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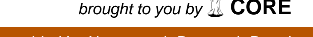

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Published in:
Journal of Chromatography A

DOI:
10.1016/j.chroma.2014.06.065

Publication date:
2014

Citation for published version (APA):
Stentoft, C., Vestergaard, M., Løvendahl, P., Kristensen, N. B., Moorby, J. M., & Jensen, S. (2014). Simultaneous quantification of purine and pyrimidine bases, nucleosides and their degradation products in cow plasma by HPLC tandem mass spectrometry. *Journal of Chromatography A*, *1356*, 197-210. https://doi.org/10.1016/j.chroma.2014.06.065

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1 **Simultaneous quantification of purine and pyrimidine bases, nucleosides and their**

2 **degradation products in bovine blood plasma by high performance liquid chromatography**

3 **tandem mass spectrometry**

4

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21

22

23

24 **Abstract**

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

47 an artery and the portal hepatic, hepatic and gastrosplenic veins and, with a few exceptions, also for
48 other species such as chicken, pig, mink, human and rat.

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

50 **1. Introduction**

51 The global efficiency of nitrogen in animal production is only slightly over 10%, with the result that
52 102 Tg (10^{12} gram) nitrogen is excreted annually (1998 figures) by domesticated animals globally
53 [1]. The nitrogen efficiency in dairy cows is generally low [2], and not only the environment, but
54 also the productive efficiency, would benefit from an optimization of diet and metabolism to
55 improve nitrogen efficiency and utilization [1, 3, 4]. Most research hitherto has focused on refining
56 protein and amino acid utilization, but this has only led to minor improvements in efficiency [4-6].
57 A better understanding of the quantitative absorption and intermediary metabolism of other
58 nitrogenous products such as the purines and pyrimidines (PP), the building blocks of nucleic acids
59 and main constituents of DNA/RNA, could uncover new ways of improving dairy cow nitrogen
60 use-efficiency and propose new feeding strategies [7, 8]. So far, the possible significance of
61 microbial PP in the nutritional physiology of ruminants has not been investigated, regardless of the
62 fact that they correspond to more than 20% of the total microbial nitrogen supply [7-9]. Little is
63 known about the quantitative aspects of PP metabolism. What is known, however, is that the
64 purines go through an effective multistep degradation to uric acid and allantoin, and the pyrimidines
65 are similarly degraded to β-alanine, before excretion [8, 10].

66 Quantitative analysis of PP in dairy cattle research has almost solely focused on purines in urine, as
67 excretion of purine derivatives can be used as an indirect measure of rumen microbial synthesis [11-
68 14]. Most published methods have thus been developed for purine metabolites in urine. Only
69 recently, Boudra et al. (2012) published a method able to quantify the pyrimidine degradation
70 products (DP) β-alanine and β-aminoisobutyric acid as well [14].

71 Different analytical separation methods have been used for determining PP in biological matrices of
72 which the majority has applied high performance liquid chromatography (HPLC) [15-17] or
73 capillary electrophoresis chromatography [17-20]. When high separation selectivity and sensitivity
74 were essential, electrokinetic techniques [16] or ultra high performance liquid chromatography [21]
75 have been used. Concerning detection, spectrometric, electrochemical or mass spectrophotometric
76 detection methods have been used, with ultra violet detection coupled to HPLC being the most
77 common one [15-17]. HPLC coupled with tandem spectrometric detection (LC-MS/MS) is
78 currently considered the method of choice for quantitative analysis of compounds in biological
79 matrices [22] and LC-MS/MS has been shown to be capable of quantifying PP and their derivatives
80 accurately in urine.
81 For this study, we wanted to develop and validate an LC-MS/MS method for quantification of a
82 range of PP and their derivatives in cow blood plasma. Into this procedure, we wanted to
83 incorporate matrix-matched calibration standards as well as stable isotopically-labelled reference
84 compounds (SIL). As no appropriate pre-treatment procedure was identified in the literature, we
85 also wanted to develop a good, stable, simple, component-specific, and repeatable pre-treatment
86 protocol for the plasma samples.
87 Several sets of plasma samples from experiments that attempted to manipulate urea-recycling and
88 increase nitrogen utilization using multicatheterized Danish Holstein cows were employed in the
89 development of this method [23] because these were representative of the types of samples that this
90 method is likely to be used for in the future.
91
92 **2. Materials and Methods**

93 *2.1 Chemicals, reagents and materials*

94 Water quality was at all times secured by treatment on a Millipore Synergy® UV water treatment
95 system from Millipore A.S. (Molsheim, France). Methanol (MeOH) from Poch S.A. (Gliwice,
96 Poland) and ethanol (EtOH 99.9% vol.) from Kemetyl A/S (Køge, Denmark) were of HPLC grade.
97 Formic acid (98-100%) (HCOOH), acetic acid (100%) (CH_3COOH), and ammonium solution
98 (25%) (NH_4OH) from Merck (Darmstadt, Germany) were of analytical reagent grade. Sodium
99 hydroxide (NaOH), also from Merck, was prepared in a 0.01 M aqueous solution. Tricholoroacetic
100 acid (≥99.0%) from Sigma-Aldrich (Brøndby, Denmark) was prepared in a 12% v/v aqueous
101 solution (TCA) daily. Contamination between samples was minimized by the use of disposable
102 materials (vials, bottles etc.) where practicable, or through the use of lab equipment that was
103 cleaned without the use of detergents.
104
105 *2.2 Standards*
106 The following compound standards (bases (BS), nucleosides (NS), DP) were obtained from Sigma-
107 Aldrich (Brøndby, Denmark): adenine, guanine, cytosine, thymine, uracil, adenosine, guanosine,
108 cytidine, uridine, inosine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, thymidine, 2'-
109 deoxyuridine, 2'-deoxyinosine, xanthine, hypoxanthine, uric acid, allantoin, β-alanine, β-
110 ureidopropionic acid and β-aminoisobutyric acid. β-ureidoisobutyric acid, one important
111 intermediate pyrimidine derivate metabolite, was not commercially available and could not be
112 included. No traces of either adenosine or 2'-deoxyadenosine were identified during method
113 development in plasma or urine samples. 2'-deoxycytidine was present in trace amounts but even
114 after extensive optimization the sensitivity remained too low for quantification. These three
115 components were therefore not pursued further. The chemical structures of the targeted metabolites
116 are shown in Table 1.

