Validation of 2 urine pH measuring techniques in a prepartum negative dietary cation-anion difference diet and the relationship with production performance

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Summary
Acidified diets prepartum are utilized in dairy cows to decrease the likelihood of hypocalcemia; however, the effect of the extent of acidification on cow performance is still debated. Our objective was to validate the accuracy of 2 pH strips to measure urine pH (categorized as low, medium, or high) in dairy cows consuming an acidified diet prepartum and the association of urine pH with production performance. We determined that both pH strips are an accurate and affordable method to determine urine pH. Additionally, varying urine pH was not associated with altered dry matter intake when cows consumed an acidified diet; however, milk yield was moderately affected during week 1 postpartum when average urine pH prepartum was <5.67.

Highlights
- Accurate and cheap measurement of urine pH is desirable for the dairy industry.
- The two urine pH strips tested were accurate in measuring urine pH in prepartum cows.
- Varying urine pH was not associated with altered dry matter intake prepartum.
Validation of 2 urine pH measuring techniques in a prepartum negative dietary cation-anion difference diet and the relationship with production performance

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Abstract: Negative dietary cation-anion difference (DCAD) diets have been implemented to combat hypocalcemia, a common peripartal disease in dairy cows; however, the extent of compensatory metabolic acidosis necessary and the subsequent effects on performance are still debated. Additionally, there is a need for an inexpensive, accurate method to measure urine pH on farm during the prepartum period to assess the extent of metabolic acidosis achieved by negative DCAD diets. Therefore, this experiment was conducted to determine the accuracy of Fisher pH sticks (pHF; ThermoFisher Scientific) and pHion balance test strips (pH H; pHion Balance) compared with a portable pH meter (pHP; Accumet AP115, ThermoFisher Scientific) in measuring urine pH (UpH) and the effect of UpH on pre- and postpartum dry matter intake (DMI), milk, and milk composition yields. Cows consumed a total mixed ration with a DCAD of −118 mEq/kg for 4 wk prepartum and 397 mEq/kg for 4 wk postpartum. Prepartum UpH measurements (n = 75) for each cow were averaged and used to classify cows in terms of urine pH as low (UpH ≤5.54; mean ± standard deviation; 5.44 ± 0.07), medium (UpH >5.54 and ≤5.90; 5.67 ± 0.09), or high (UpH >5.90; 6.42 ± 0.36). Cows were milked twice a day, and milk samples were taken on d 7 ± 1.3, 14 ± 1.4, and 28 ± 1.1 relative to calving. Milk yield and DMI were recorded daily and averaged weekly. Bland-Altman plots and Lin’s concordance correlation coefficient (CCC) were used to assess the agreement between pHP and pHF or pH I (n = 375). Receiver operating characteristic curves were used to determine the threshold with pHF and pH I that best discriminated between UpH >5.75 and ≤5.75 compared with pHP, and area under the curve (AUC) was used to assess the accuracy. At the UpH threshold of 5.75 for pHF and pH I, the sensitivity, specificity, and AUC were 89.5 and 87.4, 99.1 and 97.0, and 0.94 and 0.92, respectively. The CCC was 0.93 for pHF and pH I, indicating near-perfect agreement relative to calving. Milk yield and DMI were recorded daily and averaged weekly. Bland-Altman plots and Lin’s concordance correlation coefficient (CCC) were used to assess the agreement between pHP and pHF or pH I (n = 375). Receiver operating characteristic curves were used to determine the threshold with pHF and pH I that best discriminated between UpH >5.75 and ≤5.75 compared with pHP, and area under the curve (AUC) was used to assess the accuracy. At the UpH threshold of 5.75 for pHF and pH I, the sensitivity, specificity, and AUC were 89.5 and 87.4, 99.1 and 97.0, and 0.94 and 0.92, respectively. The CCC was 0.93 for pHF and pH I, indicating near-perfect agreement.
A fully acidified diet allowed for the addition of Ca at 2% of DM to the prepartum diet without compromising metabolic acidosis, resulting in improved health and reproductive success postpartum (Glosson et al., 2020; Ryan et al., 2020). To achieve the target degree of acidification on farm, accurate and inexpensive measurements of UpH are needed. Previously, the gold standard to measure pH was a portable pH meter; however, glass electrode pH meters require frequent calibration and training to ensure accuracy, thereby decreasing the popularity of this method (Constable et al., 2019). Therefore, a cheaper alternative is desired that allows for rapid determination of UpH on farm but maintains the accuracy of the gold standard.

