

Effect of Anticoagulant and Storage Conditions on Bovine Nonesterified Fatty Acid and β -Hydroxybutyrate Concentrations in Blood

T. Stokol and D. V. Nydam

Department of Population Medicine and Diagnostic Sciences,
College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

ABSTRACT

The objective of this study was to determine if nonesterified fatty acid (NEFA) and β -hydroxybutyrate (BHBA) concentrations were affected by anticoagulants or gel and clot activator tubes (serum separator tubes, SST), storage of samples as whole blood, separated plasma or serum at 24°C or 4°C for 24 to 72 h, or storage as serum at –40°C for 1 mo. Blood was collected from dairy cows into EDTA, heparin, nonanticoagulant tubes, and SST, and analyzed immediately to obtain baseline NEFA and BHBA concentrations. Portions were stored as whole blood or separated plasma or serum at 4 or 24°C and assayed daily for 24 (whole blood) and 72 (separated samples) h. Serum samples were frozen at –40°C and assayed at 24 h or weekly for 1 mo. Baseline NEFA concentrations were unaffected by anticoagulants; however, they were significantly higher in SST compared with nonanticoagulant tubes. Concentrations of NEFA were stable in all samples at 4°C, whereas they sequentially increased from 24 to 48 h at 24°C. Changes were more dramatic in heparinized samples. Serum could be stored frozen for up to 1 mo with minimal changes in NEFA concentrations. Concentrations of BHBA were stable under all conditions evaluated. Our results indicated that blood for NEFA testing should be collected into EDTA or nonanticoagulant tubes (but not SST), separated promptly from cells, and maintained at 4°C until analysis.

(Key words: NEFA and BHBA concentration, dairy cow, storage and temperature stability, anticoagulant)

Abbreviation key: SST = serum separator tube.

INTRODUCTION

Dairy cows are vulnerable to developing negative energy balance during the periparturient period. This is due to the high-energy demands of the developing fetus and ensuing milk production, coupled with a decline in

DM intake that occurs just before calving (Drackley, 1999; Grummer et al., 2004). Cows in negative energy balance are more prone to developing postparturient metabolic diseases, such as ketosis, fatty liver, and displaced abomasum (Cameron et al., 1998; Grummer et al., 2004). Moreover, an increased incidence of infectious diseases, including metritis and mastitis, has been associated with negative energy balance and subclinical ketosis (Kaneene et al., 1997; Leslie et al., 2000; Suriyasathaporn et al., 2000). This has been attributed to ketone-induced decreases in leukocyte chemotactic and bactericidal ability (Kaneene et al., 1997; Suriyasathaporn et al., 2000). These metabolic and infectious diseases negatively influence milk production, reproductive ability, and production costs (Cameron et al., 1998; Drackley, 1999; Duffield, 2000). Thus, it is vital to recognize when a state of excessive negative energy balance exists in cows within a dairy herd, such that nutritional and management programs can be modified to increase transition cow energy intake and optimize herd health and productivity.

During states of negative energy balance, lipolysis of adipose tissue liberates NEFA, which are used by the liver and mammary gland for energy and milk production, respectively. However, if NEFA delivery exceeds NEFA use, they are esterified and converted to triglycerides or metabolized to ketone bodies, particularly BHBA, within the liver. Triglycerides accumulate within hepatocytes and cause liver dysfunction and ketosis (Drackley, 1999; Herdt, 2000). Thus, high prepartum NEFA (>0.4 mmol/L) and postpartum BHBA (>1.40 mmol/L) concentrations in a proportion (>10%) of tested animals are considered biomarkers of negative energy balance and subclinical ketosis in the herd, respectively (Duffield, 2000; Oetzel, 2004). β -Hydroxybutyrate is typically measured in serum samples, whereas NEFA can be measured in serum or EDTA plasma. It is advantageous to dairy practitioners to collect only one sample (serum) for these tests, because both tests are included in postcalving metabolic profiles (NEFA and Metabolic Profile Sample Handling Guidelines, Nutrition Laboratory, University of Michigan). In human patients, BHBA concentrations are unaffected by storage temperature and time (Custer et al., 1983); however, NEFA

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Corresponding author: Tracy Stokol; e-mail: ts23@cornell.edu.