117 Stable isotopically-labelled reference compounds used as internal standards were purchased from

118 Cambridge Isotope Laboratories (Andover, USA). These were: adenine (8-^{13}C), guanine (8-

119 ^{13}C;7,9-^{15}N2), thymine (^{15}N2), uracil (U-^{13}C4;U-^{15}N2), guanosine (U-^{13}C10;U-^{15}N5), inosine (U-

120 ^{15}N4), cytidine (U-^{13}C9;U-^{15}N3), uridine (U-^{13}C9;U-^{15}N2), 2'-deoxyguanosine (U-^{15}N5), thymidine

121 (U-^{15}N2), xanthine (1,3-^{15}N2), hypoxanthine (^{15}N4), uric acid (1,3-^{15}N2), and β-alanine (U-

122 ^{13}C3;^{15}N). Cytosine (2,4-^{13}C2;^{15}N3) was purchased from Sigma-Aldrich (Brøndby, Denmark). All

123 were ^{13}C and/or ^{15}N labelled with purities of at least 95% (95-99%). Unfortunately, exact SIL were

124 not available for all metabolites studied; a suitable SIL was consequently selected on its similarity

125 to the corresponding metabolite in terms of structure, retention time, fragmentation pattern and

126 group. Individual stock solutions of all compound standards and SIL were prepared and kept at -

127 80°C. Bases and purine DP were diluted in water and NS and pyrimidine DP were diluted in 0.01 M

128 NaOH solution. Two stock concentrations of 500 and 5,000 µmol/L were made for each compound

129 standard. The exception was for uric acid and allantoin, where the stock concentration was

130 500/2,000 µmol/L and 500/40,000 µmol/L, respectively. For SIL only the low concentration stock

131 was prepared. All stocks were filtered through 0.45 µm PALL GHP Membrane syringe filters

132 purchased from VWR (Herlev, Denmark) and kept at -20°C in dark vials. Appropriate dilutions of

133 these solutions were made in water to produce standard mixtures and SIL mixtures for external

134 calibration and quantification.

135

136 **Table 1**

137

138 *2.3 Samples*

139 A number of 5 ml aliquots of heparinized plasma to be used for external calibration and quality

140 control were prepared from two liters of venous blood [23] drawn from a Danish Holstein dairy cow

141 fed a traditional total mixed ration. Experimental plasma samples were obtained from a feeding
142 experiment [24] with multicatheterized dairy cows [25,26]. This set of samples was drawn from
143 four blood vessels simultaneously, representing blood from an artery and the portal hepatic, hepatic
144 and gastrosplenic veins. Additional test plasma samples were obtained on site for relative matrix
145 effect evaluations. These samples were from five other species (chicken, pig, mink, human, and rat)
146 for between species comparisons, four multicatheterized cows (jugular vein) for intraspecies
147 comparisons, and bovine urine and milk samples for matrix effect evaluations.
148
149 *2.4 Pre-treatment*
150 Before pre-treatment, plasma samples for quantification of uric acid and uracil were diluted twenty-
151 fold (5% v/v) and four-fold (25% v/v) in water, respectively. This was, in the case of uric acid, to
152 avoid a non-linear calibration curve with the very high uric acid concentrations in all samples, and,
153 in the case of uracil, to be able to distinguish the small uracil signal from the pronounced
154 background noise. Pre-treatment: Plasma samples were defrosted and immediately put on ice. The
155 sample (300 µL) was then added to a SIL mixture and a water/standard mixture (550 µL total vol.)
156 before being precipitated with 1.8 mL ice-cold ethanol (10 min., on ice, -20°C). This was followed
157 by centrifugation (15 min., 5,500 × g, 4°C). The supernatant was ultrafiltered on a Pall Nanosep
158 10K, Omega membrane spin filter purchased from VWR. A 500 µL aliquot of filtered supernatant
159 was dried down under a flow of nitrogen on a SuperthermTM fitted with a Mini Oven for AI blocks
160 and evaporator with valves from Mikrolab A/S (Aarhus, Denmark) in conical autosampler vials
161 from VWR until dryness (app. 75 min., room temp.). The pellet was re-suspended in 100 µL cold
162 solvent (A) (30 min., 4°C) and transferred to a clean dark LC-vial. Matrix-matched external
163 calibrators were treated similarly to standard plasma. Milk samples were cleared with ice-cold TCA

164 12% (end 50% v/v) before pre-treatment. Urine samples were handled as plasma samples

165 throughout.

166

167 *2.5 LC-MS/MS analysis*

168 Chromatographic separation was performed on an Agilent 1100 series HPLC system (Agilent

169 Technologies, Hørsholm, Denmark) with a Synergi™ Hydro-RP LC Column (250 mm × 2 mm, 4

170 µm) protected by a conventional guard column of the same material purchased from Phenomenex

171 (Værløse, Denmark). Samples were analyzed in five separate runs, three in negative electrospray

172 (ESI) mode and two in positive ESI mode. The five groups of metabolites and their

173 chromatographic profiles are shown in Table 2. Separation was performed using a gradient solvent

174 system. For each run, HPLC solvents were freshly prepared and cleared on a 0.45 µm Pall

175 hydrophilic polypropylene membrane filter purchased from VWR. Both solvents (A) and (B) were

176 prepared from a 0.05 mol/L acetic acid buffer containing 10% or 50% methanol, respectively. The

177 acetic acid buffer was prepared by adjusting 0.05 mol/L acetic acid to pH 4.0 with ammonium

178 solution and readjusting to pH 2.8 with formic acid. The following elution gradient was used: initial

179 percentage of solvent B was 5%, this was raised to 100% in 8 min and kept there for 6 min, then

180 lowered to 5% in 30 sec, after which it was kept constant for 3.5 min to re-equilibrate the column

181 prior to the next injection. The flow rate was 200 µL/min and the injection volume was 5 µL. The

182 column temperature was maintained at 30°C while the auto sampler temperature was set to 4°C to

183 stabilize the samples during time-consuming analyses. The total run time was 18 min per sample.