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (#18157) and were a part of a larger study (Fehlberg et al., 2020). A detailed description of this study is provided in Fehlberg et al. (2020). Briefly, the experimental period was from September 2018 to June 2019. A total of 83 multiparous pregnant Holstein cows with BW (mean ± SD) = 791 ± 84 kg were blocked by parity (3.3 ± 1.1), previous 305-d mature-equivalent milk production (11,363 ± 1,860 kg), expected calving date, and BCS during the far-off dry period (3.76 ± 0.84).

Cows were dried off at −57 ± 21 d relative to expected calving and consumed a common straw-based diet until −30 d relative to expected calving. Cows were then moved to an enclosed ventilated barn with access to sand-bedded freestalls at −30 d relative to expected calving, where they were fed once daily at approximately 0530 h using an individual feeding system (American Calan Inc.), beginning the experimental period. Diets (DMT) were formulated using AMTS.Cattle.Pro version 4.7 (2017, AMTS LLC) to meet or exceed recommendations. The dry cow diet was formulated for cows at 694 kg of BW, a predicted DMI of 13 kg/d, and to achieve a DCAD of −118 mEq/kg, where DCAD = ([Na⁺] + [K⁺]) – ([Cl⁻] + [S²⁻]). The mean chemical composition (n = 10) of the prepartum TMR (DM basis) was 14.2 ± 0.68% CP, 28.4 ± 2.80% ADF, 44.8 ± 2.75% NDF, 14.0 ± 1.69% starch, and 1.44 ± 0.03 NE₃ (Mcal/kg of DM). The DCAD was obtained by dietary ingredients and the addition of an amionic mineral supplement (Animate; Phibro Animal Health Corp.) included at 3.85% of DM. After calving, cows were housed in a tiestall barn until 28 d relative to expected calving.

Regression and correlation analyses were carried out to estimate the association between UpH determined with pHF and pH or pH (n = 375). Correlation coefficients measured the strength of the relationship between pHF and pH or pH, not the agreement during mid-stream of urination. The tube was immediately closed to decrease exposure to CO₂ and subsequent increased pH (Bender and Staufenbiel, 2003). Urine pH was measured within 5 min of collection by a single operator using a portable pH meter (pHP; Accumet AP115, ThermoFisher Scientific) with a pH/automatic temperature compensation glass electrode (which was considered the gold standard), Fisherbrand pH sticks (pHf; ThermoFisher Scientific), and pHion balance test strips (pHfi; pHion Balance). For this study, both pHF and pH could detect a minimum pH of 5.0; the pHF scale increased by intervals of 0.5 until 9.0, whereas the pH scale increased by 0.5 until a pH of 5.5 and then by 0.25 to a pH of 9.0. Fisherbrand pH sticks and pHF were selected due to their ability to measure urine pH as low as 5.0 and for both sticks to have consistent intervals of measurement.

Daily DMI was determined for each cow by weighing refusals and total amounts fed and determining the difference on a DM basis. Cows were fed for 10% refusals to allow for ad libitum feed intake. All cows had free access to water. Milking procedures were explained in detail elsewhere (Fehlberg et al., 2020). Briefly, cows were milked 2 × per day and weights were recorded at each milking. Milk samples were collected at both a.m. and p.m. milkings at (mean ± SD) 7d ± 1.3, 14 ± 1.4, and 28 ± 1.1 relative to calving, composited in proportion to milk yield at each milking, and then shipped to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) to be analyzed for contents of fat, true protein, casein, lactose, SCC, total solids, and MUN using mid-infrared procedures (AOAC International, 1995).

