Influence of the para-aminohippuric acid analysis method on the net hepatic flux of nutrients in lactating cows

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ABSTRACT: Para-aminohippuric acid (pAH) is a marker frequently used to measure plasma or blood flow. In sheep studies, it is recognized that its determination must include a deacetylation step to counteract the hepatic acetylation of pAH. Such a procedure is not of common usage in cattle studies although a recent suggestion of hepatic pAH acetylation in dairy cows may have important consequences for hepatic nutrient fluxes. The aims of this study were to evaluate pAH analytical methods according to international procedures of metrology and confirm hepatic acetylation of pAH in dairy cows. The effect of the matrix used to prepare the standard curve was tested, and the influence of the pAH analytical method on blood flows and subsequent net hepatic fluxes of nutrients was determined. For the first objective, accuracy profiles were established and bias, indicators of precision, and limits of quantification were reported for 2 analytical methods (without and with a pAH deacetylation step) using 2 different standard matrices (water and plasma). Second, the net hepatic flux of different nutrients was determined including or not the deacetylation step and preparing the standard curves in plasma using samples collected from 5 multicatheterized lactating Jersey cows. The choice of the matrix had a significant impact on plasma pAH concentrations as illustrated by accuracy profiles. Water matrix decreased ($P < 0.01$) the slope, $y$-intercept, and the absorbance at concentration 0 mg pAH/L of the standard curve in both methods (without and with the deacetylation), supporting that standards prepared in water should not be used to analyze plasma samples. Samples collected on cows confirmed hepatic acetylation of pAH across the liver. Deacetylation, performed using plasma as the standard matrix, increased ($P < 0.05$) plasma pAH concentrations from 18.4, 26, and 23.5 to 21.4, 28.9, and 27.3 mg/L in the artery, portal vein, and hepatic vein, respectively. Deacetylation decreased the hepatic venous and arterial plasma and blood flows ($P < 0.05$) by 9 and 55%, respectively, modifying the net hepatic flux of acetate, total amino acid, and oxygen by more than 19% ($P < 0.05$). In conclusion, our results highlight the importance of including a deacetylation step in the pAH analysis method in cattle studies and of using plasma as standard matrix.

Key words: accuracy, blood flow, cattle, liver, para-aminohippuric acid

INTRODUCTION

The net hepatic metabolism of a nutrient is estimated by multiplying its concentration difference between the efferent (hepatic vein [HV]) and afferent (portal vein [PV] and artery) blood by the hepatic blood flow, usually determined by dilution of para-aminohippuric acid (pAH; e.g., Katz and Bergman, 1969). Accuracy in all measurements is crucial to obtain quantitatively reliable results. The cost of pAH makes splanchnic blood (plasma) flow quantification in cows extremely expensive. Different pAH analytical methods as well as standard matrix (Matuszewski et al., 2003) may impact both the accuracy of results and infusion rate. Hepatic acetylation of pAH has been reported in sheep (Katz and Bergman, 1969) and recently in cattle (Kristensen et al., 2009), resulting in underestimation of pAH concentrations and overestimations of the hepatic arterial and venous blood flows if no deacetylation step was introduced in the analysis. Inaccuracies in the estimation of hepatic arterial blood flow were suggested to bias net hepatic flux of metabolites (Kristensen et al., 2009).
Para-aminohippuric acid analysis on hepatic fluxes

The first objective of this work was to evaluate the analytical method of Hamburger et al. (1948) modified by Isserty et al. (1998). This method allows increasing the absorbance measured by spectrophotometry by making a more concentrated extract from plasma than previously used in sheep (Katz and Bergman, 1969) and cattle (Seal et al., 1992). Evaluation included tests for the matrix effect and the inclusion of a pAH deacetylation step. International metrology procedures (International Organization for Standardization [ISO] 5725-1 [ISO, 1994] and Joint Committee for Guides in Metrology [JCGM] 200:2012 [JCGM, 2012]) were applied. The limits of the pAH analytical procedure were then characterized without and with the inclusion of a deacetylation step and using plasma as standard matrix. Consequences of those limits on pAH infusion rate were calculated. The second objective was to confirm that pAH acetylation occurs in cattle liver and to test its impact on a wider range of metabolites.

