Simultaneous quantification of purine and pyrimidine bases, nucleosides and their degradation products in bovine blood plasma by high performance liquid chromatography tandem mass spectrometry

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Abstract

Improved nitrogen utilization in cattle is important in order to secure a sustainable cattle production. As purines and pyrimidines (PP) constitute an appreciable part of rumen nitrogen, an improved understanding of the absorption and intermediary metabolism of PP is essential. The present work describes the development and validation of a sensitive and specific method for simultaneous determination of 20 purines (adenine, guanine, guanosine, inosine, 2′-deoxyguanosine, 2′-deoxyinosine, xanthine, hypoxanthine), pyrimidines (cytosine, thymine, uracil, cytidine, uridine, thymidine, 2′-deoxyuridine), and their degradation products (uric acid, allantoin, β-alanine, β-ureidopropionic acid, β-aminoisobutyric acid) in blood plasma of dairy cows. The high performance liquid chromatography-based technique coupled to electrospray ionization tandem mass spectrometry (LC-MS/MS) was combined with individual matrix-matched calibration standards and stable isotopically-labelled reference compounds. The quantitative analysis was preceded by a novel pre-treatment procedure consisting of ethanol precipitation, filtration, evaporation and reconstitution. Parameters for separation and detection during the LC-MS/MS analysis were investigated. It was confirmed that using a log-calibration model rather than a linear calibration model resulted in lower CV% and a lack of fit test demonstrated a satisfying linear regression. The method covers concentration ranges for each metabolite according to that in actual samples e.g. guanine: 0.10-5.0 µmol/L, and allantoin: 120-500 µmol/L. The CV% for the chosen quantification ranges were below 25%. The method has good repeatability (CV% ≤25%) and intermediate precision (CV% ≤25%) and excellent recoveries (91-107%). All metabolites demonstrated good long-term stability and good stability within-runs (CV%≤10%). Different degrees of absolute matrix effects were observed in plasma, urine and milk. The determination of relative matrix effects revealed that the method was suitable for almost all examined PP metabolites in plasma drawn from
an artery and the portal hepatic, hepatic and gastroplenic veins and, with a few exceptions, also for
other species such as chicken, pig, mink, human and rat.

Key words: Nitrogen; Ruminant; Purine; Pyrimidine; Plasma, LC-MS/MS.

1. Introduction

The global efficiency of nitrogen in animal production is only slightly over 10%, with the result that
102 Tg (10^{12} gram) nitrogen is excreted annually (1998 figures) by domesticated animals globally
[1]. The nitrogen efficiency in dairy cows is generally low [2], and not only the environment, but
also the productive efficiency, would benefit from an optimization of diet and metabolism to
improve nitrogen efficiency and utilization [1, 3, 4]. Most research hitherto has focused on refining
protein and amino acid utilization, but this has only led to minor improvements in efficiency [4-6].
A better understanding of the quantitative absorption and intermediary metabolism of other
nitrogenous products such as the purines and pyrimidines (PP), the building blocks of nucleic acids
and main constituents of DNA/RNA, could uncover new ways of improving dairy cow nitrogen
use-efficiency and propose new feeding strategies [7, 8]. So far, the possible significance of
microbial PP in the nutritional physiology of ruminants has not been investigated, regardless of the
fact that they correspond to more than 20% of the total microbial nitrogen supply [7-9]. Little is
known about the quantitative aspects of PP metabolism. What is known, however, is that the
purines go through an effective multistep degradation to uric acid and allantoin, and the pyrimidines
are similarly degraded to β-alanine, before excretion [8, 10].
Quantitative analysis of PP in dairy cattle research has almost solely focused on purines in urine, as
excretion of purine derivatives can be used as an indirect measure of rumen microbial synthesis [11-
14]. Most published methods have thus been developed for purine metabolites in urine. Only
recently, Boudra et al. (2012) published a method able to quantify the pyrimidine degradation
products (DP) β-alanine and β-aminoisobutyric acid as well [14].
Different analytical separation methods have been used for determining PP in biological matrices of which the majority has applied high performance liquid chromatography (HPLC) [15-17] or capillary electrophoresis chromatography [17-20]. When high separation selectivity and sensitivity were essential, electrokinetic techniques [16] or ultra high performance liquid chromatography [21] have been used. Concerning detection, spectrometric, electrochemical or mass spectrophotometric detection methods have been used, with ultra violet detection coupled to HPLC being the most common one [15-17]. HPLC coupled with tandem spectrometric detection (LC-MS/MS) is currently considered the method of choice for quantitative analysis of compounds in biological matrices [22] and LC-MS/MS has been shown to be capable of quantifying PP and their derivatives accurately in urine.

For this study, we wanted to develop and validate an LC-MS/MS method for quantification of a range of PP and their derivatives in cow blood plasma. Into this procedure, we wanted to incorporate matrix-matched calibration standards as well as stable isotopically-labelled reference compounds (SIL). As no appropriate pre-treatment procedure was identified in the literature, we also wanted to develop a good, stable, simple, component-specific, and repeatable pre-treatment protocol for the plasma samples.

Several sets of plasma samples from experiments that attempted to manipulate urea-recycling and increase nitrogen utilization using multicatheterized Danish Holstein cows were employed in the development of this method [23] because these were representative of the types of samples that this method is likely to be used for in the future.

2. Materials and Methods

2.1 Chemicals, reagents and materials
Water quality was at all times secured by treatment on a Millipore Synergy® UV water treatment system from Millipore A.S. (Molsheim, France). Methanol (MeOH) from Poch S.A. (Gliwice, Poland) and ethanol (EtOH 99.9% vol.) from Kemetyl A/S (Køge, Denmark) were of HPLC grade. Formic acid (98-100%) (HCOOH), acetic acid (100%) (CH₃COOH), and ammonium solution (25%) (NH₄OH) from Merck (Darmstadt, Germany) were of analytical reagent grade. Sodium hydroxide (NaOH), also from Merck, was prepared in a 0.01 M aqueous solution. Trichloroacetic acid (≥99.0%) from Sigma-Aldrich (Brøndby, Denmark) was prepared in a 12% v/v aqueous solution (TCA) daily. Contamination between samples was minimized by the use of disposable materials (vials, bottles etc.) where practicable, or through the use of lab equipment that was cleaned without the use of detergents.

2.2 Standards

The following compound standards (bases (BS), nucleosides (NS), DP) were obtained from Sigma-Aldrich (Brøndby, Denmark): adenine, guanine, cytosine, thymine, uracil, adenosine, guanosine, cytidine, uridine, inosine, 2’-deoxyadenosine, 2’-deoxyguanosine, 2’-deoxycytidine, thymidine, 2’-deoxyuridine, 2’-deoxyinosine, xanthine, hypoxanthine, uric acid, allantoin, β-alanine, β-ureidopropionic acid and β-aminoisobutyric acid. β-ureidoisobutyric acid, one important intermediate pyrimidine derivate metabolite, was not commercially available and could not be included. No traces of either adenosine or 2’-deoxyadenosine were identified during method development in plasma or urine samples. 2’-deoxycytidine was present in trace amounts but even after extensive optimization the sensitivity remained too low for quantification. These three components were therefore not pursued further. The chemical structures of the targeted metabolites are shown in Table 1.
Stable isotopically-labelled reference compounds used as internal standards were purchased from Cambridge Isotope Laboratories (Andover, USA). These were: adenine (8-13C), guanine (8-13C;7,9-15N2), thymine (15N2), uracil (U-13C4;U-15N2), guanosine (U-13C10;U-15N5), inosine (U-15N4), cytidine (U-13C9;U-15N3), uridine (U-13C9;U-15N2), 2'-deoxyguanosine (U-15N5), thymidine (U-15N2), xanthine (1,3-15N2), hypoxanthine (15N4), uric acid (1,3-15N2), and β-alanine (U-13C3;15N). Cytosine (2,4-13C2;15N3) was purchased from Sigma-Aldrich (Brøndby, Denmark). All were 13C and/or 15N labelled with purities of at least 95% (95-99%). Unfortunately, exact SIL were not available for all metabolites studied; a suitable SIL was consequently selected on its similarity to the corresponding metabolite in terms of structure, retention time, fragmentation pattern and group. Individual stock solutions of all compound standards and SIL were prepared and kept at -80°C. Bases and purine DP were diluted in water and NS and pyrimidine DP were diluted in 0.01 M NaOH solution. Two stock concentrations of 500 and 5,000 µmol/L were made for each compound standard. The exception was for uric acid and allantoin, where the stock concentration was 500/2,000 µmol/L and 500/40,000 µmol/L, respectively. For SIL only the low concentration stock was prepared. All stocks were filtered through 0.45 µm PALL GHP Membrane syringe filters purchased from VWR (Herlev, Denmark) and kept at -20°C in dark vials. Appropriate dilutions of these solutions were made in water to produce standard mixtures and SIL mixtures for external calibration and quantification.