Daily DMI was condensed into weekly averages and analyzed independently each week, using the corresponding low, medium, and high classifications for each week. Prepartum UpH measurements for each cow (n = 75) were then averaged and used to classify cows as low (UpH ≤5.54; mean ± SD; 5.36 ± 0.15), medium (UpH >5.54 and ≤5.78; 5.65 ± 0.06), or high (UpH >5.78; 6.51 ± 0.57); for wk −3 as low (UpH ≤5.49; 5.35 ± 0.11), medium (UpH >5.49 and ≤5.77; 5.62 ± 0.09), or high (UpH >5.77; 6.62 ± 0.69); for wk −2 as low (UpH ≤5.37; 5.25 ± 0.10), medium (UpH >5.37 and ≤5.65; 5.50 ± 0.08), or high (UpH >5.65; 6.46 ± 0.68); and for wk −1 as low (UpH ≤5.48; 5.36 ± 0.11), medium (UpH >5.48 and ≤5.80; 5.66 ± 0.09), or high (UpH >5.66; 6.48 ± 0.54). Daily prepartum DMI was condensed into weekly averages and analyzed independently each week, using the corresponding low, medium, and high classifications for each week. Prepartum UpH measurements for each cow (n = 75) were then averaged and used to classify cows as low (UpH ≤5.54; mean ± SD; 5.44 ± 0.07), medium (UpH >5.54 and ≤5.90; 5.67 ± 0.09), or high (UpH >5.90; 6.42 ± 0.36) and used for postpartum DMI, and milk, ECM, 3.5% FCM, and milk composition yields. Postpartum DMI and milk yields were condensed to weekly averages. The model included the fixed effects of UpH, week, and their interaction. Daily DMI and milk production were condensed to weekly averages and analyzed independently each week, using the corresponding low, medium, and high classifications for each week. Prepartum UpH measurements for each cow (n = 75) were then averaged and used to classify cows as low (UpH ≤5.54; mean ± SD; 5.44 ± 0.07), medium (UpH >5.54 and ≤5.90; 5.67 ± 0.09), or high (UpH >5.90; 6.42 ± 0.36) and used for postpartum DMI, and milk, ECM, 3.5% FCM, and milk composition yields. Postpartum DMI and milk yields were condensed to weekly averages. The model included the fixed effects of UpH, week, and their interaction. Cow was the experimental unit and considered a random effect. Week was specified as repeated with cow as subject when analyzing variables measured over time. Denominator degrees of freedom was estimated using the Kenward-Roger method (Littell, 2002). Distribution of the residuals was evaluated to determine normality and homoscedasticity.
among them (Bland and Altman, 1986). Consequently, assessing diagnostic test performance with correlation coefficients only may be inappropriate. Therefore, Bland-Altman plots (Bland and Altman, 1986) and Lin’s concordance correlation coefficient (CCC; Crawford et al., 2007) were used to visualize and quantify, respectively, the agreement between the results from pHp and pHF or pHI. The Durbin-Watson coefficient was used to test for autocorrelation within residuals to determine independence among samples, with a score near 2 indicating zero autocorrelation.

Contingency 2 × 2 tables were created to obtain true-negative, true-positive, false-negative, and false-positive values. These values were used to compute the test characteristics (sensitivity, specificity, and positive and negative predictive values). Sensitivity (Se) was calculated as the proportion of urine samples with pH ≤ 5.75 correctly determined by pHF or pHI. Specificity (Sp) was calculated as the proportion of urine samples with pH > 5.75 correctly determined by pHF or pHI. Positive predictive value was calculated as the proportion of the urine samples with pH ≤ 5.75 that were correctly analyzed. Negative predictive value was calculated as the proportion of the urine samples with pH > 5.75 that were correctly analyzed. Receiver operating characteristic (ROC) curves were constructed to identify the threshold with pHF and pHI that best discriminated between urine samples with pH > 5.75 and those ≤ 5.75 based on the gold standard test. The area under

![Figure 1. Bland-Altman plot of differences between urine pH (n = 375) determined by a portable pH meter (pHp; Accumet AP115, ThermoFisher Scientific) and that determined using (A) Fisher pH sticks (pHF; ThermoFisher Scientific) and (B) pHion balance test strips (pHI; pHion Balance) plotted against their mean concentrations. The solid line in the middle represents the mean (bias), the upper and lower dashed lines represent the limit of the agreement (bias ± 1.96 SD), and the dotted line indicates bias = 0. Relationship between urine pH determined by pHp and (C) pHF or (D) pHI. (C) Adjusted R² = 0.96, r = 0.98, P < 0.0001, y = −0.54619 + 1.03552x; (D) adjusted R² = 0.96, r = 0.98, P < 0.0001, y = −0.45910 + 1.01927x. For both models, y = predicted pHF or pHI urine pH and x = pH by pHp, respectively.](image-url)
the ROC curve (AUC) was used to assess the accuracy of the pHF and pHI thresholds. Statistical significance for all analyses was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

There was excellent correlation between pHF and pHI and the gold standard pHP ($r = 0.98$; $P < 0.0001$; Figure 1). To determine optimal thresholds and Se and Sp values at varying thresholds, ROC curves were used. When the threshold was set at 5.75, Se was 89.5% (95% CI: 88.5–90.5) and Sp was 99.1% (95% CI: 98.9–100) for pHF and pHI, respectively. These values coincided with the greatest AUC used to determine the accuracy of the thresholds. When the AUC is 0.5, discrimination does not exist, whereas when AUC is 1.0, perfect discrimination exists, resulting in a true-positive rate of 1.0 at all false-positive rate values (Swets, 1988).