MATERIALS AND METHODS

The experiment was conducted in respect of the national legislation on animal care (Certificate of Authorization to Experiment on Living Animals, number 004495, Ministry of Agriculture, France).

**Procedure for Para-aminohippuric Acid Quantification**

Para-aminohippuric acid was quantified by a modified version of the method described by Hamburger et al. (1948). Plasma samples (500 μL) were deproteinized with a 1:1 dilution of trichloroacetic acid (20% wt/vol), and Milli-Q water (Millipore Corporation, Billerica, MA) was added to a final volume of 2 mL. After agitation, samples were centrifuged (13,100 × g) at 15°C for 30 min. A portion of the supernatant was removed (1 mL) and 5725-1 [ISO, 1994] and Joint Committee for Guides in Metrology (JCGM) 200:2012 (JCGM, 2012)) were applied. The limits of the pAH analytical procedure were then characterized without and with the inclusion of a deacetylation step and using plasma as standard matrix. Consequences of those limits on pAH infusion rate were calculated. The second objective was to confirm that pAH acetylation occurs in cattle liver and to test its impact on a wider range of metabolites.

**Evaluation of the Para-aminohippuric Acid Analysis Method**

The pAH analytical method was evaluated under 4 different conditions: 2 standard matrices (water and plasma) and 2 methods (without and with deacetylation). For each condition, the standard curve was analyzed 3 times a day (repetitions) over 5 d (series) by the same operator. The influence of the origin of the plasma used as matrix of the standard curve was also studied. For this purpose, separate standard curves were prepared in pAH-free PV, HV, and artery plasma as described above at 5 concentrations (0, 10, 20, 30, and 40 mg pAH/L). Each standard curve for each plasma matrix was repeated 2 times a day over 3 d for both methods (without and with the deacetylation).

**Determination of Net Hepatic Fluxes of Nutrients**

Five multiparous lactating Jersey cows (371 ± 31 kg of BW) were surgically fitted with permanent catheters at the splanchnic level as described by Ortigues et al. (1994) in sheep. Cows were then successively fed each of 4 isoenergetic diets differing in the source of carbohydrate (fiber vs. starch) and the protein to energy ratio (normal vs. low) as described by Cantalapiedra-Hijar et al. (2014). After an adaptation period to each diet of 27 d, an infusion of pAH (7.27 g/h) through both ruminal
and mesenteric veins was started 45 min before hourly blood sampling over 5 h (n = 5). Blood from PV, HV, and the mesenteric artery were simultaneously taken (9 mL, Monovette lithium heparin; Sarstedt SARL, Marney, France) to determine the packed cell volume and concentrations of oxygen (hemoximeter ABL 5; Radiometer, Copenhagen, Denmark), urea (KitS-1000; bioMérieux, Marcy l’Etoile, France), glucose, lactate, and VFA (Majdoub et al., 2003). Plasma was separated and analyzed for pAH, ammonia (Bergmeyer and Beutler, 1985), and total amino acids (Savary-Auzeloux et al., 2010) using the mean values of each nutrient concentration obtained per animal and diet. Therefore, its quantitative evaluation depends in a critical manner on stipulated conditions: within laboratory and within series (repeatability, thereafter called “within-series”) or within laboratory and between series (intermediate precision, thereafter called “between-series”) or between laboratories (reproducibility).

Between-series precision expresses the precision under conditions where the results are obtained by the same analytical procedure on identical samples in the same laboratory, by the same operator, using the same equipment, and during a short interval of time (adapted from Hubert et al., 2003). The between-series SD (SDbetween) is

\[
SD_{\text{between}} = \left\{ SD_{\text{within}}^2 + \left( \frac{\sum_{i=1}^{I} K (\overline{\mu} - \overline{\mu_j})^2}{k} - SD_{\text{within}}^2 \right) \right\}^{1/2}
\]

in which \( \overline{\mu} \) represents the arithmetic mean of all measurements in the i series, k the repetition index, \( \overline{\mu_j} \) the arithmetic mean of all measurements obtained in a j concentration level, and SDwithin the within-series SD:

\[
SD_{\text{within}} = \sqrt{\frac{\sum_{i=1}^{I} \sum_{k=1}^{K} (\mu_{ik} - \overline{\mu})^2}{i-1}}
\]

in which \( \mu \) represents the measured value at the k repetition and i series.

