

Application of a Pooled Sample Metabolic Profile for Use as a Herd Screening Tool

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Introduction

In the previous presentation application of metabolic profiling in assessing transition cow metabolic status and potential disease risk was detailed. A number of key analytes measured in blood were identified as significant predictors of periparturient disease risk whether measured prepartum or postpartum. A “metabolic profile” is defined as a series of specific analytical tests run in combination and used as a diagnostic aid (Ingraham and Kappel, 1988). Although similar samples and analytical methods are used in assessing disease diagnosis or metabolic profiling, their approaches to sampling and interpretation are different (Van Saun, 1997; Herdt et al., 2001). With disease diagnosis, one selects a small population of representative clinically affected animals for blood analysis. Results are compared to laboratory 95% reference ranges for interpretation. Disease diagnosis is based on a recognized pattern to changes in one or more blood analytes. In contrast for metabolic profiling, one collects samples from clinically “normal” individuals within certain defined physiologic groupings. Ultimately metabolic profiling is being used to evaluate disease risk in contrast to disease diagnosis, though in many situations one often slips into a disease diagnosis framework.

Methods used in sample collection and interpretation will vary by the metabolic profiling procedure used. In contrast to methods of the past, metabolic profiling should be considered just one tool in the nutritional diagnostic toolbox. In the broadest sense of the terminology, a metabolic profile should also include evaluation of herd records, animals, facilities and rations and integrate the information into a final diagnostic assessment (Van Saun and Wustenberg, 1997; Herdt et al., 2001). Results of a metabolic profile should be used to direct or focus attention of a diagnostic process (screening tool) or to help confirm presence of a herd disease process (diagnostic tool). The objective of this presentation will be to review the application and interpretation of a pooled sample metabolic profile procedure for use as a herd screening tool for assessing periparturient disease risk.

Diagnostic Profiling - Individual Sampling

In the traditional approach to metabolic profiling, a representative sampling of individuals were collected and analyses performed. With the CMP, a mean value for individuals was determined and then compared to some defined reference value. The problem with this approach was the amount of incurred costs and lack of test result sensitivity (interpretation problems). Oetzel has advocated that evaluating a proportion of individual samples relative to a given reference criteria may be of greater value in assessing disease risk (Oetzel, 2004). Analytes with a threshold value above or below which is associated with disease risk are best evaluated as a proportion rather than a mean. For example, one could determine rumen pH to diagnose subacute ruminal acidosis (SARA) in a herd. Elevated prefresh nonesterified fatty acids (NEFA) concentration (≥ 0.4 mmol/L) and postfresh β -hydroxybutyrate (BHB) concentration (≥ 1200 - 1400 μ mol/L) are recognized risk factors for

ketosis and left displaced abomasum (Duffield, 2004; Geisheiser et al., 1998, 2001; Oetzel, 2004; LeBlanc et al., 2005; LeBlanc, 2006). Low blood calcium concentration (< 2.0 mmol/L), immediately postcalving, is a risk indicator for subclinical hypocalcemia (Oetzel, 2004). Blood urea nitrogen (BUN) and urine pH have also been advocated as potential indicators for assessing herd protein status and anionic salt responsiveness, respectively (Oetzel, 2003, 2004). These two values can be evaluated by using means of individuals.

Few would argue the strength of individual analysis in metabolic profile analysis. Indeed the gold standard for analytical analysis would be to measure a large percent of the population of interest as individuals. Statisticians suggest at least 8 subsamples from a population is representative (mean analysis), though 12 or 13 samples are best for threshold analysis (Payne et al., 1970; Oetzel, 2004; LeBlanc, 2006). At the same time statisticians also state if we really wish to best characterize a population, then more samples are better. Similarly, we can reduce required sample numbers by reducing the level of confidence needed in our results. As Oetzel (2004) describes, clinical decision making does not require 95% confidence in a conclusion as for research, but 75% confidence in a result may be reasonable. In some ways this approach is a hybrid between using blood metabolite analysis for specific disease diagnosis and metabolic profiling. This is seemingly in contrast to the underlying premise of metabolic profiling, but may better reflect known disease relationships.

