediated via both physicochemical interactions in the lumen and systemic pharmacological effects.

5. Conclusions

Data collected from aspiration studies comprise the most valuable source of information with respect to characterizing the gastrointestinal environment and the properties of the gastrointestinal fluids, as well as the inter-subject variability in the associated parameters.

This study showed that sample handling procedures can significantly affect the pH and buffer capacity measurements of samples aspirated from the fasted upper gastrointestinal tract. It is therefore recommended that reporting of the physiological pH and buffer capacity values of fluids in the fasted upper gastrointestinal lumen should rely exclusively on data collected immediately upon aspiration, without prior drug treatment of the volunteers and without any additional sample handling.

There is a clear need for a standardized aspiration study protocol based on best practices to enable accuracy of the measurements and comparability of results across aspiration studies. Only data obtained in this way provide a valid basis for designing biorelevant test conditions and setting the physiological parameters in Physiologically Based Pharmacokinetic (PBPK) models.

Since both pH and buffer capacity of bicarbonate solutions up to 30mM are more sensitive to a freeze-thaw cycle than in aspirates, in addition to hydrochloric acid and bicarbonates, other substances may play a role in regulation of pH in the upper GI tract in the fasted state. In particular, further studies are needed in order to better define the role of proteins, and possibly other components, in the buffer capacity of the luminal fluids.

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561 *Table 1: Published median pH and average buffer capacity values for the gastric contents and the contents of upper intestine of fasted adults, clinical*
562 *study protocols and aspirate storage conditions and handling procedures.*

	H_2O volume (mL)	Drug pretreatment	Sample handling				Time of measurement (min)	Median/Mean pH (range/±SD)*	Average buffer capacity (±SD) (mmol/L/ΔpH)	Titrant	Reference
			Immediate measurement	Pooling	Centrifugation	Freezing					
Stomach	250	800 mg ibuprofen			✓	✓	0-420	2.6 (1.8-3.7)	4.7 (1.3)	NaOH	(23)
	240	-	✓				10	2.7 (1.9-3.9)	4.7 (4.6)	NaOH	(21)
							20	1.7 (1.3-2.0)	21.3 (11.4)		
							35	1.6 (1.1-2.4)	27.6 (15.7)		
	240	40 mg famotidine	✓				10	7.2 (6.9-7.3)	0.5 (0.2)	NaOH	(21)
							20	7.1 (6.0-7.2)	0.7 (0.2)		
							35	7.1 (4.7-7.3)	1.3 (0.7)		
	-	-		✓		✓	N/A	2.5 (1.40)	14.3 (9.5)	NaOH	(24)
	250	-	✓				20	2.4	7.0	NaOH	(20)
							40-60	1.7	18.0		
Duodenum	250	800 mg ibuprofen			✓	✓	0-420	5.1 (4.5-5.8)	1.4 (0.4)	HCl	(23)
	240	-	✓				5	6.8 (6.4-7.2)	8.4 (2.9)	HCl	(21)
							15	6.2 (2.3-7.1)	19.2 (33.7)		
							30	6.3 (3.0-7.0)	9.0 (3.8)		
							50	6.5 (2.7-7.7)	14.2 (10.5)		
	240	40 mg famotidine	✓				5	7.2 (7.1-7.6)	6.1 (0.8)	HCl	(21)
							15	7.2 (7.0-7.7)	9.0 (3.8)		
							30	7.1 (6.6-8.4)	7.7 (2.8)		
							50	7.3 (6.2-8.0)	6.9 (2.7)		
	N/A	-			✓	✓	N/A	N/A	4.0-13.0 (range)	N/A	(22)

	250	-	✓				30	6.2	5.60	HCl	(20)
Jejunum	250	800 mg ibuprofen			✓	✓	0-420	5.6 (4.9-6.1)	0.8 (0.3)	HCl	(23)
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4.0	N/A	(28)
	N/A	-	✓	✓		✓	N/A	7.1 (0.5)	3.2 (1.3)	HCl	(25)
	-	-		✓		✓	0-150	7.5	2.4	HCl	(26)
	-	-		✓		✓	0-150	7.5	2.8	NaOH	(26)