The measurement uncertainty is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand (JCGM 100:2008 [JCGM, 2008]). It was determined from between-series SD at each concentration level as described by Thompson (2011).

Accuracy profiles were built as described by the NF V03-110 (AFNOR, 2010) standard, assuming a linear model. This procedure is a graphic tool based on the notion of total error (bias + precision) to evaluate and decide if an analytical method correctly “fits its purpose” (Hubert et al., 2003; Feinberg, 2006; Rozet et al., 2007). It integrates 2 fundamental concepts of the ISO 17025 standard
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(ISO, 2005), such as the validation of the methods and the estimation of the uncertainty of measurements (Feinberg and Laurentie, 2006). The accuracy profile graphically represents the bias, the acceptance limit, and the coverage interval at different concentrations, allowing the rapid identification of the limit of quantification (LOQ) of an analytical procedure.

The acceptance limit (±λ) represents the accepted error around the reference value. By convention, it is usually considered at 1 or 2% on raw materials, 5% on pharmaceutical specialties, and 15% in bioanalysis, environment, etc. (Hubert et al., 2003). The acceptance limit may be defined by the operator depending on his requirements. In the present work, acceptance limits were defined a posteriori based on the results.

The coverage interval contains the set of true quantity values of a measurand with a stated probability, based on the information available (JCGM 200:2012 [JCGM, 2012]). For the purpose of this work the statistical tolerance interval or prediction interval is used as coverage interval. It is calculated as follows,

\[
\text{Statistical tolerance interval} = \overline{\text{\bar{y}}} \pm \text{Ktol} \times \text{SD}_{\text{between}}^{X},
\]

with Ktol = \text{t}_{(1-\pi),v} representing the coverage factor of the statistical tolerance interval, which represents the quantile of the Student t distribution with v degrees of freedom, \pi is the expected probability for the content of the tolerance interval (set at 95% in this work), \overline{\text{\bar{y}}} the arithmetic mean of all measurements obtained in a j concentration level, and X the accepted reference value for a j concentration level.

Finally, the LOQ represents the lowest amount of the targeted substance in a sample, which can be quantitatively determined under the experimental conditions prescribed with a well-defined accuracy. The LOQ is calculated as the upper intersection between 2 lines, representing the acceptance limit (a) and the coverage interval (t). The LOQ is calculated using the y-intercept values (\text{a}_0 and \text{t}_0) and the slope of the equation of both lines (Fig. 1).

**Statistical Analysis**

Matrix effects were first analyzed. Parameters of the standard curves prepared using water or plasma as matrix (the absorbance at the concentration 0 mg pAH/L as well as slopes of y-absorbance against x-concentration and y-intercepts) were tested by ANOVA (GLM procedure) in both pAH analysis methods (without and with the deacetylation) following the model \( Y_{ijk} = \mu + \alpha_i + \beta_j + e_{ijp} \) in which the main sources of variation were \mu, the overall mean, \alpha_i, the between-series effect, \beta_j, the matrix effect, and \( e_{ijp} \) the residual effects. The effect of the origin of the plasma matrix (A, PV, and/or HV) on these parameters was independently studied for each pAH method by replacing the matrix effect on the previous model. As preliminary analyses did not show different results between animals, animal was not included in the model.
Table 1. Influence of matrix (water or plasma) and of the origin of the plasma matrix (artery [A] and portal [PV] and hepatic vein [HV]) on the absorbance at concentration 0 mg para-aminomhippuric acid (pAH)/L (Abs. 0), y-intercept, and slope of the standard curve, without the inclusion of the pAH deacetylation step