Table 1

2.3 Samples

A number of 5 ml aliquots of heparinized plasma to be used for external calibration and quality control were prepared from two liters of venous blood [23] drawn from a Danish Holstein dairy cow.
fed a traditional total mixed ration. Experimental plasma samples were obtained from a feeding
experiment [24] with multicatheterized dairy cows [25,26]. This set of samples was drawn from
four blood vessels simultaneously, representing blood from an artery and the portal hepatic, hepatic
and gastroplenic veins. Additional test plasma samples were obtained on site for relative matrix
effect evaluations. These samples were from five other species (chicken, pig, mink, human, and rat)
for between species comparisons, four multicatheterized cows (jugular vein) for intraspecies
comparisons, and bovine urine and milk samples for matrix effect evaluations.

2.4 Pre-treatment

Before pre-treatment, plasma samples for quantification of uric acid and uracil were diluted twenty-
fold (5% v/v) and four-fold (25% v/v) in water, respectively. This was, in the case of uric acid, to
avoid a non-linear calibration curve with the very high uric acid concentrations in all samples, and,
in the case of uracil, to be able to distinguish the small uracil signal from the pronounced
background noise. Pre-treatment: Plasma samples were defrosted and immediately put on ice. The
sample (300 µL) was then added to a SIL mixture and a water/standard mixture (550 µL total vol.)
before being precipitated with 1.8 mL ice-cold ethanol (10 min., on ice, -20°C). This was followed
by centrifugation (15 min., 5,500 × g, 4°C). The supernatant was ultrafiltered on a Pall Nanosep
10K, Omega membrane spin filter purchased from VWR. A 500 µL aliquot of filtered supernatant
was dried down under a flow of nitrogen on a SuperthermTM fitted with a Mini Oven for AI blocks
and evaporator with valves from Mikrolab A/S (Aarhus, Denmark) in conical autosampler vials
from VWR until dryness (app. 75 min., room temp.). The pellet was re-suspended in 100 µL cold
solvent (A) (30 min., 4°C) and transferred to a clean dark LC-vial. Matrix-matched external
calibrators were treated similarly to standard plasma. Milk samples were cleared with ice-cold TCA
12% (end 50% v/v) before pre-treatment. Urine samples were handled as plasma samples throughout.

2.5 LC-MS/MS analysis

Chromatographic separation was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Hørsholm, Denmark) with a Synergi™ Hydro-RP LC Column (250 mm × 2 mm, 4 μm) protected by a conventional guard column of the same material purchased from Phenomenex (Værløse, Denmark). Samples were analyzed in five separate runs, three in negative electrospray (ESI) mode and two in positive ESI mode. The five groups of metabolites and their chromatographic profiles are shown in Table 2. Separation was performed using a gradient solvent system. For each run, HPLC solvents were freshly prepared and cleared on a 0.45 μm Pall hydrophilic polypropylene membrane filter purchased from VWR. Both solvents (A) and (B) were prepared from a 0.05 mol/L acetic acid buffer containing 10% or 50% methanol, respectively. The acetic acid buffer was prepared by adjusting 0.05 mol/L acetic acid to pH 4.0 with ammonium solution and readjusting to pH 2.8 with formic acid. The following elution gradient was used: initial percentage of solvent B was 5%, this was raised to 100% in 8 min and kept there for 6 min, then lowered to 5% in 30 sec, after which it was kept constant for 3.5 min to re-equilibrate the column prior to the next injection. The flow rate was 200 μL/min and the injection volume was 5 μL. The column temperature was maintained at 30°C while the auto sampler temperature was set to 4°C to stabilize the samples during time-consuming analyses. The total run time was 18 min per sample.

Table 2
A Waters (Hedehusene, Denmark) micromass triple quadrupole mass spectrometer was used for electrospray mass spectrometric analyses using massLynx 4.0 (Waters) software for data collection and processing. Capillary voltage was set to 3.2 kV, source temperature to 120°C, and desolvation temperature to 400°C. The cone and desolvation gas flows (nitrogen and argon) were set at 29 and 628 L/hour, respectively. Fragment ion spectra were recorded in both polarities and promising selective fragment ions were tested and optimized along with the cone voltage in multiple-reaction monitoring (MRM) mode. The values of the tune parameters were optimized by separately infusing a solution (500 µmol/L) of each metabolite in its mobile phase at a flow rate of 10 µL/min. The MRM transitions and the applied cone voltages and collision energies are summarized in Table 3. Common transitions were originated from the loss of HCN, NH₃, ribose, deoxyribose, HNCO, HNCONH₂ and H₂O fragments for the various PP metabolites (Table 1). The most intense transition reaction was used for quantification (Table 3). Data were collected in centroid mode with a constant dwell time of 0.05 sec and an interscan delay of 0.02 sec.

Table 3

2.6 Calibration and quantification

Quantification was performed by matrix-matched external calibration applying standard plasma spiked with a two-fold serial dilution of mixed standard solutions to obtain seven different concentration levels of each compound. The only exception was with uracil where a two-third-fold serial dilution was applied. Standard plasma (not spiked) was used for subtraction and quality control but was not included in the regression analysis. In general, all samples and calibrators were analyzed in duplicate and a standard curve and quality control samples were analyzed at the beginning and at the end of each sequence. The response was calculated as the chromatographic
peak area for all compounds. When applying standard plasma, which contained unknown quantities
of the metabolites under investigation, the measured metabolite response was initially normalized
and the response from the standard plasma was subtracted. The mean of the measured SIL
responses/SIL area for each sample was used as the normalization factor. During method
development the focus of work was on quantifying as low concentrations of metabolite as possible.
Matrix-matched calibration curves, within the relevant concentration ranges given in Table 4, were
generated for each metabolite at four (allantoin) or seven concentration levels on five consecutive
days for determining and evaluating the calibration model. As noted previously, uric acid and uracil
were quantified from diluted samples. The coefficient of variation (CV%) for each concentrate level
was then calculated for a logarithmic and a linear calibration model to test the use of log-log
transformation. The linearity of the log calibration curves were studied with a lack of fit hypothesis
test. Subsequently, the homogeneity of variance was estimated for each concentration by plotting
the CV% against log(concentration) and the quantification range set to the lowest and highest
quantified concentration giving a CV% below 25%.

