



Short communication: Ketone body concentration in milk determined by Fourier transform infrared spectroscopy: Value for the detection of hyperketonemia in dairy cows

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ABSTRACT

The objective of this study was to evaluate Fourier transform infrared (FTIR) spectrometry to measure milk ketone bodies to detect hyperketonemic cows and compare this method with milk fat to protein ratio to detect hyperketonemia. Plasma and milk samples were obtained weekly from calving to wk 9 postpartum from 69 high-producing dairy cows. The reference test for hyperketonemia was defined as plasma concentration of β -hydroxybutyrate (BHBA) $\geq 1,200$ $\mu\text{mol/L}$. The weekly prevalence of hyperketonemia during the first 9 wk of lactation was, on average, 7.1%. Both BHBA and acetone in milk, determined by FTIR, had a higher sensitivity (80%) to detect hyperketonemia compared with milk fat to protein ratio (66%). Specificity was similar for the 3 diagnostic tests (71, 70, and 71%). In conclusion, FTIR predictions of BHBA or acetone in milk can detect cows with hyperketonemia in early lactation with a higher accuracy compared with the use of milk fat to protein ratio. Because of the high proportion of false-positive tests, there are concerns about the practical applicability of FTIR predictions of acetone, BHBA, and fat to protein ratio in milk to detect hyperketonemic cows.

Key words: acetone, β -hydroxybutyrate, sensitivity, specificity

Subclinical ketosis is characterized by an increased concentration of ketone bodies (acetone, acetoacetate, and BHBA) in body fluids in the absence of clinical signs of ketosis (Duffield, 2000). The disorder is trig-

gered by negative energy balance around calving and occurs mainly within the first 2 mo after calving. Cows are defined as hyperketonemic when the concentration of BHBA in plasma exceeds the threshold of 1,200 to 1,400 $\mu\text{mol/L}$ (Duffield et al., 2009), where the threshold level is based on an increased risk for disease. Variation in threshold level depends on stage of lactation, whereas the health impairment of hyperketonemia differs with stage of lactation (Duffield et al., 2009). Estimates for the overall prevalence of hyperketonemia range from 1.1 to 14.1% (Andersson and Emanuelson, 1985; Duffield et al., 1997; Carrier et al., 2004) and can vary largely between individual farms (Dohoo and Martin, 1984). Hyperketonemia may increase the risk for other periparturient disorders such as left displaced abomasum (Geishauser et al., 1997) and is negatively associated with reproductive performance (Walsh et al., 2007).

Various urine or milk ketone tests have been investigated and applied as semiquantitative cow-side tests to detect cows with hyperketonemia on dairy farms (e.g., Nielen et al., 1994; Geishauser et al., 1997). A disadvantage of these tests is that they may not be practical to test all cows at risk at regular intervals, which would require a more automated procedure. Currently, fat to protein ratio in test-day milk is often used to indicate hyperketonemic cows, although this variable is influenced by factors other than the ketotic state of the animal alone (e.g., nutrition) (Duffield et al., 1997). To obtain a more reliable assessment of the occurrence of hyperketonemia in a larger group of cows, several attempts have been made to validate in-line methods for routine milk analysis of ketone bodies in cows (Gustafsson and Emanuelson, 1996; Nielsen et al., 2005). Such a routine analysis can be done by Fourier transform infrared (FTIR) spectrometry (Luning et al., 1996). Hansen (1999) cross-validated FTIR

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prediction of milk acetone with reference measurement of acetone in milk and concluded that the model could differentiate “possibly ketotic” from healthy cows with no false negatives and 27% false positives. In addition, Heuer et al. (2001) developed a FTIR prediction model for milk acetone to identify subclinical ketotic cows, which resulted in positive predictive values of >76% and negative predictive values of >98% in a population with prevalence of subclinical ketosis of 10 to 30%. It can be hypothesized that FTIR predictions of BHBA and acetone in milk could be valuable in screening of dairy cows for hyperketonemia. This method, however, has not yet been validated for its ability to detect cows with hyperketonemia with the concentration of BHBA in plasma as the diagnostic reference criterion for milk ketone bodies. The determination of BHBA in plasma is considered the reference test for subclinical ketosis (Duffield, 2000). The objectives of this study were 2-fold. First, we evaluated the use of FTIR predictions of BHBA and acetone in test-day milk to detect cows with hyperketonemia. Second, we compared FTIR predictions of BHBA and acetone in milk with the use of the milk fat to protein ratio to detect cows with hyperketonemia.