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Water</th>
<th>Plasma</th>
<th>SEM^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abs. 0, nm</td>
<td>0.003^2</td>
<td>0.028^d</td>
<td>0.0024</td>
</tr>
<tr>
<td>y-intercept, nm</td>
<td>0.013^2</td>
<td>0.038^d</td>
<td>0.0023</td>
</tr>
<tr>
<td>Slope</td>
<td>0.035^5</td>
<td>0.041^d</td>
<td>0.0005</td>
</tr>
<tr>
<td>Plasma origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEM^1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.023^a</td>
<td>0.028^b</td>
<td>0.032^a</td>
</tr>
<tr>
<td>PV</td>
<td>0.025^a</td>
<td>0.028^b</td>
<td>0.030^d</td>
</tr>
<tr>
<td>HV</td>
<td>0.041^a</td>
<td>0.041^a</td>
<td>0.042^a</td>
</tr>
</tbody>
</table>

^a,bWithin a row, means without a common superscript differ (P < 0.05).
^c,dWithin a row, means without a common superscript differ (P < 0.01).
^1SEM within samples was calculated as (residual variance/n observations per group)^1/2, with n = 15.

The influence of the method on plasma pAH concentrations, blood and plasma flows, and net hepatic fluxes of nutrients was analyzed using the paired t test for a standard curve prepared in plasma matrix. Standard errors of the differences were reported. Day of sampling in each animal was considered as the experimental unit. Diet was not considered in the final statistical model because none of the diet × method interactions were significant (P > 0.05). All statistical analyses were performed using the SAS statistical package (SAS Inst. Inc., Cary, NC).

RESULTS

Evaluation of Methods

Matrix effects on the standard curves are reported in Tables 1 and 2. When plasma was used, all parameters (the absorbance at the concentration 0 mg pAH/L, y-intercepts, and slope) increased in comparison to water (P < 0.01) for both methods without and with deacetylation. The higher measured absorbance at 0 mg pAH/L with plasma was compatible with the higher calculated y-intercept (P < 0.01). The accuracy profiles were first established considering the theoretical pAH concentrations in water as the reference values (Fig. 2a, 2b, 2c, and 2d). For standards prepared in water matrix and above 5 mg pAH/L, the bias was less than ±2% without and with deacetylation (Fig. 2a and 2b). By contrast, for standards prepared in plasma matrix and above 5 mg pAH/L, the bias was higher than 10% without deacetylation and in between 2 and 10% with deacetylation (Fig. 2c and 2d). When considering as reference values the theoretical pAH concentrations in plasma, bias was reduced below 2% when analyzing standards prepared in plasma (Fig. 2e and 2f). In all cases, the coverage interval was lower without deacetylation. The origin (PV, HV, and/or artery) of the plasma matrix had no influence on the parameters of the standard curve (P > 0.05), without (Table 1) or with (Table 2) the deacetylation.

On those bases, comparisons of methods were subsequently limited using plasma as the standard matrix. The numerical results of method (without and with pAH deacetylation) evaluation are shown in Table 3 for the range of concentrations measured in cows. Bias was less than ±1.6% for pAH concentration levels ranging from 15 to 30 mg/L. Regarding the indicators of precision for the nondeacetylation method, the average within- and between-series SD were 0.18 (extremes from 0.09 to 0.24) and 0.18 (from 0.09 to 0.28), respectively. On the other hand, for the deacetylation method, the average within-series SD was 0.13 (from 0.04 to 0.22) while the average between-series SD was 0.29 (from 0.10 to 0.56).

Based on bias and indicators of precision, the coverage interval was established at 95% for all treatments. The measurement uncertainty for an average pAH concentration of 25 mg/L was numerically higher when deacetylation was included in the analysis (Table 3). On these bases, the LOQ were higher with the deacetylation when the limits of acceptance were set at 5 or 10%. For the method with deacetylation, the LOQ amounted to 28 or 15.3 mg pAH/L for an acceptance limit set at 5 or 10%, respectively.

Net Hepatic Fluxes of Nutrients

The inclusion of the deacetylation step allowed recovery of 85% of the acetylated pAH. The effect of analytical method using plasma as standard matrix for plasma pAH concentrations, plasma and blood flows, and net hepatic nutrient fluxes is summarized in Table 4. All pAH concentrations were within the validated range.
The deacetylation step increased pAH concentrations detectable by the Bratton and Marshall reaction in the plasma of all vessels, from 18.4, 26, and 23.5 to 21.4, 28.9, and 27.3 mg/L in the MA, PV, and HV, respectively ($P < 0.05$). Consequently, plasma or blood flows were 9 and 55% lower after deacetylation ($P < 0.05$) for hepatic venous and arterial plasma or blood flows, respectively. Had plasma or blood flows been calculated using standard curves with water, incorrect results would have been obtained.