184

185 **Table 2**

186

187 A Waters (Hedehusene, Denmark) micromass triple quadropole mass spectrometer was used for
188 electrospray mass spectrometric analyses using massLynx 4.0 (Waters) software for data collection
189 and processing. Capillary voltage was set to 3.2 kV, source temperature to 120°C, and desolvation
190 temperature to 400°C. The cone and desolvation gas flows (nitrogen and argon) were set at 29 and
191 628 L/hour, respectively. Fragment ion spectra were recorded in both polarities and promising
192 selective fragment ions were tested and optimized along with the cone voltage in multiple-reaction
193 monitoring (MRM) mode. The values of the tune parameters were optimized by separately infusing
194 a solution (500 µmol/L) of each metabolite in its mobile phase at a flow rate of 10 µL/min. The
195 MRM transitions and the applied cone voltages and collision energies are summarized in Table 3.
196 Common transitions were originated from the loss of HCN, NH_3, ribose, deoxyribose, HNCO,
197 $HNCONH_2$ and H_2O fragments for the various PP metabolites (Table 1). The most intense
198 transition reaction was used for quantification (Table 3). Data were collected in centroid mode with
199 a constant dwell time of 0.05 sec and an interscan delay of 0.02 sec.
200
201 **Table 3**
202
203 *2.6 Calibration and quantification*
204 Quantification was performed by matrix-matched external calibration applying standard plasma
205 spiked with a two-fold serial dilution of mixed standard solutions to obtain seven different
206 concentration levels of each compound. The only exception was with uracil where a two-third-fold
207 serial dilution was applied. Standard plasma (not spiked) was used for subtraction and quality
208 control but was not included in the regression analysis. In general, all samples and calibrators were
209 analyzed in duplicate and a standard curve and quality control samples were analyzed at the
210 beginning and at the end of each sequence. The response was calculated as the chromatographic

211 peak area for all compounds. When applying standard plasma, which contained unknown quantities

212 of the metabolites under investigation, the measured metabolite response was initially normalized

213 and the response from the standard plasma was subtracted. The mean of the measured SIL

214 responses/SIL area for each sample was used as the normalization factor. During method

215 development the focus of work was on quantifying as low concentrations of metabolite as possible.

216 Matrix-matched calibration curves, within the relevant concentration ranges given in Table 4, were

217 generated for each metabolite at four (allantoin) or seven concentration levels on five consecutive

218 days for determining and evaluating the calibration model. As noted previously, uric acid and uracil

219 were quantified from diluted samples. The coefficient of variation (CV%) for each concentrate level

220 was then calculated for a logarithmic and a linear calibration model to test the use of log-log

221 transformation. The linearity of the log calibration curves were studied with a lack of fit hypothesis

222 test. Subsequently, the homogeneity of variance was estimated for each concentration by plotting

223 the CV% against log(concentration) and the quantification range set to the lowest and highest

224 quantified concentration giving a CV% below 25%.

225

226 **Table 4**

227

228 *2.7 Validation procedure*

229 The method was validated according to reports from the “Analytical methods validation:

230 bioavailability, bioequivalence and pharmacokinetic studies” conferences held in Washington in

231 1990 [27] and 2000 [28], as described by Peters et al. [29]. It was validated with respect to

232 assessment of selectivity, stability, precision, recovery, and matrix effect.

233 *Selectivity:* Metabolite and SIL cross-talk was evaluated by analyzing the standard compounds

234 alone and together with their corresponding SIL (no blank matrix was available). Three groups were

235 studied and their signals compared; a compound standard group (10% v/v, 50 µmol/L), a SIL group
236 (10% v/v, 50 µmol/L), and a combined group (5% v/v, 25 µmol/L). Analyses of BS/DP and NS
237 were carried out separately.
238 *Stability:* For continuous evaluation of long-term storage stability, a fresh quality control sample
239 was analyzed in all analytical runs. The stability within runs (6-24 h) was evaluated in two ways.
240 First, a quality control sample was analyzed at the beginning and at the end of each sequence (data
241 not shown). Secondly, a set of spiked standard plasma samples were analyzed at five different times
242 (different vials) during a 30 hour sequence. Analysis of variance (ANOVA) using linear mixed
243 models procedures was used to test the stability over time, both with a trend element and with
244 random changes over and above the linear trend (regression line) [30,31]. Applying ANOVA, the
245 across-day variation of the PP calibration curves (intercepts and slopes as interactions with test day)
246 was assessed over five consecutive days and expressed by their P-values. The stability during
247 repeated freeze-thaw cycles was not explored since all plasma samples in the present study were
248 only thawed once.
249 *Precision and recovery:* Precision of the method, in terms of within-day variation (repeatability)
250 and across-day variation (intermediate precision), was determined by analyzing replicate sets of
251 spiked standard plasma samples on five separate days expressed as their CV%. The absolute
252 recoveries were calculated using the same set of spiked standard plasma, at one level, by comparing
253 the obtained concentrations with the initial spiked level.
254 *Matrix effect:* Early tests with spiked water, urine and plasma samples revealed large variations in
255 matrix effect-induced signal suppression and enhancement between the metabolites included in the
256 analysis. Following optimization of the pre-treatment procedure, these matrix effects were
257 evaluated as the difference between samples of water and standard plasma, urine or milk samples
258 spiked with constant amounts of SIL before pre-treatment. Thus, we took advantage of the fact that

259 the incorporated SIL should behave as their matching metabolite in the ESI source [27]. The
260 conventional strategy of spiking a blank matrix sample with a compound standard was again not
261 possible as completely blank matrices were not available for these metabolites. The applied SIL-
262 based method was a modified version of the conventional method to evaluate matrix effect
263 described by Matuszewski et al. [32]. The observed matrix effect was rendered insignificant by
264 utilizing matrix-matched external calibration.
265
266 *2.8 Application*
267 To determine the application range of the method, the relative matrix effect was evaluated by
268 comparing the response from PP SIL spiked in standard jugular vein plasma with the response in
269 test plasma samples. Four different sets of samples were assessed. First, plasma from the jugular
270 vein of four multicatheterized cows was used to investigate within-species variation. Next, plasma
271 drawn from the portal vein, the hepatic vein, the gastrosplenic vein, and an artery from a
272 multicatheterized dairy cow to represent different possible sampling sites were examined. Third,
273 plasma samples from different species (chicken, pig, mink, human, rat) were used for between-
274 species evaluation. Finally, water, urine and milk samples were used to compare different matrices.
275 The relative recovery determined which of the tested matrices were suitable for the method. For the
276 same reasons as described previously, SIL replaced compound standards. Water, urine and milk
277 samples were evaluated in the same manner as plasma samples.
278
279 **3. Results and discussion**