A second approach with individual sampling is more similar to the original metabolic profile test. To address concerns with metabolic profile testing costs, the number of determined analytes per sample is reduced. For example, the metabolic profile test offered by Michigan State University includes urea nitrogen, albumin, aspartate aminotransferase (AST), NEFA and BHB (Herdt, TH, personal communication). Discount pricing is offered for submission of multiple tests (seven or more) to help reduce costs. Analytical testing addresses key metabolites reflecting energy balance, liver function and protein status and having documented association with disease risk. Samples can be collected from immediately prefresh and postfresh animal groups, though few will take samples from more than one group (prefresh or fresh) due to costs. Again, test interpretation is on an individual basis relative to defined reference values and proportional risk assessment. This testing procedure provides more diagnostic screening capabilities compared to a single analyte diagnostic procedure. Thus, this approach may help to provide direction to the diagnostic process. However, is there another way to assess herd metabolic status and disease risk and find a balance between economics and valid diagnostics?

Herd Screening - Pooled Samples

A pooled-sample approach was an attempt to address both cost concerns and increased scope of testing in assessment of herd nutritional or disease risk status (Van Saun, 1997). In the individual approach described above, single variables are being used to assess specific disease risks. A pooled-sample approach should be considered more of a herd screening process and not a specific diagnostic test. The approach to this application is to evaluate various aspects of integrated metabolism as well as changes that occur over the transition period. Testing is broad based and includes analytes reflecting energy balance (NEFA, BHB), protein status (BUN, albumin), liver function (various enzymes, cholesterol, and triglycerides) and macromineral homeostasis (Van Saun, 1997). This can also be expanded to include microminerals and vitamins. To measure this range of analytes, one must reduce costs. One approach is to measure analytes in pooled samples and evaluate over a series of predefined physiologic states (Table 1).

In the original protocol for the CMP, mean analyte values within physiologic groupings were used for interpretation. These mean values were arithmetically determined from individual samples. Use of individual sampling resulted in the high associated costs of

Table 1. Suggested physiologic groupings for collecting blood samples in completing metabolic profile testing using individual or pooled samples.

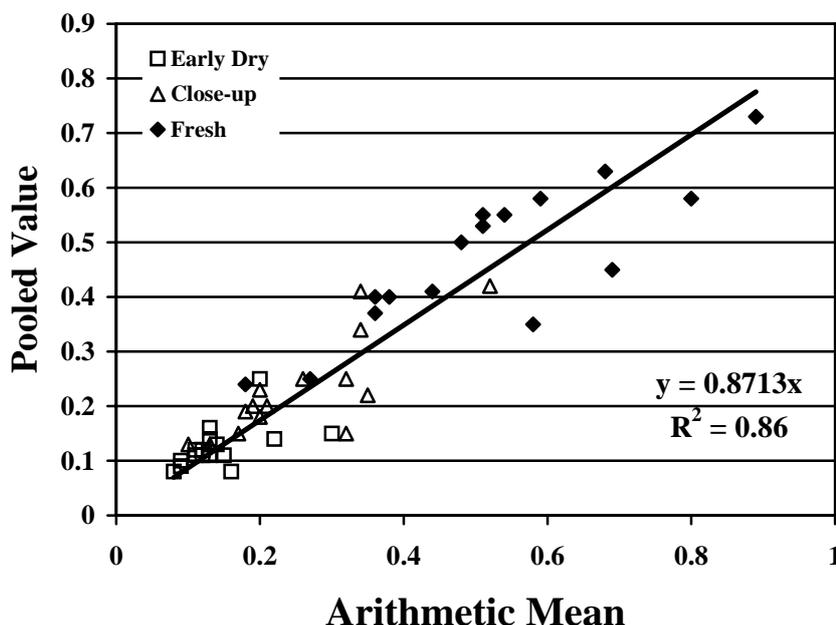
Physiologic Groups	Time relative to Calving	Parity	Disease Status
Far Off Dry	>10 days following dry off and < 30 prior to calving	Keep heifers and 2+ lactation animals separate – pool as separate parity groups within physiologic groups	Unknown
Close-up Dry	Between 3 and 21 days prior to calving (3 to 14 days best)		Unknown
Fresh	3 to 30 days in milk (7 to 21 days best)		Group cows with and without disease within lactational groups – keep days in milk similar within and between groups
Lactation groups	Define as needed based on disease conditions, production level or other problem		

this procedure. Can pooled samples be used to reduce the cost and provide some valid method of herd assessment? There are essentially two issues to be resolved in answering this question. First, can pooled samples accurately reflect the arithmetic mean of the individuals? Second, can one adequately define valid interpretation criteria for pooled sample values? The first question is the easiest to answer.