The Institutional Animal Care and Use Committee of Wageningen University approved the experimental protocol. Data and samples originated from the experiment of van Knegsel et al. (2007) assessing energy balance from calving to 9 wk postcalving. The original objective of the experiment was to study the effect of lipogenic versus glucogenic diets on negative energy balance and related metabolites of dairy cows in early lactation. The experimental design, diet composition, and analytical procedures for determination of plasma BHBA concentration were previously reported (van Knegsel et al., 2007). For the purpose of the current study, 69 cows (20 primiparous, 49 multiparous) from one herd were monitored for plasma BHBA concentration, milk fat and protein content, and milk acetone and BHBA concentrations. Plasma samples were obtained weekly from calving to wk 9 postpartum, 2 to 3 h after the a.m. feeding. From wk 1 until wk 9 postpartum, on the same day as the blood samples were collected, milk was sampled during the p.m. milking and stored at -80°C until analysis. For determination of the concentration of ketone bodies, fat and protein in milk, collected milk samples ($n = 618$ of 69 cows) were gently preheated to 40°C and mixed before analysis with a MilkoScan FT6000 (Foss Analytical A/S, Hillerød, Denmark). This FTIR was used according to the instructions of the manufacturer with operational calibrations for fat (ISO 1211; IDF 1; ISO, 1999), protein (ISO 8968; IDF 20-1; ISO, 2001), BHBA, and acetone (de Roos et al., 2007).

Receiver operating characteristic (ROC) curves (Zweig and Campbell, 1993) were produced to provide a graphic illustration of the accuracy of the 3 tests; that is, milk fat to protein ratio, milk BHBA, and milk acetone, to discriminate between nonketotic and hyperketonemic cows compared with plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$ as the diagnostic reference. Area under the curve (AUC) of the ROC curves of 3 tests was calculated using logistic regression analysis. Because cows were measured repeatedly in time, a repeated measures design was used according to Liu and Wu (2003). A first-order autoregressive covariance structure [AR(1)] fit best and was used to account for within-cow variation. In addition to the milk test results (BHBA, acetone, or fat to protein ratio), parity (1, 2, or ≥ 3), lactation week (1, 2, ... 9), and diet (lipogenic or glucogenic) were included as fixed effects in the models, but proved to be not significant ($P > 0.05$). For each test, logistic regression lines without (PROC LOGISTIC; SAS Institute, 2004) and with repeated effect of cows (PROC GLIMMIX; SAS Institute, 2004) were almost similar and AUC differed by $<0.5\%$. Because most statistical packages are able to compare AUC for ordinary logistic regression models but not repeated measures models, the AUC of the 3 milk tests were statistically evaluated using Stata 10 (ROCGOLD; StataCorp LP, College Station, TX) and were adjusted (Bonferroni) for multiple comparisons. The optimal cut-off value of each test was defined as the maximum proportion of observed agreement of correct test results [(true positive samples + true negative samples)/(total no. of samples tested)] with plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$. In addition, to quantify the added value of testing multiple variables in milk, test results (sensitivity, specificity, positive and negative predictive value (SN, SP, PV+, and PV−, respectively) at optimal cut-off values were interpreted in series or parallel. A milk sample was defined as positive in series interpretation when it tested positive in the milk BHBA, milk acetone, and milk fat to protein ratio test. A milk sample was defined positive in parallel interpretation when it tested positive to at least 1 of the 3 milk tests.