Table 4

2.7 Validation procedure

The method was validated according to reports from the “Analytical methods validation:
bioavailability, bioequivalence and pharmacokinetic studies” conferences held in Washington in
1990 [27] and 2000 [28], as described by Peters et al. [29]. It was validated with respect to
assessment of selectivity, stability, precision, recovery, and matrix effect.

Selectivity: Metabolite and SIL cross-talk was evaluated by analyzing the standard compounds
alone and together with their corresponding SIL (no blank matrix was available). Three groups were
studied and their signals compared; a compound standard group (10% v/v, 50 µmol/L), a SIL group
(10% v/v, 50 µmol/L), and a combined group (5% v/v, 25 µmol/L). Analyses of BS/DP and NS
were carried out separately.

**Stability:** For continuous evaluation of long-term storage stability, a fresh quality control sample
was analyzed in all analytical runs. The stability within runs (6-24 h) was evaluated in two ways.
First, a quality control sample was analyzed at the beginning and at the end of each sequence (data
not shown). Secondly, a set of spiked standard plasma samples were analyzed at five different times
(different vials) during a 30 hour sequence. Analysis of variance (ANOVA) using linear mixed
models procedures was used to test the stability over time, both with a trend element and with
random changes over and above the linear trend (regression line) [30,31]. Applying ANOVA, the
across-day variation of the PP calibration curves (intercepts and slopes as interactions with test day)
was assessed over five consecutive days and expressed by their P-values. The stability during
repeated freeze-thaw cycles was not explored since all plasma samples in the present study were
only thawed once.

**Precision and recovery:** Precision of the method, in terms of within-day variation (repeatability)
and across-day variation (intermediate precision), was determined by analyzing replicate sets of
spiked standard plasma samples on five separate days expressed as their CV%. The absolute
recoveries were calculated using the same set of spiked standard plasma, at one level, by comparing
the obtained concentrations with the initial spiked level.

**Matrix effect:** Early tests with spiked water, urine and plasma samples revealed large variations in
matrix effect-induced signal suppression and enhancement between the metabolites included in the
analysis. Following optimization of the pre-treatment procedure, these matrix effects were
evaluated as the difference between samples of water and standard plasma, urine or milk samples
spiked with constant amounts of SIL before pre-treatment. Thus, we took advantage of the fact that
the incorporated SIL should behave as their matching metabolite in the ESI source [27]. The conventional strategy of spiking a blank matrix sample with a compound standard was again not possible as completely blank matrices were not available for these metabolites. The applied SIL-based method was a modified version of the conventional method to evaluate matrix effect described by Matuszewski et al. [32]. The observed matrix effect was rendered insignificant by utilizing matrix-matched external calibration.

2.8 Application

To determine the application range of the method, the relative matrix effect was evaluated by comparing the response from PP SIL spiked in standard jugular vein plasma with the response in test plasma samples. Four different sets of samples were assessed. First, plasma from the jugular vein of four multicatheterized cows was used to investigate within-species variation. Next, plasma drawn from the portal vein, the hepatic vein, the gastrosplenic vein, and an artery from a multicatheterized dairy cow to represent different possible sampling sites were examined. Third, plasma samples from different species (chicken, pig, mink, human, rat) were used for between-species evaluation. Finally, water, urine and milk samples were used to compare different matrices. The relative recovery determined which of the tested matrices were suitable for the method. For the same reasons as described previously, SIL replaced compound standards. Water, urine and milk samples were evaluated in the same manner as plasma samples.

3. Results and discussion

3.1 Method development

The aim of this study was to develop a quantitative LC-MS/MS analysis and a sample pre-treatment procedure for the simultaneous analysis of several metabolites of the PP metabolism in blood
plasma of dairy cows. The chemical properties of the metabolites were polar due to high contents of 
–OH, =O and –N groups. Based on their polarity, they were roughly divided into three groups: The 
very polar group, containing β-alanine, β-aminoisobutyric acid and β-ureidopropionic acid, were all 
small molecules with similar linear polar structures, as well as the also highly polar allantoin, 
cytosine and cytidine. The polar group included the majority of the BS, such as adenine, guanine 
and uracil, as well as the intermediate DP with more base-like structures, such as uric acid, xanthine 
and hypoxanthine. Finally, the semi polar group comprised the majority of the NS with large but 
semi-polar sugar side groups, such as most of the ribonucleosides (2× -OH) and 
deoxyribonucleosides (1× -OH). Owing to their very non-polar methyl side groups, thymine and 
thymidine were also placed in the semi polar group. The very polar metabolites were poorly 
retained on the C18 column with the aqueous solvents and eluted first as expected, offering a longer 
retention time of the less polar components.

3.1.1 Pre-treatment development and evaluation
An effective clean-up procedure is crucial when performing LC-MS/MS analysis as this diminishes 
cross-talk [33,34] as well as matrix effects [35] and at the same time enhances both the selectivity 
and the sensitivity of the analysis [29]. A novel multi-step approach, consisting of protein 
precipitation, ultrafiltration, evaporation under nitrogen flow, and subsequent resolution, able to 
purify and to concentrate all of the studied metabolites from bovine plasma simultaneously, in a 
simple and efficient manner, was developed and optimized.
Initially, different solvents (acetone, acetonitrile, ethanol, methanol, sulfo-salicylic acid) were tested 
for precipitation (data not shown). Ethanol precipitation resulted in the highest recoveries and least 
noise when comparing chromatographic responses and this less harmful solvent was therefore 
chosen for the procedure. The ultrafiltration step was added as this step caused markedly lower
levels of background noise. As a consequence of the approximately eight-fold dilution during pre-
treatment, evaporation and reconstitution steps were included. Overall this resulted in a 1.4 times
concentration effect. To try to reduce degradation and instability of the samples caused by reactive
oxygen species or enzyme activities during pre-treatment, all centrifugations and incubations were
performed at 4°C and samples, stocks, and solvents etc. were kept at -4°C or on ice. Only during
evaporation were the samples maintained at room temperature. Other types of pre-treatment
methods such as simple dilution (impractical), solid phase extraction (different chemical properties)
[36,37] and accelerated solvent extraction [38] were also investigated (data not shown) but were not
found useful.

The effectiveness of the pre-treatment and the stability of the metabolites during the multiple steps
were evaluated during validation of the method, described in section 3.3, and demonstrated the
ability of this pre-treatment to purify and concentrate all of the targeted PP simultaneously in an
easy and efficient manner without significant losses. To our knowledge, no other publications have
presented a similar and effective pre-treatment procedure, as most other approaches include dilution
of the samples.

3.1.2 LC-MS/MS procedure

Based on the chemical properties of the targeted metabolites, experiences from similar studies
[14,39], and available equipment, a reversed-phase C_{18} column known to be able to quantify the
majority of the studied metabolites from urine was applied with an acetic acid buffer/methanol
HPLC solvent system.

To achieve adequate separation and elution order, a series of conditions were modified and
implemented. The composition of the acetic acid buffer and the methanol extraction solvent was
based on the work of Hartmann et al. (2006) [39], and no other types of solvent were tested. Having
tested several acetic acid buffer to methanol ratios (95%, 90%, 85%, and 80% v/v), assessing peak separation and shapes, it was concluded that the best separation was accomplished with a 90% v/v solvent (A) and 50% v/v solvent (B). The chosen injection volume, 5 µL, and flow rate, 200 µL/min, was found by assessing the same parameters, testing first injections of 5, 10, 20 µL and then flow rates of 100, 200, 300 and 400 µL/min. Concerning the elution gradient, we strived to make it as short as possible, while still achieving as good a peak separation as possible. Different elution profiles were tested, with more or less steep gradients. The final profile, described in section 2.5, gave a total run time of 18 min. By adding a small amount of methanol to the otherwise aqueous solvent (A), and, by keeping the baseline at 5% solvent (B), the solvent mixing became more smooth and transitions between runs became more stable. A major improvement in precision between runs was achieved by maintaining the column temperature at 30°C instead of 25°C. An improvement in the sample stability during the time-consuming analyses was achieved by cooling the auto-sampler to 4°C. In the end, useful combinations of retention times and peak shapes of each metabolite were achieved with the parameters described, and the method was therefore adapted and brought on to further validation.