280 *3.1 Method development*
281 The aim of this study was to develop a quantitative LC-MS/MS analysis and a sample pre-treatment
282 procedure for the simultaneous analysis of several metabolites of the PP metabolism in blood

283 plasma of dairy cows. The chemical properties of the metabolites were polar due to high contents of
284 –OH, =O and –N groups. Based on their polarity, they were roughly divided into three groups: The
285 *very polar group*, containing β-alanine, β-aminoisobutyric acid and β-ureidopropionic acid, were all
286 small molecules with similar linear polar structures, as well as the also highly polar allantoin,
287 cytosine and cytidine. The *polar group* included the majority of the BS, such as adenine, guanine
288 and uracil, as well as the intermediate DP with more base-like structures, such as uric acid, xanthine
289 and hypoxanthine. Finally, the *semi polar group* comprised the majority of the NS with large but
290 semi-polar sugar side groups, such as most of the ribonucleosides (2× -OH) and
291 deoxyribonucleosides (1× -OH). Owing to their very non-polar methyl side groups, thymine and
292 thymidine were also placed in the semi polar group. The very polar metabolites were poorly
293 retained on the C_{18} column with the aqueous solvents and eluted first as expected, offering a longer
294 retention time of the less polar components.
295
296 *3.1.1 Pre-treatment development and evaluation*
297 An effective clean-up procedure is crucial when performing LC-MS/MS analysis as this diminishes
298 cross-talk [33,34] as well as matrix effects [35] and at the same time enhances both the selectivity
299 and the sensitivity of the analysis [29]. A novel multi-step approach, consisting of protein
300 precipitation, ultrafiltration, evaporation under nitrogen flow, and subsequent resolution, able to
301 purify and to concentrate all of the studied metabolites from bovine plasma simultaneously, in a
302 simple and efficient manner, was developed and optimized.
303 Initially, different solvents (acetone, acetonitrile, ethanol, methanol, sulfo-salicylic acid) were tested
304 for precipitation (data not shown). Ethanol precipitation resulted in the highest recoveries and least
305 noise when comparing chromatographic responses and this less harmful solvent was therefore
306 chosen for the procedure. The ultrafiltration step was added as this step caused markedly lower

307 levels of background noise. As a consequence of the approximately eight-fold dilution during pre-
308 treatment, evaporation and reconstitution steps were included. Overall this resulted in a 1.4 times
309 concentration effect. To try to reduce degradation and instability of the samples caused by reactive
310 oxygen species or enzyme activities during pre-treatment, all centrifugations and incubations were
311 performed at 4°C and samples, stocks, and solvents etc. were kept at -4°C or on ice. Only during
312 evaporation were the samples maintained at room temperature. Other types of pre-treatment
313 methods such as simple dilution (impractical), solid phase extraction (different chemical properties)
314 [36,37] and accelerated solvent extraction [38] were also investigated (data not shown) but were not
315 found useful.
316 The effectiveness of the pre-treatment and the stability of the metabolites during the multiple steps
317 were evaluated during validation of the method, described in *section 3.3*, and demonstrated the
318 ability of this pre-treatment to purify and concentrate all of the targeted PP simultaneously in an
319 easy and efficient manner without significant losses. To our knowledge, no other publications have
320 presented a similar and effective pre-treatment procedure, as most other approaches include dilution
321 of the samples.
322
323 *3.1.2 LC-MS/MS procedure*
324 Based on the chemical properties of the targeted metabolites, experiences from similar studies
325 [14,39], and available equipment, a reversed-phase C_{18} column known to be able to quantify the
326 majority of the studied metabolites from urine was applied with an acetic acid buffer/methanol
327 HPLC solvent system.
328 To achieve adequate separation and elution order, a series of conditions were modified and
329 implemented. The composition of the acetic acid buffer and the methanol extraction solvent was
330 based on the work of Hartmann et al. (2006) [39], and no other types of solvent were tested. Having

331 tested several acetic acid buffer to methanol ratios (95%, 90%, 85%, and 80% v/v), assessing peak
332 separation and shapes, it was concluded that the best separation was accomplished with a 90% v/v
333 solvent (A) and 50% v/v solvent (B). The chosen injection volume, 5 µL, and flow rate, 200
334 µL/min, was found by assessing the same parameters, testing first injections of 5, 10, 20 µL and
335 then flow rates of 100, 200, 300 and 400 µL/min. Concerning the elution gradient, we strived to
336 make it as short as possible, while still achieving as good a peak separation as possible. Different
337 elution profiles were tested, with more or less steep gradients. The final profile, described in *section*
338 *2.5*, gave a total run time of 18 min. By adding a small amount of methanol to the otherwise
339 aqueous solvent (A), and, by keeping the baseline at 5% solvent (B), the solvent mixing became
340 more smooth and transitions between runs became more stable. A major improvement in precision
341 between runs was achieved by maintaining the column temperature at 30°C instead of 25°C. An
342 improvement in the sample stability during the time-consuming analyses was achieved by cooling
343 the auto-sampler to 4°C. In the end, useful combinations of retention times and peak shapes of each
344 metabolite were achieved with the parameters described, and the method was therefore adapted and
345 brought on to further validation.
346
347 *3.2 The log-calibration model and quantification range*
348 Calibration curves were prepared by linear regression of log(area) against log(concentration) (log-
349 calibration) and by linear regression in linear units on both axes (linear calibration) to verify the use
350 of the log-calibration model. Initially, the normality of residuals around the calibration lines were
351 inspected visually (Q-Q plot) and found to be approximately normal. The CV% for each
352 concentration level for both the log-calibration and the linear calibration is illustrated in figure 1. A
353 large group of the PP (panel I) considerably improved their CV% profiles using the log-calibration,
354 especially in the low ranges. However, a smaller group of PP (panel II) did not benefit from the log