Cows produced, on average (mean \pm SD), 39.2 ± 8.2 kg of milk per day, containing $4.61 \pm 0.75\%$ fat and $3.22 \pm 0.27\%$ protein from wk 1 to 9 in lactation. The average plasma BHBA concentration was 756 ± 352 $\mu\text{mol/L}$, with a range from 270 to 3,590 $\mu\text{mol/L}$. With the defined threshold of plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$, the prevalence of hyperketonemia during the first 9 wk of lactation was, on average, 7.1% per week with a range from 2.9 to 13.0%. Receiver operating characteristic curves for milk BHBA, milk acetone, and milk fat to protein ratio are shown in Figure 1. Areas under the curve were 74.8% for milk fat to protein ratio, 81.8%

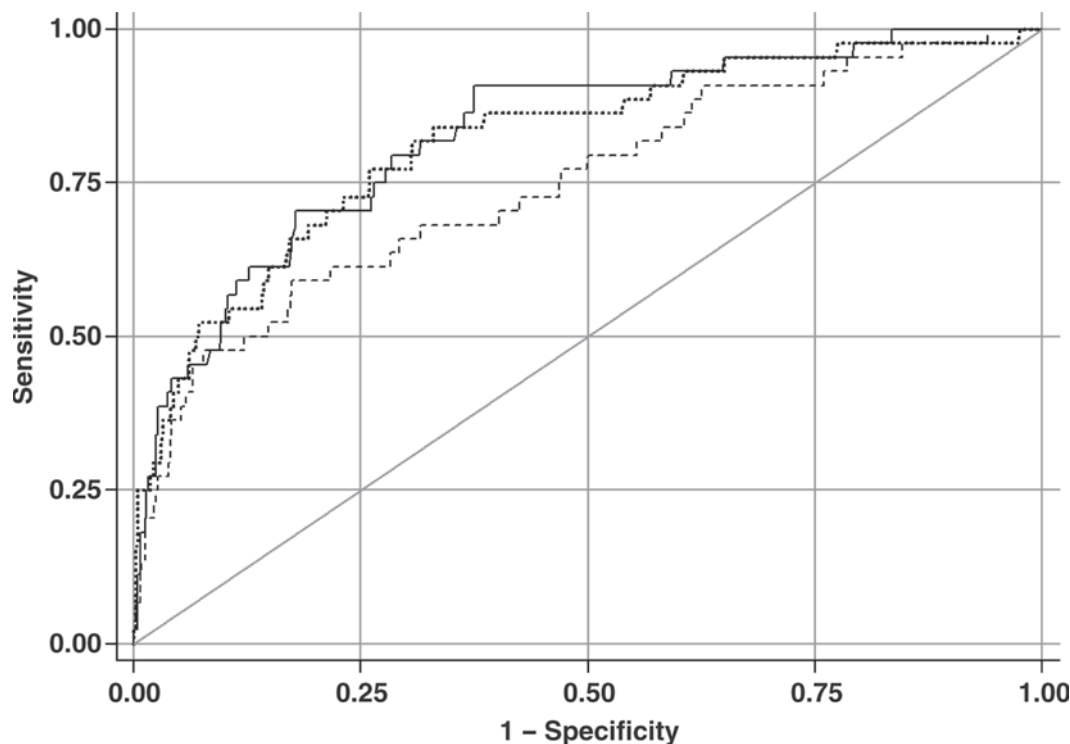


Figure 1. Receiver operating characteristic (ROC) curves for milk fat to protein ratio (----), milk BHBA (—), and milk acetone (....) at a series of cut-off values as test variable for hyperketonemia (plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$) in a herd of 69 dairy cows in early lactation (wk 1 to 9 postpartum).