3.2 The log-calibration model and quantification range

Calibration curves were prepared by linear regression of log(area) against log(concentration) (log-calibration) and by linear regression in linear units on both axes (linear calibration) to verify the use of the log-calibration model. Initially, the normality of residuals around the calibration lines were inspected visually (Q-Q plot) and found to be approximately normal. The CV% for each concentration level for both the log-calibration and the linear calibration is illustrated in figure 1. A large group of the PP (panel I) considerably improved their CV% profiles using the log-calibration, especially in the low ranges. However, a smaller group of PP (panel II) did not benefit from the log
transformation; and the transformation did not weaken as their CV% profiles either. Exceptions were with allantoin, β-ureidopropionic acid, cytosine and β-alanine, their CV% at the high end of their profiles were better without the log-log transformation. Given that quantification at low concentrations was considered to be most important, these findings validated the use of log-log transformation in the analysis of all the applied PPs. Performing a lack of fit test, the linearity of the PP calibration curves were evaluated and expressed by their P-values (Table 4). None of the PP curves resulted in a significant lack of fit except uridine, which had a very low sensitivity in the analysis, demonstrating a satisfying log-log regression.

The homogeneity of variance for the different concentration levels is illustrated in Figure 2 and the quantification ranges (CV<25%) in Table 4. Focusing on the lower concentration range, most of the PP demonstrated a typical precision profile where the CV% decreased with higher concentration levels. All purines had acceptable variation levels around the lowest concentration levels except allantoin, which should not be quantified at concentrations below ~100 µmol/L. The pyrimidine BS and cytidine and uridine had larger CV%’s with acceptable lower concentration levels from 0.66-5.15 µmol/L. Thymidine and 2’-deoxyuridine demonstrated a very large variation with CV%’s above 25% over the entire concentration range. In the case of the pyrimidine DP, they were reasonably stable over their concentration ranges, not counting β-alanine which only had a CV%<25% at its highest calibrator. The upper part of the quantification range was in all cases the highest quantified calibrator.

**Figure 1, Figure 2**

### 3.3 Method validation
Once the pre-treatment, LC-MS/MS procedure, and calibration model had been set, the performance characteristics of the method were established by validation with spiked standard plasma. In terms of quantification purposes, selectivity, stability, precision, recovery, and matrix effects were evaluated.

The most intensive fragment ion from each precursor ion was selected as the transition ion for detection and quantification. Positive identification was based on the correlation of retention time with the standards and the selected precursor/product transition. Less intensive second transitions were used for confirmation. All metabolites generated single peak shapes.

### 3.3.1 Selectivity

A blank sample for selectivity evaluation was not available for these naturally occurring plasma metabolites. Hence, the presence of chromatographic peaks from standard plasma at the same retention times as the targeted metabolites could not be excluded; such endogenous peaks would be expected to be present. Instead, the absence of standard compound/SIL cross-talk contributions was verified by comparing chromatographic responses for standards and SIL alone and in a mixture (data not shown). It was important to assess cross-talk contributions, as some of the applied SIL (Table 3) had less than three mass unit differences (3-8) to the natural metabolite, which is normally recommended as the lowest mass unit difference for LC-MS/MS analysis [33,34].

### 3.3.3 Stability

Good stability was achieved by optimizing the pre-treatment and LC-MS/MS parameters as described in section 3.1. Long-term storage stability was tested by comparing chromatographic profiles of quality control standard plasma on a daily basis. Within-run stability was evaluated by analyzing a control sample at the beginning and end of each sequence. Long sequence run times
have been of concern and the within-run stability was consequently also evaluated by performing ANOVA for measurements made at times 0, 7, 15, 22 and 29 hours, during a 30 hour sequence with triplicate determinations at each time-point, using either a slope model: $y_{ij} = \text{intercept} + b \times \text{time\_hour} + \varepsilon_{ij}$, or a combined model: $y_{ij} = \text{intercept} + \text{time}_i + b \times \text{time\_hour} + \varepsilon_{ij}$, where $y_{ij}$ is the area measured in the sample at time $i$, replicate $j$, and $b$ is the slope of the area change per hour, and $\varepsilon_{ij}$ is the random error term. Significance of the time effects were tested using an F-test with type 1 sum of squares. Residual mean square error was calculated as the square of the residual variance estimate and expressed as CV%. The metabolite responses were normalized as usual but the SIL responses were not since they could not be used to normalize themselves. The results are given in Table 5.

In general, the combined model resulted in lower CV%’s than the slope model, as the irregular time effect was also taken into consideration in the combined model. All but a few metabolites demonstrated very stable profiles over the 30 hour time span with CV% $\leq 10\%$. Exceptions were thymidine (136%), 2’-deoxyuridine (46%) and β-alanine (13%), where especially the former two were found to be unstable. This was probably due to low sensitivities in the analysis. The SILs were found to be equally or more stable than their corresponding metabolites probably due to their higher spike concentrations. As expected, thymidine (U-15N2) and β-alanine (U-13C3;15N) had the same instability issues as their partners. No 2’-deoxyuridine SIL was applied in this analysis. Surprisingly, the uracil and cytidine SIL had CV%’s above 10%. In the case of uracil (13%), excessive degradation was avoided by always placing uracil samples in the beginning of a sequence.

To assess the stability of the calibration curves between run-days, ANOVA was conducted determining the across-day (intermediate precision) precision (Table 4). Most PP demonstrated a significant ($P<0.05$) difference between test days on either curve intercept or slope, or at least a
tendency ($P<0.1$). Exceptions were with allantoin, cytosine, uridine, thymidine and 2’-deoxyuridine, all of which revealed reasonably stable curves over test days. These results demonstrated the need for renewing calibration curves on a daily basis.

### Table 5

#### 3.3.4 Precision and recovery

To ensure correct quantification and to evaluate analytical precision, within-day and across-day variation was determined by studying replicate sets of spiked standard plasma samples ($n = 8$, samples) on five separate days ($m = 5$, days). Here, precision was defined as the degree to which repeated measurements under unchanged conditions showed the same result, expressed as the CV%. Absolute recoveries were identified by using the same set of spiked standard plasma samples, comparing the recovered quantified concentrations with the initial spiked concentrations. Since linearity ranges were short and close to zero, a single, instead of the traditional three, recovery concentration levels was chosen. Precision and recovery outcomes are given in Table 6. The obtained results showed very good extraction efficiency and precision. The recoveries were between 91% and 107%, except for uric acid with a lower recovery of 78%. Also, the low sensitivity and accompanying instability of cytidine, thymidine and 2’-deoxyuridine was again highlighted with recoveries of 162%, 121%, and 149%, respectively. In general, the within- and across-day variations mirrored the recovery results. The exceptions were with allantoin and cytosine, both of which had good recoveries, 107% and 103%, but exhibited large CV%’s, within-day variation 34% and 21%, and across day variation 49% and 24%, respectively.