In a number of preliminary studies, individual samples were used to determine if pooled sample results for any given analyte were different from the arithmetic mean of the individuals within the pool (Lehwenich, 1999; Tornquist and Van Saun, 1999; Van Saun, 2004, 2005, 2006a). Using a variety of statistical methods, we found that pooled samples do accurately represent individual means from pool sizes ranging from 5 to 20. When one uses sample sizes of 25 to 30 we found more statistical differences between arithmetic mean and pooled values, but numerical values were not greatly different. This statistical difference may have been more of a “statistical artifact” due to the larger “n” and smaller error term. In plotting arithmetic mean versus pooled value for various analytes most of the graphs showed a nearly 1-to-1 relationship (Figure 1). These results are consistent with another study looking at a pooled sampling technique (Lehwenich, 1999).

Does this mean pooled values precisely represent the mean of individuals? No, there is some variation, though it depends upon the analyte. Fortunately, key metabolic indicators for transition cows showed less than a 5% difference between arithmetic mean and pooled values (Van Saun, 2004, 2005). In our experience, pooled sample values are always in the same direction relative to the reference population mean, but may vary in magnitude in distance away from the mean. Samples that were not collected and handled under the best conditions (various degrees of hemolysis present) resulted in increasing differences between pooled and mean values. We have also found that pooled samples held at room temperature for a period of time (hours) resulted in analytical problems resulting from sample gelling. Herds that were experiencing more disease problems had more statistical differences between pooled and arithmetic mean values (Tornquist and Van Saun, 1999; Van Saun, 2005). From the preliminary studies, it would seem feasible that analyte analysis of a pooled sample does reasonably represent the arithmetic mean of the sampled individuals.

Figure 1. Relationship between NEFA concentrations in transition dairy cows measured as individuals and averaged for a pool and a pooled sample of the same individuals.



Pooled Profile Procedure

Proper sample selection and pooling technique are keys to success with pooled samples. In selecting samples we need to ensure that true herd or nutritional differences will be borne out and not masked by random or other sources of controllable variation (Herdt, 2000). Samples can be pooled by appropriate physiologic states (Table 1) to allow interpretation of dynamic changes in "population" means over a period of time (Van Saun, 1997). To address a fresh cow problem, pooled samples should be collected from recently dry cows (>10 days following dry-off up to 30 prior to calving), close-up dry cows (3 to 21 days prior to calving) and fresh cows (3 to 21 days in milk). Other appropriate sample pools can be determined given the specific problem to be addressed. For example, if a herd was experiencing increased prevalence of retained fetal membranes (RFM), you could pool both clinical and non-clinical animals within the fresh cow group for comparison. Obviously you would not know which animals go on to have RFM prior to calving. Also within these groupings, first lactation animals are kept separate from second and greater lactation animals. This physiologic grouping strategy is based on findings of significant differences in mean analyte concentrations across these groups in either healthy or sick cows (Van Saun, 2004). Most clinical pathology laboratories do not alter their reference ranges relative to physiologic criteria, thus confounding interpretation of results and limiting usefulness of metabolic profiling.

Sample collection and handling are important if one is to expect useful diagnostic information in return. Blood samples should be taken from either jugular or coccygeal veins with a minimal amount of stress. Blood samples from the mammary veins are not appropriate given the loss of nutrients into the mammary gland. Lower concentrations of phosphorus and potassium have been documented in jugular compared to coccygeal blood samples as a result of salivary gland uptake (Rowlands, 1980; Maas, 1983). Time of sampling relative to feeding and feeding management may also influence metabolite concentrations and should be considered in the decision process of when to sample. If herds are being repeatedly sampled as a monitoring tool, samples should be taken at approximately the same time of day to minimize diurnal and prandial variation.

Vacuum tubes are the most common and easiest form of sample collection. A variety of vacuum tubes are available. Vacuum tubes are colored coded for specific diagnostic test procedures based on the specific anticoagulant or additive present in the tube. Most commonly a single serum (red top, no anticoagulant) or plasma (green top, sodium heparin) sample is collected; however, in some cases a whole blood sample may be desired. Extreme efforts should be taken to prevent hemolysis of the sample. All samples should be properly identified with animal and group identification and date of collection. Other pertinent information for interpretation of the metabolic profile would include: animal age, lactation number, milk production level, milk composition, days in milk, pregnancy status and body condition score.