for milk acetone, and 83.0% for milk BHBA (Table 1). With pairwise comparisons milk BHBA ($P = 0.05$) had a higher accuracy to detect hyperketonemic cows compared with milk fat to protein ratio, whereas there was no difference between milk BHBA and milk acetone ($P = 0.50$). Optimal cut-off values were 23 $\mu\text{mol/L}$ for milk BHBA and 70 $\mu\text{mol/L}$ for milk acetone. The optimal cut-off value for milk fat to protein ratio in this study (1.5) was higher than earlier reported (1.3) in a Canadian study (Duffield et al., 1997). The SN, SP, PV+, and PV– of each of the tests, when interpreted at their optimal cut-off values, are shown in Table 2. Sensitivity ranged from 66% for fat to protein ratio to 80% for both BHBA and acetone. Specificity was similar for the 3 tests at approximately 70%. Predictive values of a negative test were high for all 3 tests, implying that 96 to 98% of all animals that tested negative were truly negative. Predictive values of a positive test were very low, at 15 to 18%, implying that of all animals that tested positive, less than 20% were truly positive. When the 3 milk tests (BHBA, acetone, and fat to protein ratio) were interpreted in series, SP increased to 89%. Predictive values of both negative and positive test results did not change when tests were combined in parallel or series, except when the 3 tests were interpreted in series, the PV+ increased to 28%.

This is still a very low value, and a high proportion of false positives limits the practical applicability of FTIR predictions of acetone, BHBA, and fat to protein ratio in milk to detect hyperketonemic cows if the prevalence is relatively low (7% in this study).

It may be useful to increase the optimal cut-off value when the prevalence is low. This would increase the SP and PV+ of the test and increase the practical applicability of the test to detect cows with hyperketonemia. On the other hand, increasing the optimal cut-off value would increase the proportion of false-negative test results. Therefore, the degree by which the cut-off value should be increased depends on the economic consequences of a large proportion of false negatives (Zweig and Campbell, 1993). To our knowledge, little is known about the economic costs associated with hyperketonemia in dairy cows. In addition to the incidence and treatment of hyperketonemia itself, the positive relation of hyperketonemia with secondary diseases and milk yield loss (Dohoo and Martin, 1984) results in a negative effect of hyperketonemia on net herd returns (Kossaibati and Esslemont, 1997).

This study is the first to report the value of FTIR predictions for milk BHBA and acetone to detect sub-clinical ketotic cows with plasma BHBA concentration $\geq 1,200$ $\mu\text{mol/L}$ as the reference test. This resulted in

Table 1. Receiver operating characteristic (ROC) curves¹ analysis for milk BHBA, milk acetone, and milk fat to protein ratio as test variables for hyperketonemia (plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$) in a herd of 69 dairy cows in early lactation

ROC curve	AUC, ² %	SE	95% CI	P-value	Bonferroni P-value
BHBA	82.95	3.31	76.46–89.45	0.05	0.09
Acetone	81.80	3.62	74.71–88.89	0.12	0.23
Fat to protein ratio	74.81	4.25	66.47–83.15	Reference	

¹ROC curves are presented in Figure 1.²Area under the curve.

similar sensitivities but lower specificities than the previously reported studies that used wet chemical analysis of milk ketone bodies as the reference test (de Roos et al., 2007; Heuer et al., 2001). It seems likely, however, that FTIR predictions for acetone and BHBA in milk correspond better to wet chemical analysis of BHBA in milk than to BHBA concentration in plasma. This discrepancy between milk and plasma BHBA could be attributed to timing of sampling (late morning blood sample was compared with afternoon milking) or intermediary ketone body metabolism, wherein plasma BHBA can be used for the synthesis of short- and medium-chain fatty acids in the mammary gland (Bauman and Griinari, 2003).

The advantage of cow-side tests is the rapid diagnosis of individual suspected cows, thus facilitating direct treatment and prevention of clinical disease. On the other hand, screening cows for hyperketonemia through FTIR spectrometry could easily be included in regular milk recordings and added to the test-day information. Herewith, within-herd prevalence could be estimated more accurately and supply an additional tool for evaluation of herd health status. The relatively high proportion of false-positives, however, is a limitation for the practical applicability of FTIR predictions of acetone, BHBA, and fat to protein ratio in milk to detect hyperketonemia. Furthermore, as illustrated by Figure 2, lactation week affects the ratio between