### Table 6
3.3.5 Absolute matrix effect

It is useful to distinguish between two types of matrix effects: absolute matrix effect, which is the difference in response between an undiluted solution and a post-extraction spiked sample, and relative matrix effect (section 3.4), which is the difference between various lots of post-extraction spiked samples [32]. Matrix effects are very common problems when applying LC-MS/MS analysis on biological samples [22, 35, 40]. The term describes the effect molecules originating from the sample matrix can have on the ionization process in the mass spectrometer when co-eluting with the compound of interest. It theoretically occurs in either the solution or the gaseous phase and the main cause is a change in droplet solution properties caused by the presence of nonvolatile and less volatile solutes that change the efficiency of droplet formation and evaporation, which in turn affects the amount of charged ions in the gas phase that ultimately reach the detector [35]. As the effect occurs in the ESI source before detection, it is hard to compensate for by mass spectrometry alone [41,42]. In this analysis, the matrix effect was quantified by comparing the response of SIL in spiked matrix samples before extraction with the response obtained in water. Matrix effects for all SILs are illustrated in Figure 3.

Recognizing that the nature of matrix effects is varying and the sensitivity between metabolites are very different the sizes of the bars are relative indicators of the degree of suppression or enhancement. Signal enhancement was observed in plasma for almost all metabolites, and only a few, such as inosine, cytidine, β-alanine and cytosine, had their signals suppressed. These metabolites did not share any obvious similarities in polarity or structure; however, matrix effects are known to be very compound-dependent [22]. In contrast to the signal enhancement generally encountered in plasma, in urine all metabolite signals were suppressed. This demonstrates the different matrix effects a given component experience when present in different matrices in LC-
MS/MS analysis. In milk, only the purines had a common pattern, i.e., signal suppression, and the
remaining metabolites were neither suppressed nor enhanced.

Matrix effects can vary between measurements, hence, it is not possible to test for matrix effects
only once and consider it to be constant [43]. Matrix effects were largely eliminated in the analysis
first of all by making the external calibrators matrix-matched, hence, quantifying calibrators and
sample metabolites under the same conditions, secondly, by implementing a very effective pre-
treatment [33, 44], and thirdly, by implementing SIL [22, 42]. Matrix-matching is necessary when
specific SILs are not available for all metabolites [42]. These initiatives compensated quite well for
the signal suppression or enhancement in the plasma samples, thereby achieving accurate
quantification.

**Figure 3**

### 3.4 Analytical application (relative matrix effect)

This LC-MS/MS analysis was established for quantification of 20 target metabolites of the PP
metabolism in blood plasma samples from multicatheterized cows. Since jugular vein plasma
(representing systemic circulating blood) was used for method development and because
quantification relied on matrix-matched calibration (jugular vein plasma), the relative matrix effect
was evaluated in alternative types of plasma. The relative matrix effect was evaluated by comparing
the response from SIL spiked in standard jugular vein plasma with the response in tested plasma
samples. A relative recovery between 85% and 115% was considered good and between 75% and
125% acceptable, hence, tested samples exerted the same matrix effect on the metabolite as the cow
jugular vein plasma sample. The generosity of 75-125% was due to the small sample size (n = 2
samples) inevitably resulting in less precision. The PP responses given as recovery (%) are depicted in Table 7.

First of all, it was confirmed that within-species variation was not an issue with any of the metabolites examined, except for uridine. Secondly, the results demonstrated that all the examined metabolites, evaluated in all four plasma types from feeding experiments with multicatheterized cows with this particular type of cow model, could appropriately be quantified with the developed LC-MS/MS method. Only xanthine (67%), uridine (135%/148%/127%) and thymidine (132%) displayed recoveries outside the acceptable range of 75-125% and especially thymidine will be hard to quantify with this method due to other issues anyway. Surprisingly, the between-species range was very broad and most metabolites could be evaluated in plasma from other species tested with a few exceptions. Further confirmed was also the results from section 3.3.5, concluding that matrix effects varied significantly between different types of matrices such as water, plasma, urine and milk. Hence, it is necessary to design, optimize and validate a specific LC-MS/MS method for each applied matrix.

Table 7

4. Conclusions

This work presents the development and validation of a new method for simultaneous and accurate quantification of 20 targeted metabolites of PP metabolism with different structures and physiochemical properties in blood plasma from dairy cows. Exceptions were with cytidine, thymidine and 2’-deoxyuridine, where the method’s sensitivity for these three PP metabolites was so low that they caused imprecise quantification over the examined concentration ranges. The metabolites were purified and concentrated using a novel multi-step pre-treatment procedure consisting of protein
precipitation, ultrafiltration, evaporation under nitrogen flow, and subsequent reconstitution. This procedure ensured efficient recoveries for most investigated metabolites and efficient removal of interfering matrix components. The method is selective, sensitive, stable, and precise. The potential application of the method was demonstrated by evaluating its range of use in different types of blood plasma from multicatheterized cows, here, only uridine, showed undesirable matrix effects. The method is adaptable and can be further developed for the quantitative detection of the same metabolites in other matrices such as urine or milk.

Acknowledgements

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References


**Figure captions**

Fig. 1. The coefficient of variation (CV%) for each concentration level using linear regression of area against concentration (linear calibration) and using linear regression of log(area) against log(concentration) (log-calibration). Panel I present the 13 purines and pyrimidines that considerably improved their CV% profiles using the log-calibration. Panel II, present the seven purines and pyrimidines that did not benefit from the log transformation. Abbreviations for the 20 metabolites: Ade, adenine; Gua, guanine; Guo, guanosine; Ino, inosine; dGuo, 2’-deoxyguanosine; dIno, 2’-deoxyinosine; Xan, xanthine; Hyp, hypoxanthine; Uac, uric acid; All, allantoin; Ur,

28
Fig. 2. The homogeneity of variance for the different concentration levels of the purine and pyrimidine calibration curves divided into bases, nucleosides and degradation products (CV%).

Abbreviations for the 20 metabolites: Ade, adenine; Gua, guanine; Guo, guanosine; Ino, inosine; dGuo, 2'-deoxyguanosine; dIno, 2'-deoxyinosine; Xan, xanthine; Hyp, hypoxanthine; Uac, uric acid; All, allantoin; Urd, uridine; β-ure, β-ureidopropionic acid; β-ami, β-aminoisobutyric acid; Cyt, cytosine; Thy, thymine; Ura, uracil; Cyd, Cytidine; Thd, thymidine; dUrd, 2’-deoxyuridine; β-ala, β-alanine.

Fig. 3. Matrix effects in plasma, urine and milk expressed as response relative to water (area).
Highlights

- Simultaneous quantification of 20 purines and pyrimidines
- LC-MS/MS method developed and validated for bovine blood plasma
- Novel metabolite concentrating pre-treatment
- Matrix-matched calibration standards and stable isotopically-labelled references
- The method is simple, sensitive and specific
Figure 1

Linear calibration I
- Ade
- Gua
- Guo
- Ino
- dGuo
- dlno
- Xan
- Hyp
- Uac
- All
- Urd
- β-ure
- β-ami

Log-calibration I
- Ade
- Gua
- Guo
- Ino
- dGuo
- dlno
- Xan
- Hyp
- Uac
- All
- Urd
- β-ure
- β-ami

Linear calibration II
- Cyt
- Thy
- Ura
- Cyd
- Thd
- dUrd
- β-ala

Log-calibration II
- Cyt
- Thy
- Ura
- Cyd
- Thd
- dUrd
- β-ala
Figure 2

**Purine bases**
- Ade
- Gua
- Xan
- Hyp

**Purine nucleosides**
- Guo
- Ino
- dGuo
- dIno

**Purine degradation products**
- Uac
- All

**Pyrimidine bases**
- Cyt
- Thy
- Ura

**Pyrimidine nucleosides**
- Cyd
- Urd
- Thd
- dUrd

**Pyrimidine degradation products**
- β-ala
- β-ure
- β-ami
Figure 3

Purine bases and nucleosides
- Adenine (8-13C)
- Guanine (8-13C, 7,9-15N2)
- Guanosine (U-13C10; U-15N5)
- Inosine (U-15N4)
- 2’-deoxyguanosine (U-15N5)
- Xanthine (1,3-15N2)