563 *N/A: not available information*

564 **In the Hens et al.,*(23) *Litou et al.,*(21) *Pedersen et al.*(24) *and Kalantzi et al.*(20) *studies pH was measured immediately upon aspiration. In the*
565 *studies of Persson et al.*(26) *and Fadda et al.*(25) *the exact timepoint of the measurement of the pH is not specified.*

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567

Table 2: The impact of freezing and of centrifuging and freezing on the pH and buffer capacity of bicarbonate buffer systems as a function of concentration.

Buffer concentration (mM)	pH			Buffer capacity		
	Upon preparation	After freezing	After centrifugation and freezing	Upon preparation	After freezing	After centrifugation and freezing
10	6.50	7.08 p>0.05	7.28 p<0.05	5.83	5.33 p=0.140	4.67 p=0.004
20	6.50	7.22 p<0.001	7.23 p<0.001	11.4	10.93 p>0.05	10.13 p>0.05
30	6.50	7.28 p<0.001	7.32 p<0.001	17.33	15.27 p<0.001	14.67 p<0.001
50	6.50	7.30 p=0.071	7.33 p=0.071	27.2	23.60 p=0.05	24.00 p=0.05
100	6.50	7.30 p=0.05	7.36 p=0.05	51.44	44.30 p>0.05	42.33 p>0.05

571 *Table 3: The impact of keeping the sample on the bench for 4 or 24 h on the pH and buffer capacity of bicarbonate buffer systems as a function of*

572 *concentration*.

Buffer concentration (mM)	pH			Buffer capacity		
	Upon preparation	**After 4 h**	**After 24 h**	**Upon preparation**	**After 4 h**	**After 24 h**
10	6.50	6.78 p<0.001	7.10 p<0.001	5.83	5.57 p<0.001	5.83 vs 4.70 0.005
20	6.50	6.99 p<0.001	7.15 p<0.001	11.4	11.20 p>0.05	9.70 p>0.05
30	6.50	6.89 p<0.001	7.04 p<0.001	17.33	17.27 p > 0.05	15.13 p=0.008
50	6.50	6.87 p<0.001	7.03 p<0.001	27.2	26.80 p>0.05	21.07 p<0.001
100	6.50	6.89 p>0.05	7.04 p=0.004	51.44	50.80 p>0.05	49.67 p>0.05

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Figure Captions

Figure 1: Data on the buffer capacity of gastric contents in fasted healthy adults vs. the corresponding pH values previously published by Litou et al.(21) (●, individual data), by Kalantzi et al.(20) (■, individual data), and by Pedersen et al.(24) (◆, mean ± SD data). (A) Data measured immediately upon aspiration; (B) data measured after one freeze-thaw cycle. The inserts in the Figure represent the linear relationship between the buffer capacity, measured immediately upon aspiration or after one freeze-thaw cycle, with the concentration of hydrogen ions.

Figure 2: Data on the buffer capacity of gastric contents of fasted healthy adults after pretreatment with famotidine vs. the corresponding pH values previously published by Litou et al.(21) (A) data measured immediately upon aspiration; (B) data measured after one freeze-thaw cycle.

Figure 3: Data on the buffer capacity of contents of upper intestine of fasted healthy adults vs. the corresponding pH values published previously by Litou et al.(21) (●), by Kalantzi et al.(20) (■), and by Persson et al.(26) (×). (A) data measured immediately upon aspiration; (B) data measured after one freeze-thaw cycle.

Figure 4: (A) Data on the buffer capacity of fasted adult gastric contents vs. the corresponding pH values collected without prior treatment [Litou et al.(21) (●),and Pedersen et al.(24) (◆)], and after administration of 800 mg ibuprofen just before initiation of aspirations [Hens et al.(23) (▲)].