are far less stable. Nonesterified fatty acid values are affected by anticoagulants, and increase with time, particularly at higher storage temperatures (Rogiers, 1978; Gleeson, 1987; McGann and Hodson, 1991; Menendez et al., 2001). The current protocol for blood sampling for NEFA testing is to collect blood into gel and clot activator or serum separator tubes (SST), allow the blood to clot, centrifuge the sample, transfer the serum into another tube, then ship the serum chilled or frozen overnight for analysis (NEFA and Metabolic Profile Sample Handling Guidelines). A previous study in dairy cows showed that NEFA concentrations were slightly higher in EDTA plasma than in serum in 8 of 10 cows (Brookes et al., 1984). However, to our knowledge, there have been no published reports on the effect of other anticoagulants (e.g., heparin), use of SST, or other pre-analytical variables, including delayed separation of serum or plasma from cells, storage temperature, and duration of storage, on NEFA and BHBA concentrations in dairy cows.

We performed this study to provide recommendations to dairy practitioners and researchers on how best to collect and store samples for NEFA and BHBA concentrations to obtain accurate results. We hypothesized that both analytes would be unaffected by anticoagulants or use of SST and would be most stable if separated from cells and stored cool until analysis. The objectives of this study were to determine if NEFA and BHBA concentrations are influenced by: 1) use of anticoagulants and SST; 2) delays in separating serum or plasma from cells; 3) maintaining samples at 24°C or 4°C after collection; 4) storing samples for up to 72 h at these temperatures; and 5) frozen storage.

MATERIALS AND METHODS

Sample Collection and Handling

For measuring the effect of anticoagulant, storage time, and storage temperature on NEFA and BHBA concentrations, blood was collected from the tail veins of 10 periparturient dairy cows (5 animals each from 2 herds) into EDTA- (purple-top tube; Becton Dickinson, Franklin Lakes, NJ), heparin-(green-top tube; Becton Dickinson), and nonanticoagulant (red-top tube; Becton Dickinson) tubes. An aliquot was centrifuged immediately after collection or clotting at $3000 \times g$ for 10 min at 24°C, and then the serum or plasma was assayed to obtain baseline concentrations. Portions of the sample were then maintained as whole blood or were separated from cells and stored as plasma (EDTA or heparin) or serum. Whole blood samples were stored at 24 (room temperature) or 4°C, centrifuged, and the serum or plasma assayed at 24 h, whereas separated samples were stored at 24 or 4°C, then assayed at 24, 48, and

72 h. Whole blood samples were stored for only 24 h because we reasoned that veterinarians would be able to separate samples within this period after collection.

To determine if collecting blood into SST affected serum NEFA and BHBA concentrations, blood samples were collected from 18 periparturient cows from a single herd into SST and nonanticoagulant tubes. Samples were kept cool (4°C) and the serum was separated from cells and analyzed within 4 to 6 h after collection.

For assessing NEFA and BHBA concentrations in serum after freeze-thawing, blood samples were collected from 30 periparturient cows (18 and 12 each from 2 herds) into nonanticoagulant tubes, separated immediately from cells, and assayed to obtain baseline values. The samples were then frozen at -40°C, thawed, and assayed 24 h later (experiment 1). In a second experiment (experiment 2), blood samples were collected from 11 dry cows from a single herd into nonanticoagulant tubes, the serum was separated from cells, and analyzed for baseline concentrations. Portions of the serum were then frozen at -40°C, then thawed, and analyzed at 7, 14, and 28 d after collection.

Analysis of NEFA and BHBA Concentrations

Nonesterified fatty acids (NEFA-C, Wako Chemicals USA, Inc., Richmond, VA; Custer et al., 1983) and BHBA (β -HBA, Catachem Inc., Bridgeport, CT; Morris et al., 2002) concentrations were measured using colorimetric enzymatic reactions with an automated wet chemistry analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, IN). The analyzer was calibrated and controls assayed daily according to manufacturers' recommendations, to ensure acceptable assay performance.

Statistical Analyses

Median values in different sample types, over time, or at different temperatures were compared with a Wilcoxon signed-rank test (2 medians) or Friedman 1-way ANOVA test (>2 medians), with a Bonferroni adjustment for the numbers of pairwise comparisons. Alpha was set at $P \leq 0.05$.