Pyrimidine bases and nucleosides
- Thymine (15N2)
- Uracil (U-13C4; U-15N2)
- Cytidine (U-13C9; U-15N3)
- Uridine (U-13C9; U-15N2)
- Thymidine (U-15N2)

Purine degradation products
- Uric acid (1,3-15N2)
- Hypoxanthine (15N4)

Cytosine and β-alanine
- β-alanine (U-13C3; 15N)
- Cytosine (2,4-13C2; 15N3)
<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Names, types, empirical formulae and suggestions for fragmentations of the compounds analyzed by the LC-MS/MS method</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purines</th>
<th>Pyrimidines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Type</td>
</tr>
<tr>
<td>Adenine</td>
<td>Base</td>
</tr>
<tr>
<td>Guanine</td>
<td>Base</td>
</tr>
<tr>
<td>Guanosine</td>
<td>NS</td>
</tr>
<tr>
<td>Inosine</td>
<td>NS</td>
</tr>
<tr>
<td>2'-deoxyguanosine</td>
<td>NS</td>
</tr>
</tbody>
</table>
2'-deoxyinosine  NS

Thymidine  Frag. 7  NS

Xanthine  Base  /DP

2'-deoxyuridine  NS

Hypoxanthine  Base  /DP

β-alanine  Frag. 8  DP

Uric acid  DP

β-ureidopropionic acid  DP

Allantoin  Frag. 6  DP

β-aminoisobutyric acid  DP

NS, nucleoside; DP, degradation product.
Illustrated with lines are the eight types of suggested metabolite fragmentations.
Table 2

The 20 metabolites were divided into five groups and run according to ESI -/+ mode and structure

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine (1)</td>
<td>Cytosine (6)</td>
<td>Guanosine (12)</td>
<td>Uracil (20)</td>
<td></td>
</tr>
<tr>
<td>Guanine (2)</td>
<td>Thymine (7)</td>
<td>Cytidine (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthine (3)</td>
<td>Hypoxanthine (8)</td>
<td>Uridine (14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allantoin (4)</td>
<td>β-alanine (9)</td>
<td>Inosine (15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(Uric acid 1,3-15N2)</em> (5)</td>
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<td>Thymidine (16)</td>
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</tr>
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<td><strong>Group 5: Uric acid (ESI -)</strong></td>
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<td>2'-deoxyguanosine (17)</td>
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<td></td>
</tr>
<tr>
<td>Uric acid (5)</td>
<td></td>
<td>2'-deoxyinosine (18)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2'-deoxyuridine (19)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DP, degradation product; NS, nucleoside.

Plasma samples and standard plasma for quantification and external calibration of uracil and uric acid were diluted 25% and 5% v/v, respectively, in water. A group 5 chromatographic profile (uric acid) is not illustrated in the table since uric acid (1,3-15N2) can be observed with group 1 (same peak, same shape, same RT).
Table 3

Transition reactions monitored by LC-MS/MS, cone voltages and collision energy for the metabolite/stable isotopically-labelled reference compound (SIL) analyzed, and suggested corresponding fragments lost

<table>
<thead>
<tr>
<th>Metabolite/SIL</th>
<th>Mw (g/mol)</th>
<th>Retention time (min)</th>
<th>Precursor ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Product ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Neutral loss (NL)</th>
<th>Fragmentation 1-8</th>
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<td><strong>Purines</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>135.13</td>
<td>3.81</td>
<td>134</td>
<td>35</td>
<td>107</td>
<td>16</td>
<td>27</td>
<td>- HCN 1</td>
</tr>
<tr>
<td>Guanine/</td>
<td>151.13</td>
<td>3.86</td>
<td>150</td>
<td>28</td>
<td>133</td>
<td>13</td>
<td>17</td>
<td>- NH₃ 2</td>
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<td>Guanosine/</td>
<td>283.24</td>
<td>6.18</td>
<td>282</td>
<td>33</td>
<td>150</td>
<td>19</td>
<td>132</td>
<td>- deoxyribose 3</td>
</tr>
<tr>
<td>Inosine/</td>
<td>268.23</td>
<td>5.81</td>
<td>267</td>
<td>26</td>
<td>135</td>
<td>20</td>
<td>132</td>
<td>- deoxyribose 3</td>
</tr>
<tr>
<td>Inosine (U-¹³N⁴)</td>
<td>272.20</td>
<td>5.81</td>
<td>271</td>
<td>26</td>
<td>139</td>
<td>20</td>
<td>116</td>
<td>- ribose 4</td>
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<tr>
<td><strong>Pyrimidines</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine/</td>
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<td>2.91</td>
<td>112</td>
<td>29</td>
<td>95</td>
<td>20</td>
<td>17</td>
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<td>Cytidine (2,4-¹³C₂;¹⁵N³)</td>
<td>116.08</td>
<td>2.91</td>
<td>112</td>
<td>30</td>
<td>99</td>
<td>19</td>
<td>18</td>
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<td>6.21</td>
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<td>7</td>
<td>17</td>
<td>- NH₃ 2</td>
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<td>27</td>
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<td>18</td>
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<td>3.97</td>
<td>113</td>
<td>26</td>
<td>96</td>
<td>7</td>
<td>17</td>
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<td>Uracil (U-¹³C₄;¹⁵N²)</td>
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<td>3.97</td>
<td>119</td>
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<td>3.19</td>
<td>242</td>
<td>23</td>
<td>109</td>
<td>14</td>
<td>133</td>
<td>- deoxyribose 3</td>
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<tr>
<td>Cytidine (U-¹³C₉;¹⁵N³)</td>
<td>255.13</td>
<td>3.19</td>
<td>254</td>
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<td>116</td>
<td>15</td>
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<td>255.12</td>
<td>4.50</td>
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<td>16</td>
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<td>242.23</td>
<td>8.52</td>
<td>241</td>
<td>25</td>
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<td>26</td>
<td>153</td>
<td>11</td>
<td>43</td>
<td>- HNCO 5</td>
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<td><strong>β-alanine/</strong></td>
<td>89.09</td>
<td>2.91</td>
<td>90</td>
<td>13</td>
<td>72</td>
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<td>**β-alanine (U-¹³C₃;¹⁵N)</td>
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<td>76</td>
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<td>Metabolite</td>
<td>Retention Time</td>
<td>Specific Retention</td>
<td>HPLC M/z</td>
<td>Mass Difference</td>
<td>Confirmation</td>
<td>Species</td>
<td>SIL Information</td>
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<td>-----------------------------------------------</td>
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<td>--------------</td>
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<tr>
<td>β-ureidopropionic acid</td>
<td>3.77</td>
<td>+</td>
<td>11</td>
<td>-13</td>
<td>115</td>
<td>10</td>
<td>18</td>
<td>- H_2O</td>
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<tr>
<td>β-alanine (U-13C3;15N) #</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- H_2O</td>
</tr>
<tr>
<td>β-aminoisobutyric acid</td>
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<td>13</td>
<td>+13</td>
<td>86</td>
<td>10</td>
<td>18</td>
<td>- H_2O</td>
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<td>β-alanine (U-13C3;15N) #</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- H_2O</td>
</tr>
</tbody>
</table>

SIL, stable isotopically-labelled reference compound.

All metabolites had a specific retention time and generated single peak shapes.