By pooling samples you are obtaining information from a greater number of animals for much less cost. Rather than the standard 21 samples to calculate 3 group means, you may submit 3 pooled samples, which represent means of 10 to 20 animals each. For data from pooled samples to be relevant, all cows should be equally represented. In preparing pooled samples one must be meticulous in precisely measuring equal amounts of serum from each individual to be included in the pooled sample. Depending upon the total number to be included, typically between 100 and 500 μl (0.1 to 0.5 ml) from each individual are mixed into a new clean test tube (7 to 10 ml capacity). This process is best completed with use of a micropipetter or a TB syringe for precision. Pooled samples should be adequately mixed then directly submitted to the laboratory or frozen and shipped.

Interpretation of Pooled Samples

The real challenge for metabolic profiling is defining appropriate and sensitive reference values. This is even more of a concern in interpreting mean samples, especially pooled values. This has been the stumbling block for adoption of this procedure. Some have advocated that mean values are totally worthless diagnostically (LeBlanc, 2006). One must recognize that this process, however well refined, will never be as sensitive as individual animal analysis. Interpretation criteria for pooled samples are still in the process of development and refinement, but basic concepts will be discussed.

For individual animals, metabolite values are compared to standard, laboratory-dependent reference values. These reference values generally represent a 95% confidence interval. This means that 95% of normal animals should have a given metabolite concentration within this range. This also suggests that 5% of the population will be outside of this reference range and still be normal, emphasizing the need to clinically evaluate the animal. A number of factors, most notably physiologic state and age, have been shown to influence blood metabolite concentrations. Most reference ranges do not account for these differences and thus may confound direct interpretation. Having a thorough understanding of the physiologic regulation of a given nutrient is crucial to interpretation.

In contrast to individual animal samples, pooled mean metabolite values can not be directly compared to reference ranges in the same way. When interpreting pooled samples one needs to remember that measured value represents a population with individuals above and below the mean. As a general rule, means of pooled samples should be near the midpoint of the reference range to be considered normal. For example, if serum total calcium (Ca) concentration for fresh cows is 2.25 mmol/L and the reference range is between 2.25 and 3.0 mmol/L, this might be interpreted to suggest a potential problem with subclinical hypocalcemia whereas it would be considered normal in an individual. The measured mean (2.25 mmol/L) represents a population with approximately 50% of the individual values above and below, assuming a normal distribution to the population. This suggests that a number of individuals would have serum Ca concentrations below the normal range. Of course interpretation of metabolic profile results has to be considered in light of presenting

problems in the herd. If the herd is experiencing clinical signs consistent with subclinical hypocalcemia, e.g., slow increase in feed intake and milk production, displaced abomasum and ketosis problems, this would be supportive evidence of the metabolic profile results.

Without population variance determinations in pooled samples, you cannot really determine how significant mean differences are. Yet, with many metabolites, like BUN, calcium, magnesium or glucose, you can eliminate the possibility that a single sample was sufficiently low or high to skew the mean. For low BUN values, it is difficult to have values approaching zero whereas for other metabolites, if the sampled cow had an extremely skewed value, it would have been exhibiting clinical signs and would not have been sampled. Metabolites with high variability (wide range of values; liver enzymes) will be of less diagnostic value as compared to low variability metabolites (minerals).

In following the original concept promoted in interpreting CMP samples, pooled samples can be compared to an expected healthy population mean (or median) and the associated population variance (Van Saun, 2005, 2006b). Population statistics were determined for various blood analytes in dairy cows having no evidence of periparturient disease at different defined periods relative to calving (far off dry, close-up dry and fresh) (Van Saun, 2004). The number of standard deviations away from the healthy population mean was determined for a number of pooled samples, using both arithmetic means and pooled values (Table 2). The percent of abnormal values within a pool was determined using individual samples, compared to standard reference values for respective analytes. A linear relationship, though specific for each analyte, was found between percent abnormal values within a pool and number of standard deviations different from the mean resulting (Figure 2). In general, if a pooled sample value was less than 0.25 (range 0.1-0.5) standard deviations from the healthy population mean, then < 10% of the individuals had abnormal values. Analyte-specific criteria can then be developed to estimate number of abnormal values with a pooled sample. This analysis is consistent with previous work on defining criteria for mean values and what many laboratories are using for metabolic profile evaluation criteria. Further work to establish specific pooled sample criteria is ongoing.