355 transformation; and the transformation did not weaken as their CV% profiles either. Exceptions
356 were with allantoin, β-ureidopropionic acid, cytosine and β-alanine, their CV% at the high end of
357 their profiles were better without the log-log transformation. Given that quantification at low
358 concentrations was considered to be most important, these findings validated the use of log-log
359 transformation in the analysis of all the applied PPs. Performing a lack of fit test, the linearity of the
360 PP calibration curves were evaluated and expressed by their *P*-values (Table 4). None of the PP
361 curves resulted in a significant lack of fit except uridine, which had a very low sensitivity in the
362 analysis, demonstrating a satisfying log-log regression.
363 The homogeneity of variance for the different concentration levels is illustrated in Figure 2 and the
364 quantification ranges (CV<25%) in Table 4. Focusing on the lower concentration range, most of the
365 PP demonstrated a typical precision profile where the CV% decreased with higher concentration
366 levels. All purines had acceptable variation levels around the lowest concentration levels except
367 allantoin, which should not be quantified at concentrations below ~100 µmol/L. The pyrimidine BS
368 and cytidine and uridine had larger CV%'s with acceptable lower concentration levels from 0.66-
369 5.15 µmol/L. Thymidine and 2'-deoxyuridine demonstrated a very large variation with CV%'s
370 above 25% over the entire concentration range. In the case of the pyrimidine DP, they were
371 reasonably stable over their concentration ranges, not counting β-alanine which only had a
372 CV%<25% at its highest calibrator. The upper part of the quantification range was in all cases the
373 highest quantified calibrator.
374
375 **Figure 1, Figure 2**
376
377 *3.3 Method validation*

378 Once the pre-treatment, LC-MS/MS procedure, and calibration model had been set, the
379 performance characteristics of the method were established by validation with spiked standard
380 plasma. In terms of quantification purposes, selectivity, stability, precision, recovery, and matrix
381 effects were evaluated.
382 The most intensive fragment ion from each precursor ion was selected as the transition ion for
383 detection and quantification. Positive identification was based on the correlation of retention time
384 with the standards and the selected precursor/product transition. Less intensive second transitions
385 were used for confirmation. All metabolites generated single peak shapes.
386
387 *3.3.1 Selectivity*
388 A blank sample for selectivity evaluation was not available for these naturally occurring plasma
389 metabolites. Hence, the presence of chromatographic peaks from standard plasma at the same
390 retention times as the targeted metabolites could not be excluded; such endogenous peaks would be
391 expected to be present. Instead, the absence of standard compound/SIL cross-talk contributions was
392 verified by comparing chromatographic responses for standards and SIL alone and in a mixture
393 (data not shown). It was important to assess cross-talk contributions, as some of the applied SIL
394 (Table 3) had less than three mass unit differences (3-8) to the natural metabolite, which is normally
395 recommended as the lowest mass unit difference for LC-MS/MS analysis [33,34].
396
397 *3.3.3 Stability*
398 Good stability was achieved by optimizing the pre-treatment and LC-MS/MS parameters as
399 described in *section 3.1*. Long-term storage stability was tested by comparing chromatographic
400 profiles of quality control standard plasma on a daily basis. Within-run stability was evaluated by
401 analyzing a control sample at the beginning and end of each sequence. Long sequence run times

402 have been of concern and the within-run stability was consequently also evaluated by performing
403 ANOVA for measurements made at times 0, 7, 15, 22 and 29 hours, during a 30 hour sequence with
404 triplicate determinations at each time-point, using either a slope model: y_{ij} = intercept +
405 b*time_hour + ε_{ij}, or a combined model: y_{ij} = intercept + time_i + b*time_hour + ε_{ij}, where y_{ij} is the
406 area measured in the sample at time i, replicate j, and b is the slope of the area change per hour, and
407 ε_{ij} is the random error term. Significance of the time effects were tested using an F-test with type 1
408 sum of squares. Residual mean square error was calculated as the square of the residual variance
409 estimate and expressed as CV%. The metabolite responses were normalized as usual but the SIL
410 responses were not since they could not be used to normalize themselves. The results are given in
411 Table 5.

412 In general, the combined model resulted in lower CV%'s than the slope model, as the irregular time
413 effect was also taken into consideration in the combined model. All but a few metabolites
414 demonstrated very stable profiles over the 30 hour time span with CV%≤10%. Exceptions were
415 thymidine (136%), 2'-deoxyuridine (46%) and β-alanine (13%), where especially the former two
416 were found to be unstable. This was probably due to low sensitivities in the analysis. The SILs were
417 found to be equally or more stable than their corresponding metabolites probably due to their higher
418 spike concentrations. As expected, thymidine (U-$^{15}N_2$) and β-alanine (U-^{13}C3;^{15}N) had the same
419 instability issues as their partners. No 2'-deoxyuridine SIL was applied in this analysis.
420 Surprisingly, the uracil and cytidine SIL had CV%'s above 10%. In the case of uracil (13%),
421 excessive degradation was avoided by always placing uracil samples in the beginning of a
422 sequence.

423 To assess the stability of the calibration curves between run-days, ANOVA was conducted
424 determining the across-day (intermediate precision) precision (Table 4). Most PP demonstrated a
425 significant (P<0.05) difference between test days on either curve intercept or slope, or at least a