(B) Data on the buffer capacity of fasted adult contents of upper intestine vs. the corresponding pH values collected without prior treatment of the volunteers [Litou et al.(21) (●),and Persson et al.(26) (×)] and after administration of 800 mg ibuprofen just before initiation of aspirations [Hens et al.(23) (▲)]. All data were collected after one freeze-thaw cycle and/or centrifugation and a freeze-thaw cycle.

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Figure 1

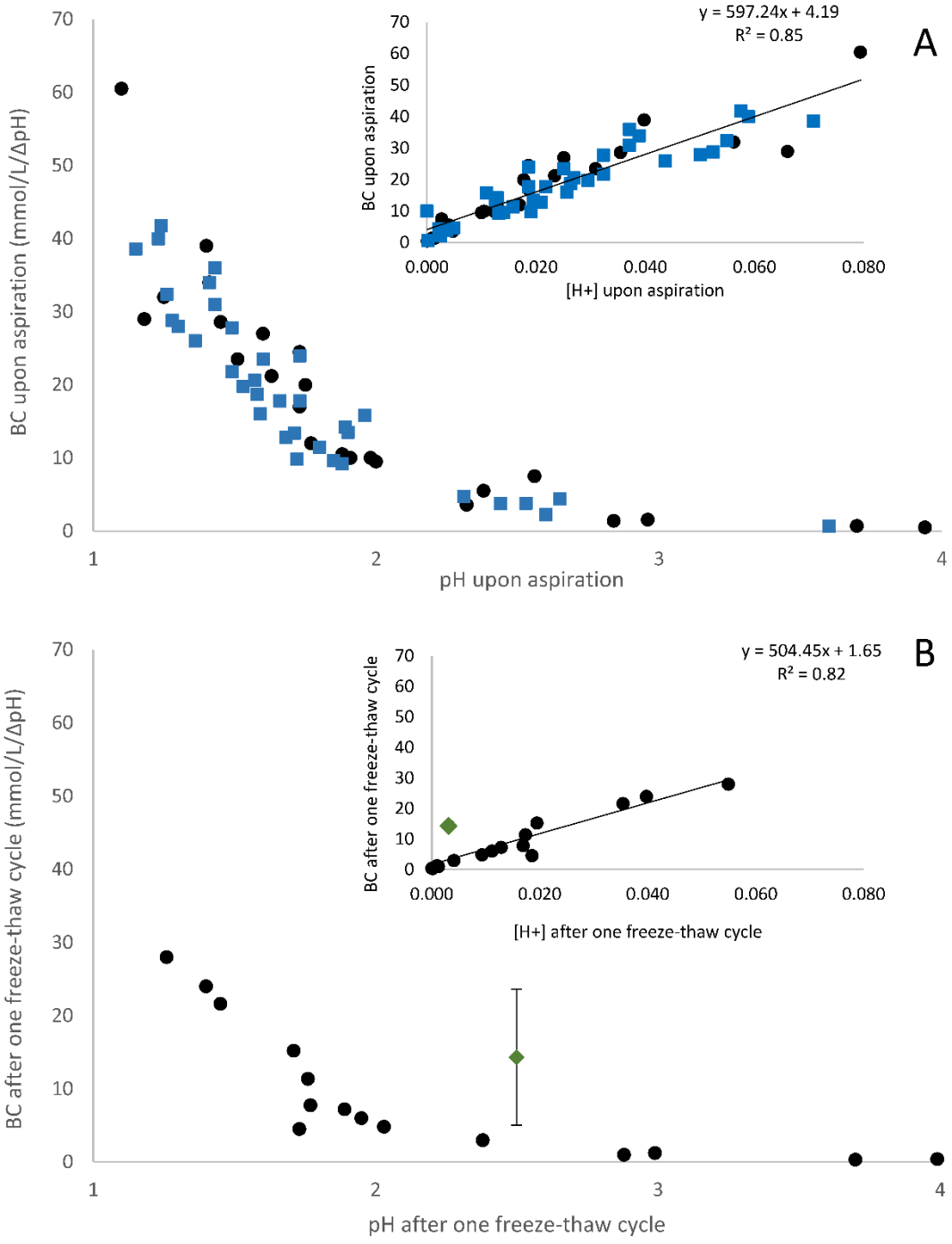

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Figure 2

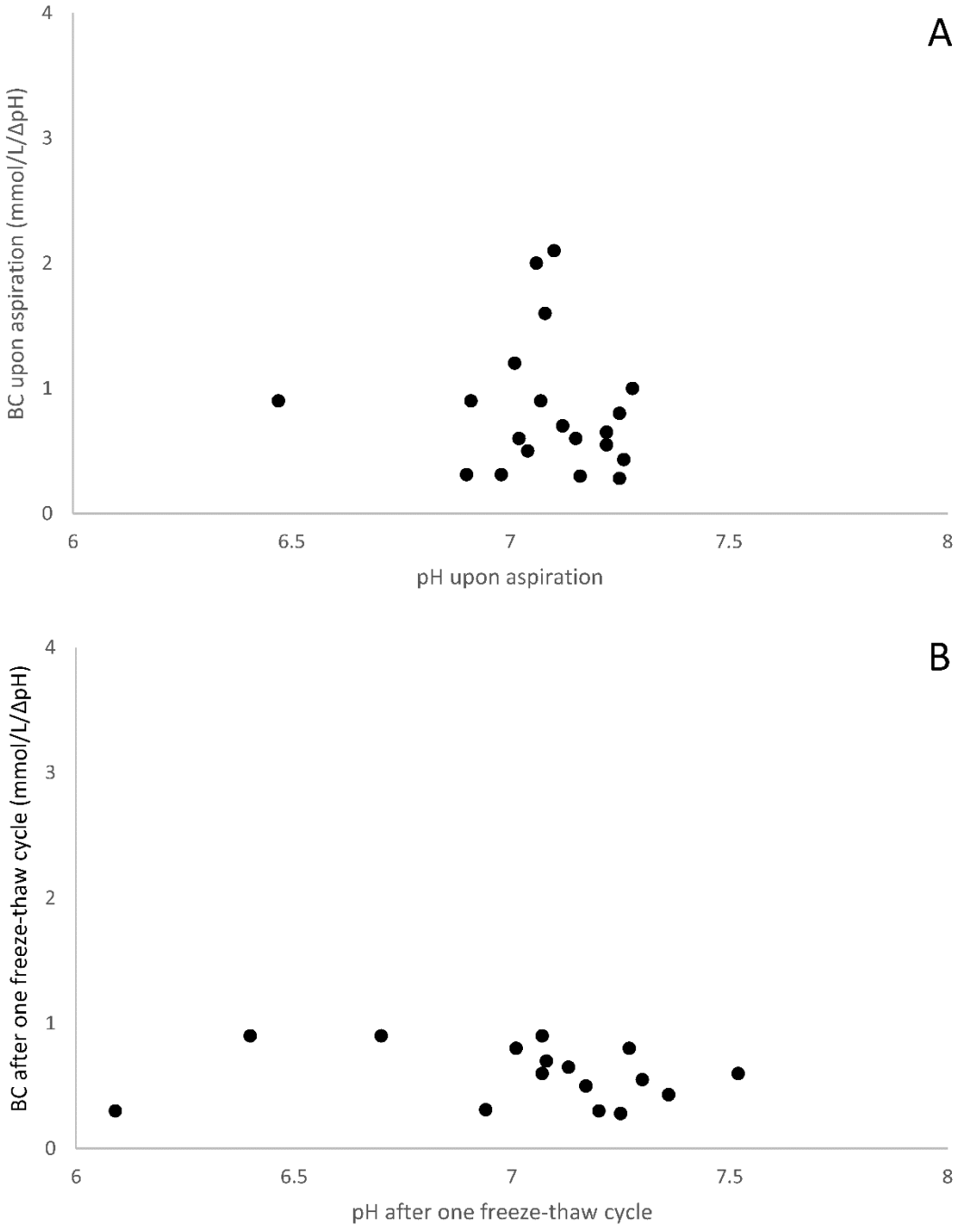

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Figure 3