milk BHBA and acetone and fat to protein ratio, which could affect the diagnostic value of these variables to predict the risk for subclinical ketosis. The applicability of these FTIR predictions could be increased by including cow characteristics that have been related to the susceptibility to hyperketonemia (e.g., body condition, lactation week, or milk production level; Nielsen et al., 2005; Duffield et al., 2009) to increase the accuracy of the predicted risk for hyperketonemia per individual recorded cow. The current data, however, contained too little variation in stage of lactation and milk production level between cows to test this approach. Validation of FTIR predictions of BHBA and acetone in milk compared with BHBA in plasma using data of different farms with more variation in cow characteristics and diets could help researchers assess the value of such a diagnostic model.

In conclusion, FTIR predictions of BHBA or acetone in milk can detect cows with hyperketonemia in early lactation with a higher accuracy compared with the use of milk fat to protein ratio. Furthermore, specificity increases to 89% when cows for all 3 milk tests (BHBA, acetone, and fat to protein ratio) were classified as hyperketonemic. Because of the high proportion of false-positive test results, there are concerns about the practical applicability of FTIR predictions of acetone, BHBA, and fat to protein ratio in milk to detect hyperketonemic cows.

Table 2. Sensitivity (SN), specificity (SP), and positive (PV+) and negative predictive value (PV–) for BHBA, acetone, and fat to protein ratio in milk, interpreted separately and combined in serial or parallel testing, to identify hyperketonemic cows (plasma BHBA $\geq 1,200$ $\mu\text{mol/L}$) in a herd of 69 dairy cows in early lactation

Test variable ¹ (optimal cut-off value)	SN, % (95% CI)	SP, % (95% CI)	PV+, % (95% CI)	PV–, % (95% CI)
BHBA (≥ 23 $\mu\text{mol/L}$) (B)	80 (65 to 90)	71 (68 to 75)	18 (13 to 24)	98 (96 to 99)
Acetone (≥ 70 $\mu\text{mol/L}$) (A)	80 (65 to 90)	70 (66 to 73)	17 (12 to 22)	98 (96 to 99)
Fat to protein ratio (≥ 1.5) (F)	66 (50 to 80)	71 (67 to 74)	15 (10 to 20)	96 (94 to 98)
B and A and F (serial test)	55 (39 to 70)	89 (86 to 92)	28 (19 to 39)	96 (94 to 98)
B and A (serial test)	75 (60 to 87)	76 (72 to 79)	19 (14 to 26)	98 (96 to 99)
B or A or F (parallel test)	91 (78 to 97)	51 (46 to 55)	12 (9 to 16)	99 (96 to 99)
B or A (parallel test)	84 (70 to 93)	65 (61 to 69)	16 (11 to 21)	98 (97 to 100)

¹Serial test = a cow is classified positive if all tests are positive, otherwise negative; parallel test = a cow is classified negative if all tests are negative, otherwise positive.

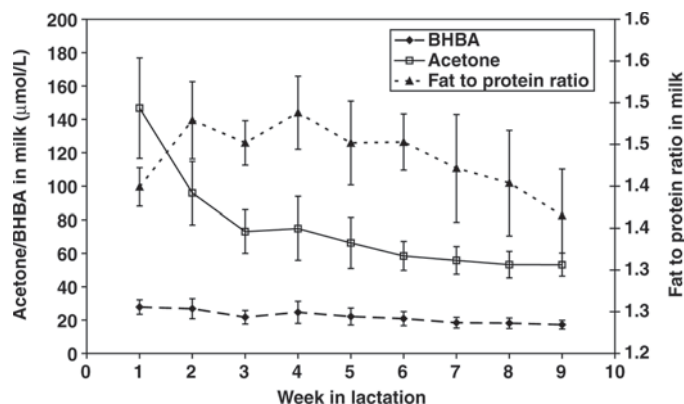


Figure 2. Milk acetone, milk BHBA, and milk fat to protein ratio of dairy cows ($n = 69$) during wk 1 to 9 in lactation. Values represent means \pm SEM.

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