426 tendency (P<0.1). Exceptions were with allantoin, cytosine, uridine, thymidine and 2'-
427 deoxyuridine, all of which revealed reasonably stable curves over test days. These results
428 demonstrated the need for renewing calibration curves on a daily basis.
429
430 **Table 5**
431
432 *3.3.4 Precision and recovery*
433 To ensure correct quantification and to evaluate analytical precision, within-day and across-day
434 variation was determined by studying replicate sets of spiked standard plasma samples (n = 8,
435 samples) on five separate days (m = 5, days). Here, precision was defined as the degree to which
436 repeated measurements under unchanged conditions showed the same result, expressed as the CV%.
437 Absolute recoveries were identified by using the same set of spiked standard plasma samples,
438 comparing the recovered quantified concentrations with the initial spiked concentrations. Since
439 linearity ranges were short and close to zero, a single, instead of the traditional three, recovery
440 concentration levels was chosen. Precision and recovery outcomes are given in Table 6. The
441 obtained results showed very good extraction efficiency and precision. The recoveries were
442 between 91% and 107%, except for uric acid with a lower recovery of 78%. Also, the low
443 sensitivity and accompanying instability of cytidine, thymidine and 2'-deoxyuridine was again
444 highlighted with recoveries of 162%, 121%, and 149%, respectively. In general, the within- and
445 across-day variations mirrored the recovery results. The exceptions were with allantoin and
446 cytosine, both of which had good recoveries, 107% and 103%, but exhibited large CV%'s, within-
447 day variation 34% and 21%, and across day variation 49% and 24%, respectively.
448
449 **Table 6**
450

451 *3.3.5 Absolute matrix effect*

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

466 Recognizing that the nature of matrix effects is varying and the sensitivity between metabolites are
467 very different the sizes of the bars are relative indicators of the degree of suppression or
468 enhancement. Signal enhancement was observed in plasma for almost all metabolites, and only a
469 few, such as inosine, cytidine, β-alanine and cytosine, had their signals suppressed. These
470 metabolites did not share any obvious similarities in polarity or structure; however, matrix effects
471 are known to be very compound-dependent [22]. In contrast to the signal enhancement generally
472 encountered in plasma, in urine all metabolite signals were suppressed. This demonstrates the
473 different matrix effects a given component experience when present in different matrices in LC-

474 MS/MS analysis. In milk, only the purines had a common pattern, i.e., signal suppression, and the
475 remaining metabolites were neither suppressed nor enhanced.
476 Matrix effects can vary between measurements, hence, it is not possible to test for matrix effects
477 only once and consider it to be constant [43]. Matrix effects were largely eliminated in the analysis
478 first of all by making the external calibrators matrix-matched, hence, quantifying calibrators and
479 sample metabolites under the same conditions, secondly, by implementing a very effective pre-
480 treatment [33, 44], and thirdly, by implementing SIL [22, 42]. Matrix-matching is necessary when
481 specific SILs are not available for all metabolites [42]. These initiatives compensated quite well for
482 the signal suppression or enhancement in the plasma samples, thereby achieving accurate
483 quantification.
484
485 **Figure 3**
486
487 *3.4 Analytical application (relative matrix effect)*
488 This LC-MS/MS analysis was established for quantification of 20 target metabolites of the PP
489 metabolism in blood plasma samples from multicatheterized cows. Since jugular vein plasma
490 (representing systemic circulating blood) was used for method development and because
491 quantification relied on matrix-matched calibration (jugular vein plasma), the relative matrix effect
492 was evaluated in alternative types of plasma. The relative matrix effect was evaluated by comparing
493 the response from SIL spiked in standard jugular vein plasma with the response in tested plasma
494 samples. A relative recovery between 85% and 115% was considered good and between 75% and
495 125% acceptable, hence, tested samples exerted the same matrix effect on the metabolite as the cow
496 jugular vein plasma sample. The generosity of 75-125% was due to the small sample size (n = 2

497 samples) inevitably resulting in less precision. The PP responses given as recovery (%) are depicted
498 in Table 7.
499 First of all, it was confirmed that within-species variation was not an issue with any of the
500 metabolites examined, except for uridine. Secondly, the results demonstrated that all the examined
501 metabolites, evaluated in all four plasma types from feeding experiments with multicatheterized
502 cows with this particular type of cow model, could appropriately be quantified with the developed
503 LC-MS/MS method. Only xanthine (67%), uridine (135%/148%/127%) and thymidine (132%)
504 displayed recoveries outside the acceptable range of 75-125% and especially thymidine will be hard
505 to quantify with this method due to other issues anyway. Surprisingly, the between-species range
506 was very broad and most metabolites could be evaluated in plasma from other species tested with a
507 few exceptions. Further confirmed was also the results from section 3.3.5, concluding that matrix
508 effects varied significantly between different types of matrices such as water, plasma, urine and
509 milk. Hence, it is necessary to design, optimize and validate a specific LC-MS/MS method for each
510 applied matrix.
511
512 **Table 7**
513
514 **4. Conclusions**
515 This work presents the development and validation of a new method for simultaneous and accurate
516 quantification of 20 targeted metabolites of PP metabolism with different structures and physio-
517 chemical properties in blood plasma from dairy cows. Exceptions were with cytidine, thymidine
518 and 2'-deoxyuridine, where the method's sensitivity for these three PP metabolites was so low that
519 they caused imprecise quantification over the examined concentration ranges. The metabolites were
520 purified and concentrated using a novel multi-step pre-treatment procedure consisting of protein

521 precipitation, ultrafiltration, evaporation under nitrogen flow, and subsequent reconstitution. This
522 procedure ensured efficient recoveries for most investigated metabolites and efficient removal of
523 interfering matrix components. The method is selective, sensitive, stable, and precise. The potential
524 application of the method was demonstrated by evaluating its range of use in different types of
525 blood plasma from multicatheterized cows, here, only uridine, showed undesirable matrix effects.
526 The method is adaptable and can be further developed for the quantitative detection of the same
527 metabolites in other matrices such as urine or milk.
528

529 **Acknowledgements**
530 We gratefully acknowledge Lis Sidelmann, Birgit Hørdum Løth and the barn staff at Department of
531 Animal Science, Aarhus University, Foulum, Denmark for skillful technical assistance. Steven
532 Lock, Application manager EMEA at ABSCIEX, is recognized for his assistance in assessing
533 MS/MS fragmentation patterns. We also thank senior scientists Torben Larsen and Peter Lund for
534 supplying plasma samples for analytical application experiments. C. Stentoft holds a PhD
535 scholarship co-financed by the Faculty of Science and Technology, Aarhus University and a
536 research project supported by the Danish Milk Levy Fond, c/o Food and Agriculture, Aarhus N,
537 Denmark. Funding for the cow animal experiments from which some of the plasma samples were
538 obtained was partly provided by the Commission of the European Communities (Brussels, Belgium;
539 Rednex project FP7, KBBE-2007-1).
540

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653

654 **Figure captions**

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

662 uridine; β-ure, β-ureidopropionic acid; β-ami, β-aminoisobutyric acid; Cyt, cytosine; Thy, thymine;

663 Ura, uracil; Cyd, Cytidine; Thd, thymidine; dUrd, 2'-deoxyuridine; β-ala, β-alanine.

664

665 Fig. 2. The homogeneity of variance for the different concentration levels of the purine and

666 pyrimidine calibration curves divided into bases, nucleosides and degradation products (CV%).