The net hepatic flux of nutrients was modified by the inclusion of the pAH deacetylation step ($P < 0.05$), except for the flux of urea and lactate ($P > 0.05$). The percentage change varied with the nutrient. Deacetylation modified the net hepatic flux of acetate, total amino acid, and oxygen by more than 19% while the flux of the other nutrients was modified between 2 and 19%.

**DISCUSSION**

The international procedures of metrology are a useful tool to characterize the strengths and limits of the analytical methods used in each laboratory (Rozet et al., 2007; Marlet and Lognay, 2010) with multiple applications, such as the reduction in the number of analyses (Lemosquet et al., 2004). As pointed out by Feinberg and Laurentie (2006), the accuracy profile is able to account for heterogeneity of variances as a function of concentra-
Table 3. Bias (%), indicators of precision (intra- and between-series SD), uncertainty, and limit of quantification (LOQ) for the 2 methods (without [ND] or with para-aminohippuric acid (pAH) deacetylation [D]) using plasma as standard matrix and considering the theoretical concentrations of pAH as the reference values.

<table>
<thead>
<tr>
<th>Concentration level, mg pAH/L</th>
<th>ND</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bias</td>
<td>0.61</td>
<td>1.26</td>
</tr>
<tr>
<td>Within-series SD</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>Between-series SD</td>
<td>0.24</td>
<td>0.47</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bias</td>
<td>0.71</td>
<td>1.55</td>
</tr>
<tr>
<td>Within-series SD</td>
<td>0.16</td>
<td>0.19</td>
</tr>
<tr>
<td>Between-series SD</td>
<td>0.17</td>
<td>0.28</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bias</td>
<td>–0.02</td>
<td>–1.08</td>
</tr>
<tr>
<td>Within-series SD</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>Between-series SD</td>
<td>0.18</td>
<td>0.56</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bias</td>
<td>–0.47</td>
<td>–0.32</td>
</tr>
<tr>
<td>Within-series SD</td>
<td>0.21</td>
<td>0.12</td>
</tr>
<tr>
<td>Between-series SD</td>
<td>0.21</td>
<td>0.39</td>
</tr>
<tr>
<td>LOQ, mg pAH/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOQ at 5% of Accept. L.</td>
<td>7.0</td>
<td>28.6</td>
</tr>
<tr>
<td>LOQ at 10% of Accept. L.</td>
<td>2.5</td>
<td>15.3</td>
</tr>
<tr>
<td>LOQ at 15% of Accept. L.</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Uncertainty at 25 mg pAH/L</td>
<td>0.20</td>
<td>0.38</td>
</tr>
</tbody>
</table>

1 Accept. L. = acceptance limit.

Table 4. Influence of method (without [ND] or with deacetylation [D]) on the plasma para-aminohippuric acid (pAH) concentrations in the artery (A) and portal (PV) and hepatic vein (HV), plasma and blood flows in the hepatic artery (HA), PV, and HP, and the net hepatic flux of nutrients, using plasma as matrix for the standard curve.

| Plasma pAH concentration, mg/L | ND | D | SEM
|-------------------------------|----|----|----|
| A                             | 18.4<sup>a</sup> | 21.4<sup>b</sup> | 0.62
| PV                            | 26.0<sup>a</sup> | 28.9<sup>b</sup> | 0.70
| HV                            | 23.5<sup>a</sup> | 27.3<sup>b</sup> | 0.58

| Plasma flow, L/min | ND | D | SEM
|-------------------|----|----|----|
| HA                | 4.3<sup>a</sup> | 1.9<sup>b</sup> | 0.28
| PV                | 16.7<sup>a</sup> | 17.2<sup>b</sup> | 0.45
| HV                | 21.0<sup>a</sup> | 19.1<sup>b</sup> | 0.48

| Blood flow, L/min | ND | D | SEM
|-------------------|----|----|----|
| HA                | 5.7<sup>a</sup> | 2.6<sup>b</sup> | 0.36
| PV                | 22.1<sup>a</sup> | 22.8<sup>b</sup> | 0.50
| HV                | 27.9<sup>a</sup> | 25.4<sup>b</sup> | 0.57