When the AUC was slightly less in the current study. Bland-Altman plots were used to visually assess the agreement between pHF and pHI. The Bland-Altman method calculates the bias estimate, which is the mean difference between 2 methods of measurement and the 95% CI of agreement ($\pm 1.96$ SD). The Bland-Altman plot demonstrated that pHF and pHI measured UpH 0.34 points greater than pHP (Figure 1), as indicated by the mean difference represented by the solid line. The 95% CI of agreement was $-0.10$ to 0.78 for pHF and pHI, represented by the 2 dashed lines. This is similar to the findings of Constable et al. (2019), in which the mean bias was 0.28 when comparing Multistix-10-SG to a pH meter and 0.10 when comparing Hydron to a pH meter. In a similar study, when Multistix-SG was used, the mean bias was 0.20 compared with a pH meter (Constable et al., 2019), although AUC was slightly less in the current study.

Data were collected daily and consolidated to weekly averages for 4 wk prepartum and 4 wk postpartum.

Treatments consisted of UpH collected from cows weekly and classified, by terciles, as low, medium, or high.

Terciles for prepartum data were determined independently by week. Terciles for wk −4 (n = 53): low (UpH ≤5.55; mean ± SD: 5.36 ± 0.15), medium (UpH >5.55 and ≤5.80; 5.62 ± 0.09), or high (UpH >5.77; 6.62 ± 0.69). Terciles for wk −1 (n = 83): low (UpH ≤5.48; mean ± SD: 5.36 ± 0.11), medium (UpH >5.48 and ≤5.80; 5.66 ± 0.06), or high (UpH >5.78; 6.46 ± 0.68).

Terciles for postpartum data were determined by averaging UpH for the 4 wk before calving for each cow (n = 75) and then classified as low (UpH ≤5.54; mean ± SD: 5.34 ± 0.11), medium (UpH >5.54 and ≤5.77; 5.65 ± 0.06), or high (UpH >5.77; 6.48 ± 0.54).

There was excellent correlation between pHF and pHI and the gold standard pHP ($r = 0.98$; $P < 0.0001$; Figure 1). To determine agreement between pHF and pHI, and pHP and pHI, the CCC was used. The CCC assigns values from −1 to 1, in which agreement is excellent (AUC >0.90; Swets, 1988). The greatest AUC for pHF and pHI at the threshold of 5.75 was 0.94 (95% CI: 0.91–0.97) and 0.92 (95% CI: 0.89–0.95), respectively. Therefore, when the threshold is set at 5.75, the AUC is considered excellent (AUC >0.90; Swets, 1988) for pHF and pHI. This agrees with a previous study in which a urine dipstick (Multistix-10-SG; Siemens) and pH paper (Hydron; MicroEssential Laboratory) had excellent agreement, with AUC = 0.991 and 0.995, respectively, compared with a pH meter (Constable et al., 2019), although AUC was slightly less in the current study.

Bland-Altman plots were used to visually assess the agreement between pHF and pHI, and the gold standard pHP ($r = 0.98$; $P < 0.0001$; Figure 1). To determine agreement between pHF and pHI, and pHP and pHI, the CCC was used. The CCC assigns values from −1 to 1, in which agreement is excellent (AUC >0.90; Swets, 1988). The greatest AUC for pHF and pHI at the threshold of 5.75 was 0.94 (95% CI: 0.91–0.97) and 0.92 (95% CI: 0.89–0.95), respectively. Therefore, when the threshold is set at 5.75, the AUC is considered excellent (AUC >0.90; Swets, 1988) for pHF and pHI. This agrees with a previous study in which a urine dipstick (Multistix-10-SG; Siemens) and pH paper (Hydron; MicroEssential Laboratory) had excellent agreement, with AUC = 0.991 and 0.995, respectively, compared with a pH meter (Constable et al., 2019), although AUC was slightly less in the current study.
−1 is perfect disagreement and 1 is perfect agreement. The CCC was 0.933 (95% CI: 0.92–0.94; P < 0.01) for pHF and 0.926 (95% CI: 0.913–0.938; P < 0.01) for pHI. This indicates near-perfect agreement between pHF and the 2 sticks (pHF and pHI) used to measure UpH. The Pearson correlation coefficient, representing a linear relationship between 2 methods of measurement, also indicated near perfect agreement at 0.981 (95% CI: 0.976–0.984) for pHF and 0.978 (95% CI: 0.973–0.982) for pHI when each were individually compared with pHF. Based on these findings, either pHF or pHI would allow accurate and inexpensive measurement of UpH in dairy cows, although pHI is the least expensive option. Multistix-10-SG may be more accurate to measure UpH, likely because of the smaller interval of measurement; however, it is not readily available to producers and is more expensive than pHion balance test strips (pHI), which are easily accessible. Additionally, pHI uses a dual pad indicator on a plastic strip, which does not bleed, whereas a common issue with pH paper (e.g., Hydrion) is color bleeding once dipped in liquid.