667 Abbreviations for the 20 metabolites: Ade, adenine; Gua, guanine; Guo, guanosine; Ino, inosine;

668 dGuo, 2'-deoxyguanosine; dIno, 2'-deoxyinosine; Xan, xanthine; Hyp, hypoxanthine; Uac, uric

669 acid; All, allantoin; Urd, uridine; β-ure, β-ureidopropionic acid; β-ami, β-aminoisobutyric acid; Cyt,

670 cytosine; Thy, thymine; Ura, uracil; Cyd, Cytidine; Thd, thymidine; dUrd, 2'-deoxyuridine; β-ala,

671 β-alanine.

672

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

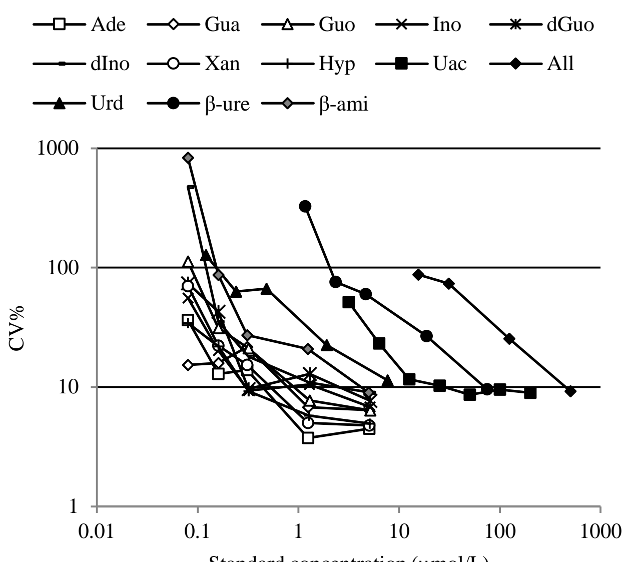