| Net hepatic flux, mmol/min | ND | D | SEM
|---------------------------|----|----|----|
| Blood glucose             | 7.5<sup>a</sup> | 6.8<sup>b</sup> | 0.23
| Blood acetate             | 3.0<sup>a</sup> | 0.2<sup>b</sup> | 0.81
| Blood propionate          | –7.7<sup>a</sup> | –7.9<sup>b</sup> | 0.24
| Blood butyrate            | –1.8<sup>a</sup> | –1.9<sup>b</sup> | 0.10
| Blood urea                | 2.5<sup>a</sup> | 2.6<sup>b</sup> | 0.61
| Blood lactate             | –2.6<sup>a</sup> | –2.6<sup>b</sup> | 0.15
| Blood oxygen              | –30.8<sup>a</sup> | –25.0<sup>b</sup> | 3.47
| Plasma ammonia            | –4.6<sup>a</sup> | –4.7<sup>b</sup> | 0.23
| Plasma total AA           | –2.3<sup>a</sup> | –2.9<sup>b</sup> | 0.37

<sup>a,b</sup>Within a row, means without a common superscript differ (P < 0.05).

<sup>1</sup>SEM within samples was calculated as (residual variance/n observations per group)<sup>1/2</sup>, with n = 15.
been necessary either to improve the analytical methods to decrease the LOQ or to increase the pAH infusion rate in cows (and the associated cost) to produce concentrations in samples above the LOQ.

Second, acetylation of pAH in cattle liver is thus confirmed by our results, as reported previously by Kristensen et al. (2009). After deacetylation, which was almost complete (85% recovery measured) with an acetylated pAH control as in Isserty et al. (1998), and using plasma as matrix, plasma pAH concentrations significantly increased by 11 to 16%. Correcting for loss of the pAH marker by including a deacetylation step in the analytical method reduced HA plasma or blood flow by 55%, on average. Reductions by 60 and 51% had been reported in sheep (Lobley et al., 1996; Isserty et al., 1998) and lactating Danish Holstein cows (Kristensen et al., 2009), respectively. This reduction was a consequence of a 9% lower HV plasma or blood flow after deacetylation, in agreement with the aforementioned studies (13%, Isserty et al., 1998; 16%, Kristensen et al., 2009). The arterial contribution to total hepatic plasma or blood flow was therefore halved (from 20 to 10%) resulting in a similar contribution to that measured using radionuclide-labeled plastic microspheres in sheep (Apatu and Barnes, 1991) or electromagnetic blood flow probes in calves (Durand et al., 1988). These data highlight the importance of including a pAH deacetylation step to obtain accurate hepatic blood flows in cattle as already proposed in sheep.

Based on deacetylation and use of plasma as the standard matrix, a difference of 2.6 L/min was then detected between PV and HV blood flow. Under these conditions, the measurement uncertainty at 25 mg pAH/L represents 14.6% of the blood flow difference. Of course, this evaluation does not account for the other sources, which also influence accuracy of blood flow measurements, such as the correct location of the catheter tip or inadequate pAH mixing in the blood (Isserty and Ortigues, 1994).

Consequently, the calculated net hepatic flux of nutrients depended on the method used. Deacetylating pAH and using plasma as standard matrix changed some nutrient fluxes by more than 19%. In the case of the acetate, this change was more than 90%. No significant correlations between these changes and the blood or plasma nutrient concentrations or the arteriovenous concentration differences were detected.

In conclusion, metrology procedures can be applied in each laboratory as a tool to evaluate and/or optimize the experimental conditions. It is strongly advisable to evaluate analytical methods according to these procedures before any experiment to clearly quantify the strengths and the limits of each method. Second, it is necessary to include a pAH deacetylation step for blood and plasma flow determination in lactating dairy cows. Third, it is highly recommended to take extra blood samples during sample collection to prepare the standards in the same matrix as the samples. Finally, if the present pAH analytical method is applied, a pAH infusion rate greater than 7 g/h is recommended to provide minimal plasma pAH concentrations of 15 mg/L.

**LITERATURE CITED**