RESULTS

Effect of Anticoagulant, Storage Temperature, and Storage Time on NEFA Concentrations

Baseline concentrations in different anticoagulant tubes or SST. Baseline NEFA concentrations were not significantly different in EDTA, heparin, or nonanticoagulated whole blood samples (Figure 1, $P = 0.117$). In contrast, NEFA concentrations were slightly, but significantly, higher when collected into SST (0.16

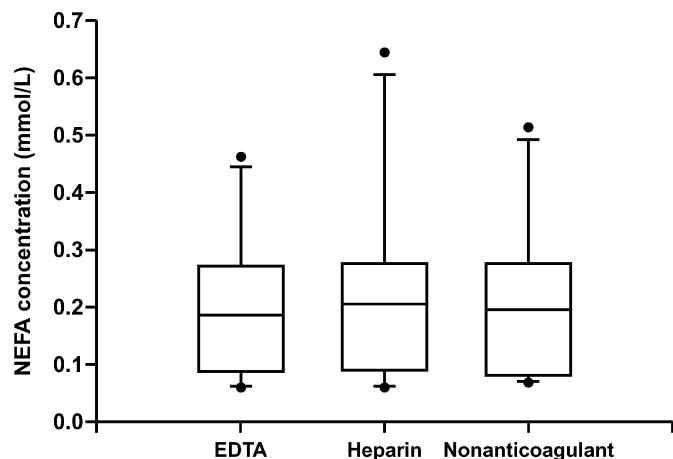


Figure 1. Box and whisker plot of baseline NEFA concentrations in blood samples collected from dairy cows into EDTA, heparin, and nonanticoagulant tubes ($n = 10$). Median NEFA concentrations were not significantly different between sample types ($P = 0.117$).

mmol/L, range: 0.09 to 0.49 mmol/L) compared with nonanticoagulant tubes (0.13 mmol/L, range: 0.06 to 0.46 mmol/L) ($P = 0.003$). The median difference between these samples (SST minus nonanticoagulant) was 0.02 mmol/L (range: -0.04 to 0.06 mmol/L).

Maintaining samples as whole blood or separating plasma or serum from cells. Comparisons between whole blood and serum or plasma were performed only for the 24-h samples. There was no significant difference in NEFA concentrations between whole blood and plasma (regardless of anticoagulant) or serum samples at 24 h when samples were maintained at 4°C. Similarly, there was no significant difference in NEFA concentrations between whole blood and plasma (EDTA or heparin) after 24 h at 24°C. In contrast, in nonanticoagulated samples, NEFA concentrations were signifi-

cantly higher after 24 h in whole blood compared with serum at 24°C (Table 1) ($P = 0.027$).

Effect of anticoagulant, storage temperature, and storage time. When samples were stored at 4°C, NEFA concentrations changed little over 24 (whole blood) or 72 (plasma or serum) h in any sample. In addition, there was no significant difference in NEFA concentrations of the different samples at this temperature at any time ($P > 0.017$ with Bonferroni adjustment). In contrast, at 24°C, NEFA concentrations increased significantly in whole blood within 24 h and in plasma and serum within 24 and 48 h, respectively (Table 1). Indeed, NEFA concentrations increased sequentially in serum or plasma with time at this temperature. There were, however, differences between anticoagulants, with larger increases occurring in samples collected into heparin (Table 1). At each time point (24, 48, or 72 h), NEFA concentrations were significantly higher ($P < 0.017$ with Bonferroni adjustment) in heparin- than EDTA-anticoagulated plasma or serum, whereas EDTA-anticoagulated plasma and serum yielded similar values.

Effect of freeze-thaw. Concentrations of NEFA did not change significantly ($P = 0.366$) when serum was frozen at -40°C , then thawed, and analyzed 24 h later (experiment 1, Table 2). Minor changes occurred in NEFA concentrations when serum was stored frozen for up to 1 mo (experiment 2, Table 2).

Effect of Anticoagulant, Storage Temperature, and Storage Time on BHBA Concentrations

Baseline concentrations in different anticoagulant tubes or SST. Baseline BHBA concentrations were not significantly different in EDTA, heparin, or nonanticoagulated whole blood samples (Figure 2) ($P = 0.248$). Similarly, there was no significant difference in

Table 1. Nonesterified fatty acid concentrations (mmol/L) in dairy cow blood collected into EDTA, heparin, or nonanticoagulated tubes and maintained as whole blood, separated plasma, or separated serum at 4 or 24°C over 72 h ($n = 10$).