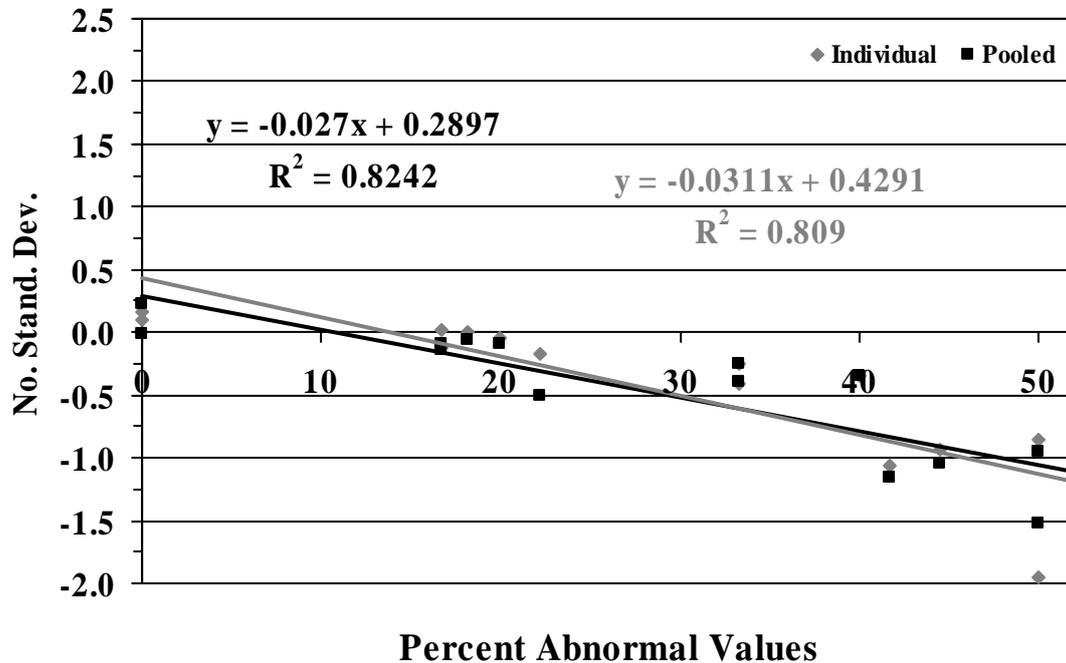
Table 2. Preliminary reference criteria for pooled samples of selected analytes based on various sigma levels around the healthy population median.

Analyte	Slope ¹	Sigma Level Range ²				Units
		0.25	0.5	0.75	1	
Albumin	0.233	34.1-35.9	33.2-36.8	32.3-37.7	31.4-38.6	g/l
BHB	0.311	0.6-0.9	0.46-1.0	0.31-1.2	0.16-1.3	mmol/l
BUN	-0.203	9.9-11.6	9.0-12.4	8.1-13.3	7.3-14.1	mmol/l
Calcium	0.50	2.3-2.4	2.2-2.5	2.1-2.54	2.01-2.6	mmol/l
Glucose	0.105	2.7-3.1	2.5-3.3	2.2-3.5	2.0-3.8	mmol/l
NEFA	0.257	285-435	209-511	134-586	58-662	µmol/l

¹Slope value was obtained from linear models regressing number of SD per 10% of abnormal values within a sampled population.

²Healthy population median ± sigma times the standard deviation for a given analyte. Light and darker shaded cells represent >20% and >40% abnormal values, respectively, within a pooled sample if the sample value is outside previous range and within highlighted ranges.

Figure 2. Relationship between percent abnormal values and number of standard deviations the sample value (mean of individuals or pooled sample) deviates from healthy population median β -hydroxybutyrate (BHB) concentrations in sampled fresh cows.



A second method to use and interpret pooled samples is by a modification of statistical process control. In larger herds, where sampling can be done more repetitively, one could collect samples on a monthly or bimonthly basis and plot the pooled sample results over time. Standard deviations from a healthy population as described above could be used to initially generate various sigma levels for evaluation. One can then graphically monitor metabolic status using one or more key analytes over time to assess potential risk for periparturient disease.

Conclusions

Though some variation may be masked, pooled sampling may be used as an economic approach to a herd metabolic status screening tool. Most important measures of metabolic status (Albumin, BUN, NEFA, BHB) showed minimal differences between pooled and individual samples. Pooled samples are statistically equivalent to the mean of pooled individuals. Observed effect of herd on sample differences may suggest poor sample handling or processing practices. The real challenge to using pooled samples is interpretation. Empirically one can interpret pooled samples by determining how far they deviate from the midpoint of the reference range for healthy individuals. A new approach to pooled samples was described and requires further refinement and validation, but is a reasonable starting point. Use of statistical process control (SPC) may be a reasonable approach to using pooled samples for on-going herd monitoring, especially for larger herds that are more likely to complete repetitive sampling. Pooled samples may provide an economic alternative to traditional metabolic profiling.

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