Results of prepartum DMI and postpartum DMI, milk yield, and milk composition are in Table 1. Prepartum, the effect of UpH on DMI was determined by week due to variations in UpH weekly. There were no differences in DMI due to UpH prepartum (P ≥ 0.14) or postpartum DMI (P = 0.72). Previous research indicated that metabolic acidosis characterized by UpH ranging from 5.5 to 7.0 (Constable et al., 2009) may decrease DMI during the 3 wk before calving compared with cows not in compensated metabolic acidosis (Zimpel et al., 2018; Glosson et al., 2020). In the current study, the average UpH for the top one-third of cows (i.e., the high UpH group) was 6.42 compared with 5.44 (low) and 5.54 (medium), indicating that all cows were likely in metabolic acidosis, which was expected according to the study design. Our data indicate no advantage or disadvantage in terms of DMI to have a greater UpH while still being within the classification of induced metabolic acidosis. There was a tendency for an UpH × week interaction for milk yield (P = 0.09; Figure 2A). Greater milk yield at wk 1 postpartum for cows in the high UpH group may indicate a detrimental carryover effect if UpH is averaged at <5.54 (cows in the low and medium groups) and may suggest that UpH ranging from 6.0 to 7.5, deeming the diet partially acidified (Cardoso et al., 2020), may be more beneficial for milk yield. However, yields of ECM and 3.5% FCM were not affected by UpH (P ≥ 0.26). Additionally, milk composition content and yields were not different for cows characterized as having low, medium, or high UpH (P ≥ 0.12), excluding total solids. There was a tendency for a UpH × week interaction for total solids content (P = 0.06; Figure 2B). Greater milk total solids for cows in the low group is likely due to lesser milk yield in those cows (Fuertes et al., 1998).

Further analysis of the data revealed variability in the standard deviations within the UpH terciles. For all terciles, the group characterized as high had the greatest variability, as indicated by greater standard deviations. For example, when UpH was averaged for each cow prepartum, the standard deviation for the high group was 0.36, whereas it was 0.07 for the low group and 0.09 for the medium group. This greater variability for the high UpH group may correspond to greater difficulty ensuring that the UpH of cows stays within the range indicated for this group. This is likely due to acid–base balance, in which the amount of strong acid within the UpH (as H+ ions) exceeded the buffering capacity (Jørgensen, 1957), resulting in a decrease in pH that was sustained for cows in the low and medium groups or those with an average UpH <5.54. An additional consideration regarding classification of metabolic acidosis based exclusively on DCAD level, which has previously been used to estimate metabolic acidosis (Lean et al., 2019), rather than UpH, is that determining the DCAD only may not be adequate and could lead to increased variability (Cardoso et al., 2020). In the current study, all cows consumed the same negative DCAD diet but UpH ranged from an average of 5.44 to 6.42, suggesting that many factors other than DCAD affect UpH. However, it should also be noted that according to Constable et al. (2019), UpH is only an adequate estimation of net acid excretion when >6.11, although UpH <6.11 does accurately predict Ca excretion of ≥4 g/d.

Based on the results obtained from this study, we conclude that Fisherbrand pH strips and pHiOn strips are both accurate and
inexpensive methods to measure UpH. Additionally, the degree of metabolic acidosis, as characterized by UpH, does not affect prepartum or postpartum DMI. However, there may be unfavorable effects on milk yield during wk 1 postpartum if average UpH is <5.54 during the prepartum period, which may be economically detrimental to producers because of the increased cost associated with a greater anion inclusion rate needed to achieve that UpH.

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