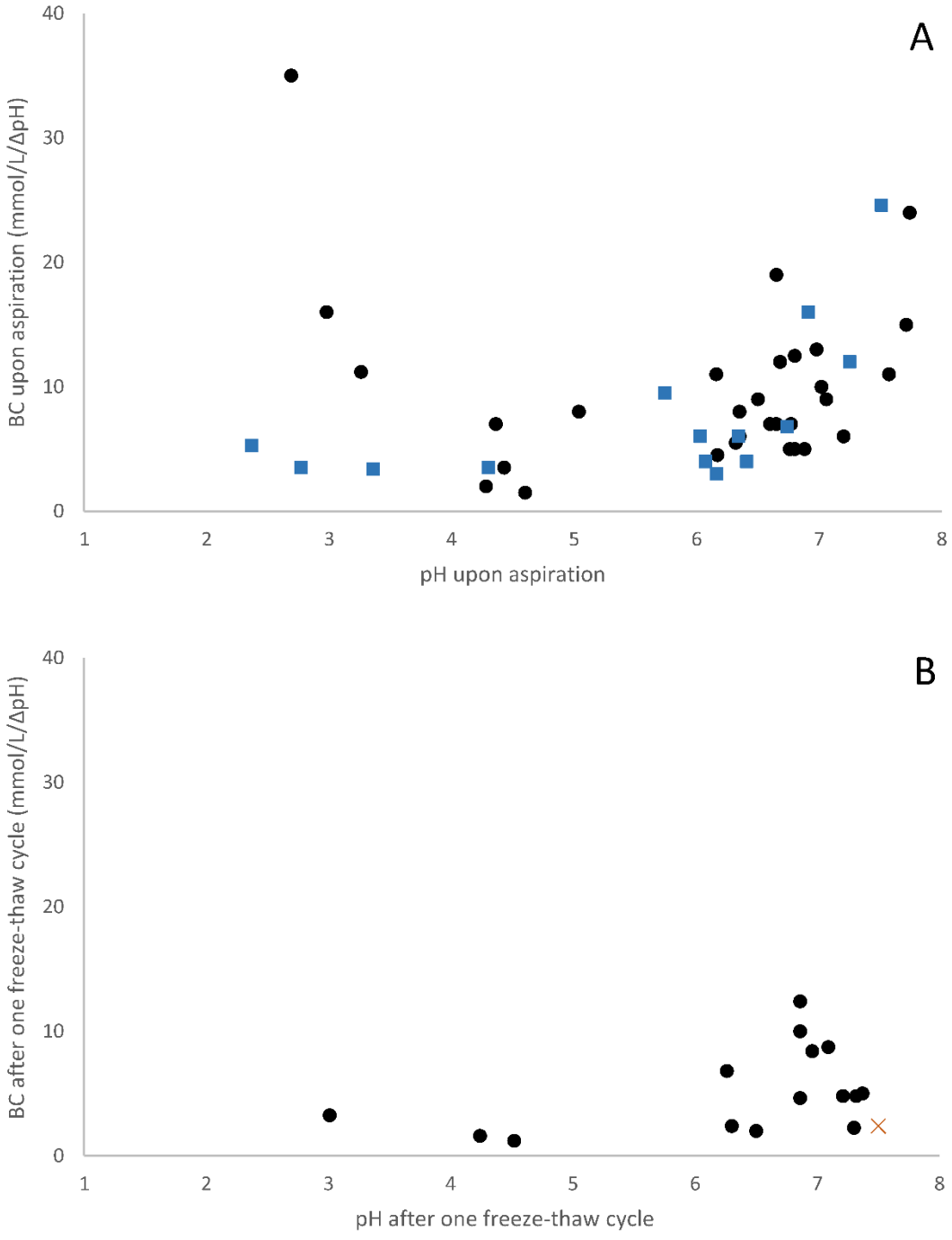

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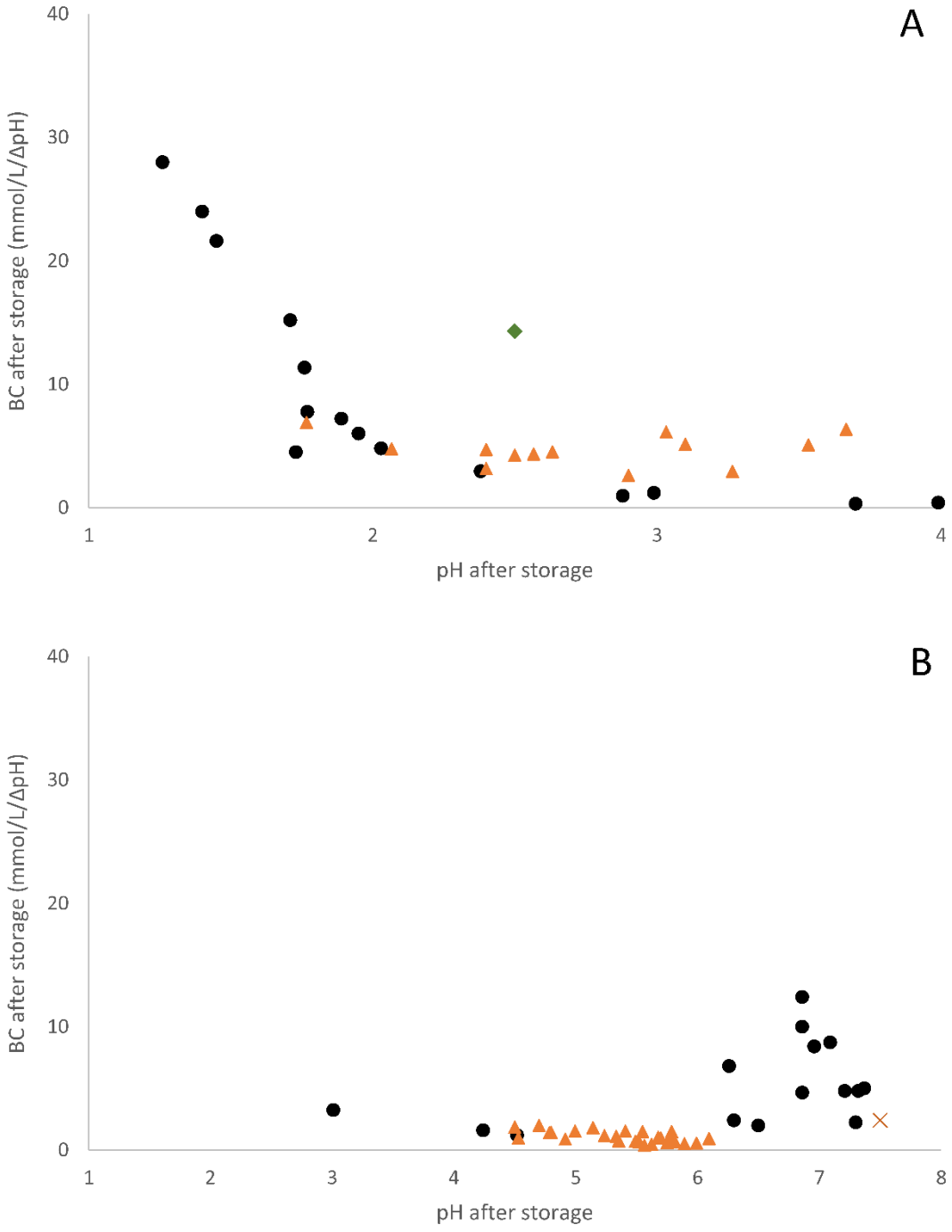
Figure 4



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