Storage time (h)	4°C						24°C					
	EDTA		Heparin		Nonanticoagulant		EDTA		Heparin		Nonanticoagulant	
	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Serum	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Serum
0	0.19	0.19	0.21	0.21	0.20	0.20	0.19	0.19	0.21	0.21	0.20	0.20
24	0.20	0.19	0.22	0.20	0.19	0.19	0.21 ^a	0.21 ^a	0.26 ^a	0.25 ^{ac}	0.23 ^{ab}	0.20
48		0.19		0.22		0.19		0.23 ^a		0.29 ^{ac}		0.22 ^a
72		0.18		0.21		0.18 ^a		0.23 ^a		0.34 ^{ac}		0.24 ^a

^aMedians significantly different at each time point compared with baseline values for each sample type ($P < 0.008$ with Bonferroni adjustment).

^bMedian significantly different in whole blood vs. separated serum at 24 h ($P < 0.027$).

^cMedians significantly different at each time point in heparin- vs. EDTA-anticoagulated plasma or serum ($P < 0.017$ with Bonferroni adjustment).

Table 2. Concentrations of NEFA and BHBA in serum from dairy cows with storage at -40°C .

Storage time (d)	NEFA (mmol/L)		BHBA (mmol/L)	
	Experiment 1 (n = 30)	Experiment 2 (n = 11)	Experiment 1 (n = 30)	Experiment 2 (n = 11)
0	0.17 ^a	0.49 ^{ab}	0.67 ^a	0.96 ^a
1	0.17 ^a	...	0.67 ^a	...
7	...	0.52 ^c	...	0.96 ^a
14	...	0.51 ^b	...	0.96 ^a
28	...	0.49 ^a	...	0.86 ^a

^{a,b,c}For each analyte within each experiment, medians with common superscripts are not significantly different ($P > 0.05$ for experiment 1 and $P > 0.008$ for experiment 2 with Bonferroni adjustment).

BHBA concentrations in blood collected into SST (0.76 mmol/L, range: 0.19 to 1.35 mmol/L) or nonanticoagulant tubes (0.73 mmol/L, range: 0.19 to 1.25 mmol/L) ($P = 0.557$). The median difference between these samples (SST minus nonanticoagulant) was 0.03 mmol/L (range: -0.19 to 0.38 mmol/L).

Maintaining samples as whole blood or separating plasma or serum from cells. Concentrations of BHBA were not significantly different in whole blood compared with separated plasma or serum by 24 h of collection at 4 ($P = 0.142$) or 24°C ($P = 0.620$) (Table 3).

Effect of anticoagulant, storage temperature, and storage time. Concentrations of BHBA did not change significantly ($P > 0.017$ with Bonferroni adjustment) over time, regardless of anticoagulant, storage temperature, or storage time (Table 3).

Effect of freeze-thaw. Concentrations of BHBA did not change significantly ($P = 0.640$) when serum was frozen for 24 h at -40°C , then thawed (experiment 1, Table 2). Similarly, BHBA concentrations were stable

when serum was frozen at -40°C for 1 mo after collection ($P = 0.210$) (experiment 2, Table 2).

DISCUSSION

Our results indicated that NEFA and BHBA concentrations can be measured accurately in bovine blood samples collected into EDTA, heparin, or nonanticoagulant tubes as long as the plasma or serum is separated from cells within 24 h of collection and samples are maintained at 4°C at all times. Moreover, these analytes are stable in frozen serum for up to 1 mo after collection. Nonesterified fatty acid, but not BHBA, concentrations will increase with time when anticoagulated plasma and serum are not kept cool (4°C). Nonesterified fatty acid concentrations are slightly, but significantly, higher in serum samples obtained from SST.

Previous studies in human patients using the same analytical technique have shown that NEFA concentrations are higher (4 to 10%) in serum than in EDTA plasma (McGann and Hodson, 1991; Menendez et al., 2001). In our study, NEFA concentrations were similarly higher (by 5%) in serum, although this was not significant or clinically relevant. Higher values in serum have been attributed to activation of de-esterifying enzymes (e.g., lecithinases) or lipolytic enzymes during clotting or possible EDTA inhibition of the reaction (McGann and Hodson, 1991). In contrast, NEFA concentrations were higher (albeit not significantly) in EDTA plasma compared with serum in sheep (Morris et al., 2002), and in a previous study in 10 dairy cows (Brookes et al., 1984). A reason for the discrepancies between these results and our data is not readily apparent, but could be related to differences in sample handling and processing, because the same analytical technique was used for these 3 studies. β -Hydroxybutyrate concentrations were similar regardless of sample type, as has been reported previously for ovine blood (Morris et al., 2002). In contrast, BHBA values were influenced by anticoagulants in human samples, with lower values seen after exogenous addition of EDTA or sodium fluo-