# This SIL was selected as the most suitable according to structure, retention time, fragmentation pattern and metabolite group.
## Table 4

Concentration level, calibration range, lack-of fit, quantification range and precision of the metabolite calibration curves

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Range$^a$</th>
<th>Type</th>
<th>Concentration levels</th>
<th>Calibration range (µmol/L)</th>
<th>Linearity</th>
<th>Precision (test-day)$^d$</th>
<th>Precision (test-day)$^d$</th>
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<td></td>
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<td></td>
<td>Lack of fit$^b$</td>
<td>Quantification range$^c$ (µmol/L)</td>
<td>Intercept $^c$</td>
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<td><strong>Purines</strong></td>
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<td>$P$-value</td>
<td>$P$-value</td>
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<td></td>
<td>0.92</td>
<td>0.16-5.0</td>
<td>&lt;0.001</td>
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<td>Base/DP</td>
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<td>0.16-5.0</td>
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<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.40</td>
<td>0.08-5.0</td>
<td>0.009</td>
</tr>
<tr>
<td>Uric acid</td>
<td>DP</td>
<td>7</td>
<td>0-200</td>
<td></td>
<td>0.99</td>
<td>3.15-200</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Allantoin</td>
<td>DP</td>
<td>4</td>
<td>15-500</td>
<td></td>
<td>0.64</td>
<td>124-500</td>
<td>0.427</td>
</tr>
<tr>
<td><strong>Pyrimidines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>Base</td>
<td>7</td>
<td>0-7.5</td>
<td></td>
<td>0.84</td>
<td>1.92-7.5</td>
<td>0.566</td>
</tr>
<tr>
<td>Thymine</td>
<td>Base</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.68</td>
<td>1.27-5.0</td>
<td>0.035</td>
</tr>
<tr>
<td>Uracil</td>
<td>Base</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.88</td>
<td>0.66-5.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cytidine</td>
<td>NS</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.70</td>
<td>5.15-5.0</td>
<td>0.086</td>
</tr>
<tr>
<td>Uridine</td>
<td>NS</td>
<td>7</td>
<td>0-7.5</td>
<td></td>
<td>0.02</td>
<td>1.91-7.5</td>
<td>0.286</td>
</tr>
<tr>
<td>Thymidine</td>
<td>NS</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.48</td>
<td>-</td>
<td>0.741</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>NS</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.35</td>
<td>-</td>
<td>0.151</td>
</tr>
<tr>
<td>β-alanine</td>
<td>DP</td>
<td>7</td>
<td>0.25-13</td>
<td></td>
<td>0.59</td>
<td>13-13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>β-ureidopropionic acid</td>
<td>DP</td>
<td>7</td>
<td>0-75</td>
<td></td>
<td>0.87</td>
<td>4.67-75</td>
<td>0.003</td>
</tr>
<tr>
<td>β-aminoisobutyric acid</td>
<td>DP</td>
<td>7</td>
<td>0-5.0</td>
<td></td>
<td>0.29</td>
<td>0.31-5.0</td>
<td>0.026</td>
</tr>
</tbody>
</table>

NS, nucleoside; DP, degradation product.

Only four curves were available for uric acid and β-ureidopropionic acid. In the case of allantoin, the three lower concentration levels were excluded to better fit the concentration range of actual samples. For uridine, one observation in one curve was considered an outlier following visual inspection and was rejected.

$^a$ External calibration was performed with seven concentrations of metabolite on five separate days (n = 5, days), except for allantoin where only four concentration levels were available. The ranges where chosen according to concentration ranges in actual samples.

$^b$ Lack of fit hypothesis test to validate the linearity of the calibration curves expressed by their $P$-values (n = 5, curves). $P < 0.05$ was considered significant.

$^c$ The quantification range was set to the lowest and highest quantified concentration giving an acceptable CV% < 25% (see Figure 2).

$^d$ The intermediate precision of the calibration curves (intercepts and slopes as interactions with test day) expressed by their $P$-values (n = 5, days). $P < 0.05$ was considered significant, $P < 0.1$ a tendency.

$^e$ Value is above the highest calibrator concentration.
### Table 5

Stability of each metabolite/stable isotopically-labelled reference compound during a 30 hour sequence

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration level (µmol/L)</th>
<th>Slope model (CV%)</th>
<th>Combined model (CV%)</th>
<th>Corresponding SIL</th>
<th>Concentration level (µmol/L)</th>
<th>Slope model (CV%)</th>
<th>Combined model (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>4</td>
<td>9</td>
<td>4</td>
<td>Adenine (8-13C)</td>
<td>7</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Guanine</td>
<td>4</td>
<td>11</td>
<td>8</td>
<td>Guanine (8-13C,7,9-15N2)</td>
<td>7</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Guanosine</td>
<td>4</td>
<td>12</td>
<td>7</td>
<td>Guanosine (U-13C10,U-15N5)</td>
<td>7</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Inosine</td>
<td>4</td>
<td>11</td>
<td>2</td>
<td>Inosine (U-15N4)</td>
<td>7</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>2'-deoxyguanosine</td>
<td>4</td>
<td>14</td>
<td>6</td>
<td>2'-deoxyguanosine (U-15N5)</td>
<td>7</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>2'-deoxyinosine</td>
<td>4</td>
<td>11</td>
<td>5</td>
<td>2'-deoxyguanosine (U-15N5)</td>
<td>-a</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>Xanthine</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>Xanthine (1,3-15N2)</td>
<td>7</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>4</td>
<td>12</td>
<td>2</td>
<td>Hypoxanthine (1-15N4)</td>
<td>7</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Uric acid</td>
<td>4</td>
<td>12</td>
<td>6</td>
<td>Uric acid (1,3-15N2)</td>
<td>35</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Allantoin</td>
<td>40</td>
<td>10</td>
<td>7</td>
<td>Uric acid (1,3-15N2)</td>
<td>35</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Pyrimidines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>4</td>
<td>26</td>
<td>3</td>
<td>Cytosine (2,4-13C2;15N3)</td>
<td>14</td>
<td>9</td>
<td>9</td>
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<tr>
<td>Thymine</td>
<td>7</td>
<td>18</td>
<td>10</td>
<td>Thymine (15N2)</td>
<td>7</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Uracil</td>
<td>4</td>
<td>18</td>
<td>6</td>
<td>Uracil (U-15C4;U-15N2)</td>
<td>14</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>Cytidine</td>
<td>4</td>
<td>11</td>
<td>9</td>
<td>Cytidine (U-15C9;U-15N3)</td>
<td>7</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Uridine</td>
<td>4</td>
<td>11</td>
<td>4</td>
<td>Uridine (U-15C9;U-15N2)</td>
<td>14</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Thymidine</td>
<td>7</td>
<td>136</td>
<td>136</td>
<td>Thymidine (U-15N2)</td>
<td>40</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>7</td>
<td>46</td>
<td>46</td>
<td>2'-deoxyuridine (U-15N5)</td>
<td>-a</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>β-alanine</td>
<td>7</td>
<td>16</td>
<td>13</td>
<td>β-alanine (U-15C3;15N)</td>
<td>28</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>β-ureidopropionic acid</td>
<td>7</td>
<td>10</td>
<td>2</td>
<td>β-alanine (U-15C3;15N)</td>
<td>-a</td>
<td>-a</td>
<td>-a</td>
</tr>
<tr>
<td>β-aminoisobutyric acid</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>β-alanine (U-15C3;15N)</td>
<td>-a</td>
<td>-a</td>
<td>-a</td>
</tr>
</tbody>
</table>

SIL, stable isotopically-labelled reference compound.