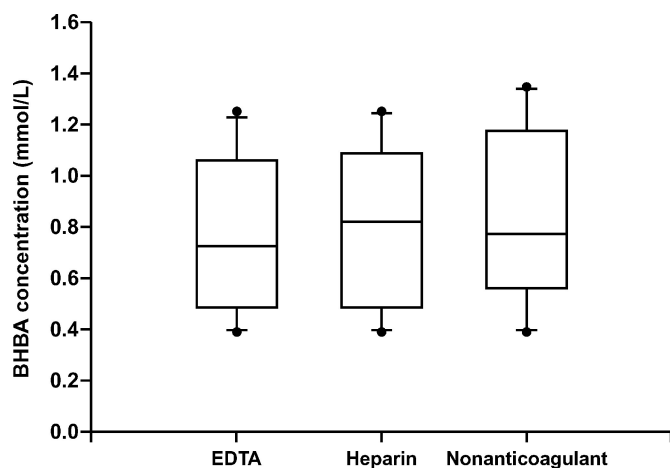


Figure 2. Box and whisker plot of baseline BHBA concentrations in blood samples collected from dairy cows into EDTA, heparin, and nonanticoagulant tubes (n = 10). Median BHBA concentrations were not significantly different between sample types ($P = 0.248$).

Table 3. Changes in BHBA concentrations (mmol/L) in dairy cow blood collected into EDTA, heparin, or nonanticoagulated tubes and stored as whole blood, separated plasma, or separated serum at 4 or 24°C for 72 h (n = 10). None of the changes were statistically significant ($P > 0.05$).

Storage time (h)	4°C						24°C					
	EDTA		Heparin		Nonanticoagulant		EDTA		Heparin		Nonanticoagulant	
	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Serum	Whole blood	Plasma	Whole blood	Plasma	Whole blood	Serum
0	0.72	0.72	0.82	0.82	0.77	0.77	0.72	0.72	0.82	0.82	0.77	0.77
24	0.77	0.72	0.77	0.77	0.77	0.72	0.82	0.77	0.77	0.82	0.72	0.77
48		0.77		0.77		0.62		0.82		0.77		0.82
72		0.77		0.72		0.67		0.77		0.77		0.62

ride/potassium oxalate, but not lithium heparin (Custer et al., 1983).

Nonesterified fatty acid concentrations were stable at 4°C, regardless of anticoagulant, for 24 h in whole blood and for 72 h in separated plasma or serum. Similarly, NEFA concentrations did not change significantly over 48 h in human serum or plasma kept at 4°C (Menendez et al., 2001). Our results are in contrast to that reported for ovine blood, where serum NEFA concentrations increased significantly by 20% over 72 h at 4°C (Morris et al., 2002). Nonesterified fatty acid concentrations were consistently higher in all samples, regardless of anticoagulant, when stored at 24°C. Significant increases were apparent after about 24 h of storage at 24°C in whole blood or anticoagulated plasma. Similar findings were reported previously in human subjects (McGann and Hodson, 1991; Menendez et al., 2001), although the increases were much greater in serum and EDTA plasma (30 to 75%) than that seen in our study (20 to 21%). It is interesting that NEFA concentrations were less stable in heparinized plasma; concentrations increased by 62% within 72 h of storage at 24°C. Similar results have been reported previously for human patients in some studies (Gleeson, 1987). Heparin also produced the largest variation in baseline NEFA concentrations (Figure 1). Therefore, heparin should be avoided as an anticoagulant for collecting blood samples for NEFA testing in dairy cows. The increase in NEFA concentrations at higher storage temperatures has been attributed to spontaneous or enzyme (lipoprotein lipase, lecithinase)-catalyzed hydrolysis of esterified fats (McGann and Hodson, 1991). This process may be accelerated in heparinized samples (Gleeson, 1987), although addition of exogenous heparin to human samples after collection in vitro did not affect NEFA concentrations when measured using the same enzymatic method used in our study (McGann and Hodson, 1991) or gas chromatography (Rogiers, 1978). Heparin does activate lipoprotein lipase; however, this enzyme is attached to capillary endothelium and is only released after intravenous boluses of hepa-

rin (Watson et al., 1995). It must be noted that, although the increases in NEFA concentrations with storage at 24°C were significant, none were of sufficient magnitude to alter result interpretation; that is, the proportion of cows with NEFA concentrations <0.4 mmol/L was the same (10%) at all time points, regardless of storage temperature or anticoagulant. Nonesterified fatty acid concentrations were also stable with frozen storage, as reported previously for human (Gleeson, 1987; McGann and Hodson, 1991; Menendez et al., 2001) and ovine (Morris et al., 2002) blood.