An appropriate concentration level was chosen for each metabolite/SIL according to their sensitivity in the analysis. The stability (significance of time) of each metabolite/SIL was expressed by their CV% using either a slope- or a combined model. The data handling was conducted with metabolite responses in area units. If the CV% ≤ 10% the stability was considered acceptable over time.

* SIL used for more than one metabolite.
Table 6
The recovery and within- and across-day variation of each metabolite investigated

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration level (µmol/L)</th>
<th>Concentrationa (µmol/L)</th>
<th>Recoveryb (%)</th>
<th>Within-day variationc (CV%)</th>
<th>Across-day variationd (CV%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>4.17</td>
<td>4.33</td>
<td>104</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Guanine</td>
<td>4.13</td>
<td>3.77</td>
<td>91</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Guanosine</td>
<td>4.15</td>
<td>4.13</td>
<td>100</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Inosine</td>
<td>4.18</td>
<td>4.10</td>
<td>98</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>2'-deoxyguanosine</td>
<td>4.15</td>
<td>4.27</td>
<td>103</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>2'-deoxyinosine</td>
<td>4.11</td>
<td>4.23</td>
<td>103</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Xanthine</td>
<td>4.12</td>
<td>4.39</td>
<td>106</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>4.11</td>
<td>4.07</td>
<td>99</td>
<td>1</td>
<td>6</td>
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<tr>
<td>Uric acid</td>
<td>4.08</td>
<td>4.38</td>
<td>78</td>
<td>16</td>
<td>55</td>
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<tr>
<td>Allantoin</td>
<td>41.44</td>
<td>45</td>
<td>107</td>
<td>34</td>
<td>49</td>
</tr>
<tr>
<td><strong>Pyrimidines</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>4.13</td>
<td>4.24</td>
<td>103</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td>Thymine</td>
<td>6.86</td>
<td>6.72</td>
<td>98</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Uracil</td>
<td>4.11</td>
<td>4.33</td>
<td>105</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Cytidine</td>
<td>4.16</td>
<td>6.75</td>
<td>162</td>
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<td>24</td>
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<td>Uracilc</td>
<td>4.12</td>
<td>3.89</td>
<td>94</td>
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<tr>
<td>Thymidine</td>
<td>6.90</td>
<td>8.35</td>
<td>121</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>2'-deoxyuridine</td>
<td>6.86</td>
<td>10</td>
<td>149</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>β-alanine</td>
<td>6.86</td>
<td>7.21</td>
<td>105</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>β-ureidopropionic acid</td>
<td>6.91</td>
<td>6.30</td>
<td>91</td>
<td>14</td>
<td>13</td>
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<tr>
<td>β-aminoisobutyric acid</td>
<td>6.83</td>
<td>6.86</td>
<td>100</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Only four curves were available for uric acid and β-ureidopropionic acid. In the case of allantoin, the three lower concentration levels were excluded to better fit the concentration range of actual samples. For uridine, one observation in one curve was considered an outlier following visual inspection and was rejected. An appropriate concentration level was chosen for each metabolite according to the metabolites sensitivity in the analysis.

a Recovered quantified concentration.
b The recovery (%) was calculated as: (mean recovery concentration/mean spiked concentration) × 100 (n = 8, samples). Recovery (%) was an average of recoveries obtained over five days (m = 5, days).
c The within-day variation (n = 8, samples) expressed as CV%.
d The across-day variation (m = 5, days) expressed as CV%. 
### Table 7
Comparison of the response from the metabolites (stable isotopically-labelled reference compounds) spiked in standard jugular vein plasma with the response obtained in tested plasma samples from four other cows, four other blood vessels, five other animal species and three other matrices, to evaluate relative matrix effect and the application range of the method

<table>
<thead>
<tr>
<th>SIL</th>
<th>Four cows</th>
<th>Four vessels</th>
<th>Five species</th>
<th>Three matrices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 P H R A C P M H R W U M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Purines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine (8-13C)</td>
<td>101 102 106 101</td>
<td>99 93 94 83</td>
<td>84 87 71 116 91</td>
<td>88 47 58</td>
</tr>
<tr>
<td>Guanine (8-13C,7-15N2)</td>
<td>94 91 106 96</td>
<td>92 88 87 79</td>
<td>74 86 67 106 36</td>
<td>94 54 54</td>
</tr>
<tr>
<td>Guanosine (U-13C10,U-15N5)</td>
<td>110 98 99 103</td>
<td>94 115 122 119</td>
<td>110 115 111 112 120</td>
<td>105 93 77</td>
</tr>
<tr>
<td>Inosine (U-15N4)</td>
<td>113 97 102 101</td>
<td>96 116 121 114</td>
<td>110 114 114 115 121</td>
<td>107 83 68</td>
</tr>
<tr>
<td>2'-deoxyguanosine (U-15N5)</td>
<td>110 101 103 105</td>
<td>95 117 121 107</td>
<td>109 116 117 114 123</td>
<td>99 62 2</td>
</tr>
<tr>
<td>Xanthine (1,3-15N2)</td>
<td>88 86 96 93</td>
<td>85 76 77 67</td>
<td>66 76 66 117 78</td>
<td>97 64 67</td>
</tr>
<tr>
<td>Hypoxanthine (15N4)</td>
<td>103 106 101 105</td>
<td>105 103 108 104</td>
<td>103 108 107 114 90</td>
<td>45 13 116</td>
</tr>
<tr>
<td>Uric acid (1,3-15N2)</td>
<td>97 86 107 97</td>
<td>111 112 107 98</td>
<td>100 99 60 102 95</td>
<td>71 68 110</td>
</tr>
<tr>
<td><strong>Pyrimidines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosine (2,4-13C2;15N3)</td>
<td>95 115 100 107</td>
<td>106 116 104 110</td>
<td>90 82 32 138 98</td>
<td>579 43 999</td>
</tr>
<tr>
<td>Thymine (15N2)</td>
<td>97 96 98 100</td>
<td>100 98 101 101</td>
<td>93 106 98 102 97</td>
<td>98 79 95</td>
</tr>
<tr>
<td>Uracil (U-13C4;U-15N2)</td>
<td>104 93 106 101</td>
<td>101 102 103 105</td>
<td>94 106 97 104 106</td>
<td>88 81 100</td>
</tr>
<tr>
<td>Cytidine (U-13C9;U-15N3)</td>
<td>97 83 96 96</td>
<td>95 122 109 104</td>
<td>124 116 99 108 99</td>
<td>170 30 48</td>
</tr>
<tr>
<td>Uridine (U-13C9;U-15N2)</td>
<td>117 107 101 141</td>
<td>98 135 148 127</td>
<td>62 176 87 80 105</td>
<td>35 14 32</td>
</tr>
<tr>
<td>Thymidine (U-15N2)</td>
<td>102 106 98 112</td>
<td>108 125 132 118</td>
<td>126 118 135 123 126</td>
<td>95 72 102</td>
</tr>
<tr>
<td>β-alanine (U-13C3;15N)</td>
<td>105 105 104 101</td>
<td>110 98 107 107</td>
<td>90 96 77 112 99</td>
<td>357 42 149</td>
</tr>
</tbody>
</table>

1, cow 1; 2, cow 2; 3, cow 3; 4, cow 4; P, portal hepatic vein; H, hepatic vein; G, gastrosplenic vein; A, artery; C, chicken; P, pig; M, mink; H, human; R, rat; W, water; U, urine; M, milk.

The relative recovery was calculated as: (tested sample(area) - jugular(area)) x 100 (n = 2, samples). A relative recovery between 85% and 115% was considered good and between 75% and 125% was considered acceptable. Shaded areas show recoveries not fulfilling these criteria.