Delayed separation (up to 24 h) of serum or plasma from cells did not affect NEFA concentrations when whole blood was stored at 4°C. Similarly, NEFA concentrations were stable when EDTA- or heparin-anticoagulated samples were stored as whole blood for 24 h at 24°C. In contrast, NEFA concentrations were significantly higher in nonanticoagulated whole blood stored at 24°C for 24 h compared with separated serum. These data are similar to those reported for human patients, in which a 24-h delay in separation increased NEFA concentrations when samples were stored at 24 but not 4°C. These increases were marked (31 to 36%) in heparin- and nonanticoagulated blood samples, but minimal in EDTA-anticoagulated samples (9%) (McGann and Hodson, 1991). These results emphasize the importance of keeping blood cool at all times after sample collection for NEFA testing.

Serum obtained from SST yielded slightly, but significantly, higher NEFA concentrations than serum obtained from nonanticoagulant tubes. The reason for this is unclear; however, it may be an artifact of the silicone gel, because the tubes were handled identically. Serum separator tubes are often favored by large-animal practitioners because they hasten clotting and facilitate serum collection by lay staff. However, although the changes were small (median difference of 0.02 mmol/L) and unlikely to be relevant, we recommend that SST not be used for NEFA evaluation. In our experience, serum in these SST is often not removed from the clot (and placed in a separate tube), because it is assumed

that the silicone barrier prevents cell-associated artifacts in biochemical parameters (e.g., hypoglycemia from glucose consumption by cells) from occurring. However, these artifacts still occur (T. Stokol, personal observations, 2005). Furthermore, if samples left in SST freeze (intentionally or inadvertently during winter), the ensuing hemolysis will cause artifactually high NEFA concentrations, substantially affecting interpretation of results (T. Stokol and D. V. Nydam, unpublished results, 2005; Morris et al., 2002). Therefore, these tubes provide little advantage over regular non-anticoagulant tubes.

In contrast to NEFA, BHBA concentrations were stable under all storage conditions tested. β -Hydroxybutyrate concentrations were similarly stable in sodium fluoride-anticoagulated ovine plasma frozen for 180 d at -20 or -80°C (Morris et al., 2002), and in human blood stored as whole blood (nonanticoagulated or fluoride/oxalate-anticoagulated) or separated plasma or serum for 7 d at 4 or 24°C (Custer et al., 1983). It is noted that the NEFA and BHBA concentrations in our cohort of peripartum cows were not high. It is possible that there would be more variation in the concentrations of these analytes due to anticoagulants or storage in samples with higher baseline values.

CONCLUSIONS

We recommend that blood for NEFA testing in dairy cows be collected into EDTA or nonanticoagulant tubes, separated from cells as soon as possible after collection, and kept cool (4°C) at all times. Samples should reach the laboratory within 72 h (preferably 24 h) of collection to ensure valid results. Serum or plasma samples do not need to be frozen to maintain stable NEFA concentrations, although frozen samples should be stable for at least 1 mo after collection (if stored in a dedicated freezer). Heparin should not be used as an anticoagulant for NEFA testing. Furthermore, we recommend that for metabolic profile testing in postparturient dairy cows, only a serum sample should be collected and handled as above (primarily to maintain NEFA concentrations). These guidelines will be useful to dairy practitioners for obtaining optimal results when investigating dairy cattle herds for problems associated with negative energy balance and subclinical ketosis.

Cornell University recently changed its supplier of the BHBA reagent from Catechem Inc. to Randox Laboratories (Antrim, UK). The enzymatic method for detecting BHBA is the same for both suppliers. In a preliminary trial, we collected blood from 10 periparturient dairy cows into nonanticoagulant tubes and compared serum BHBA concentrations with the 2 reagents. The median BHBA concentrations were stable with both

reagents in separated serum samples maintained at 4 or 24°C for 72 h.

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