674

675

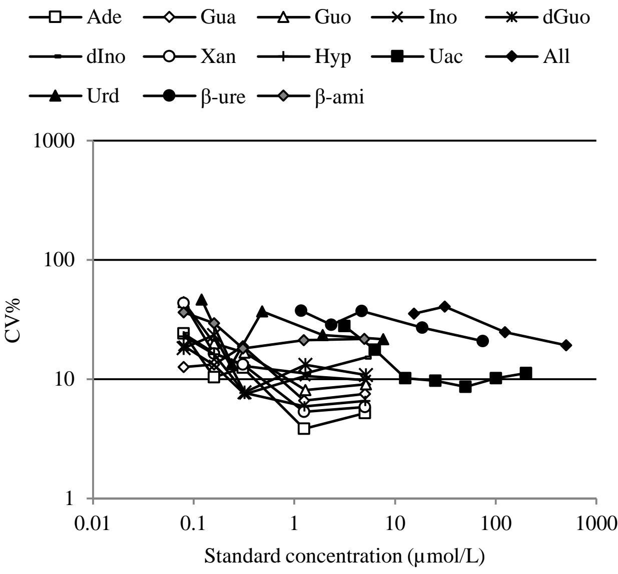
Highlights

- 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

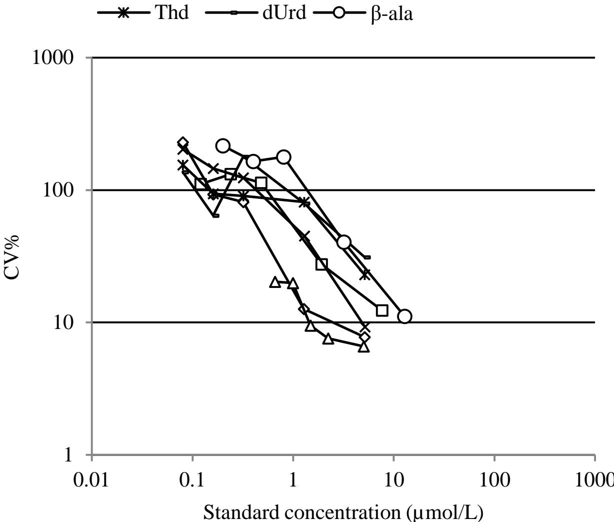
Linear calibration I



Log-calibration I



Linear calibration II



Log-calibration II

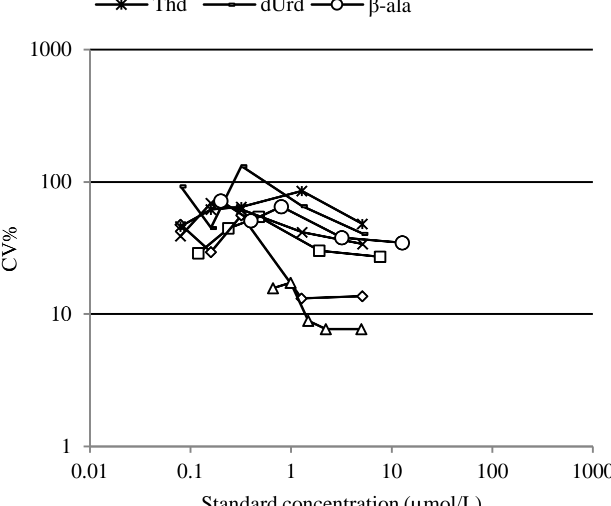


Figure 2

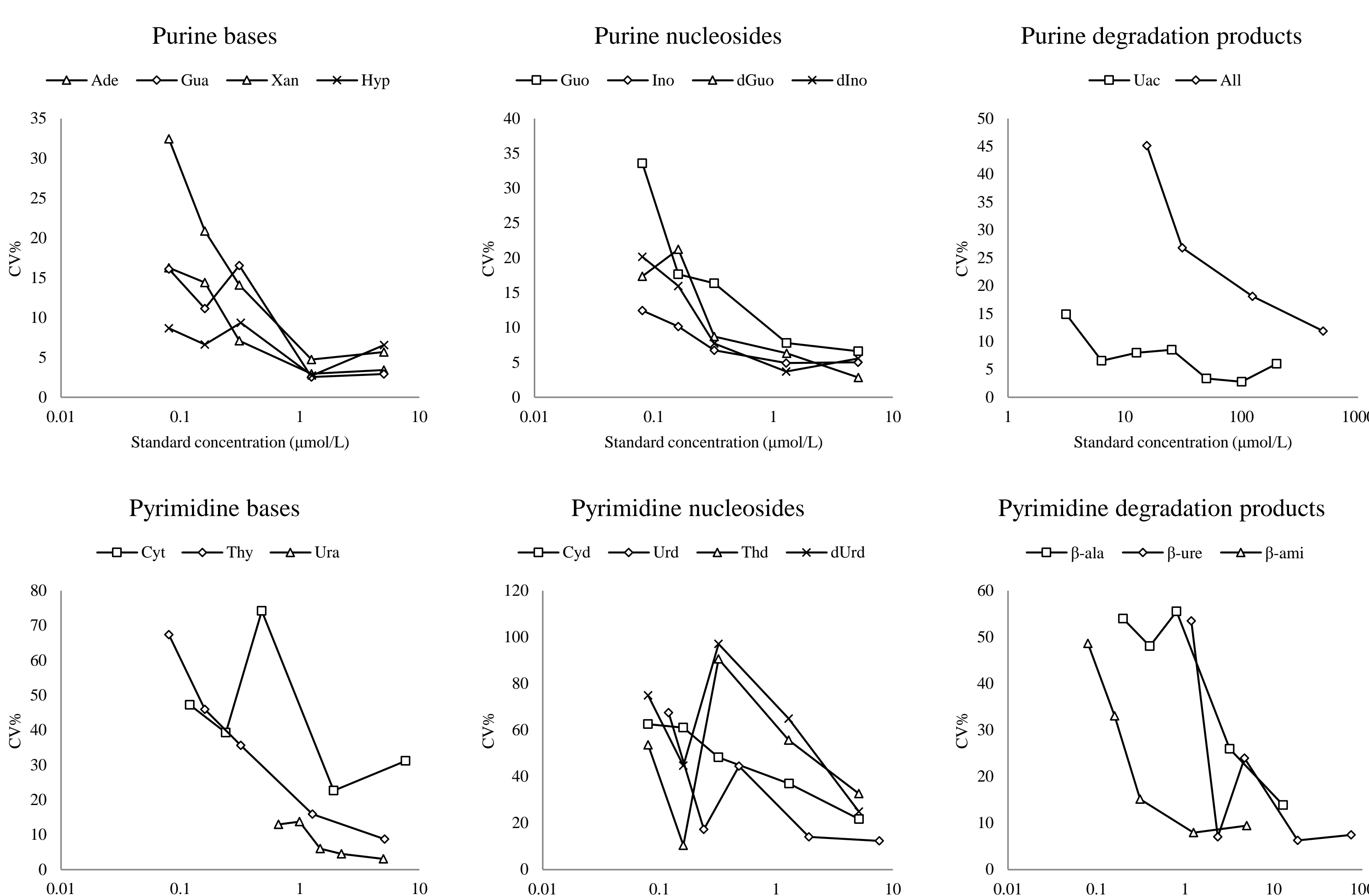
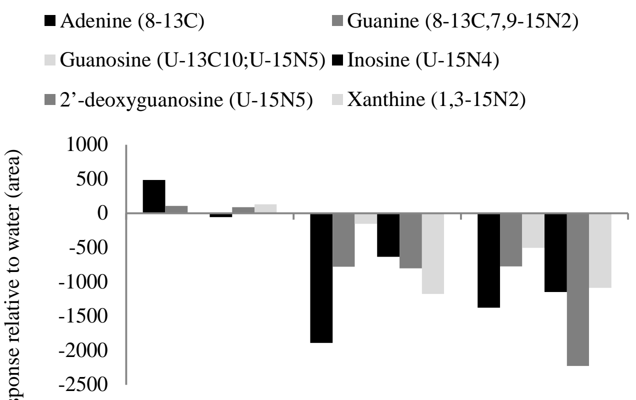
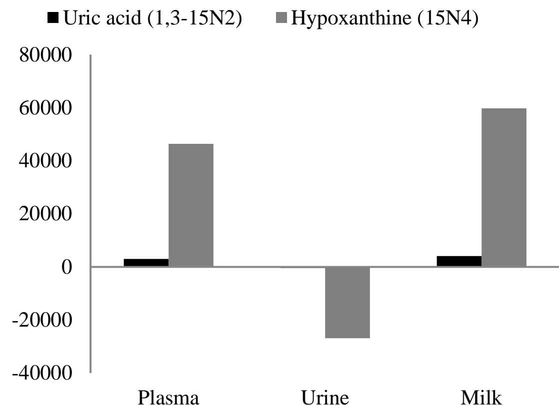
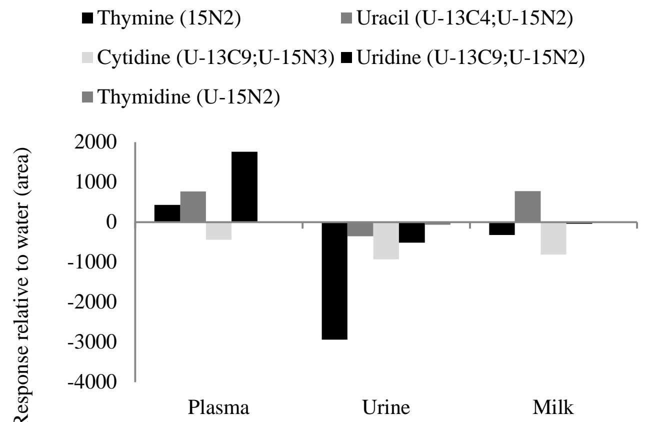


Figure 3







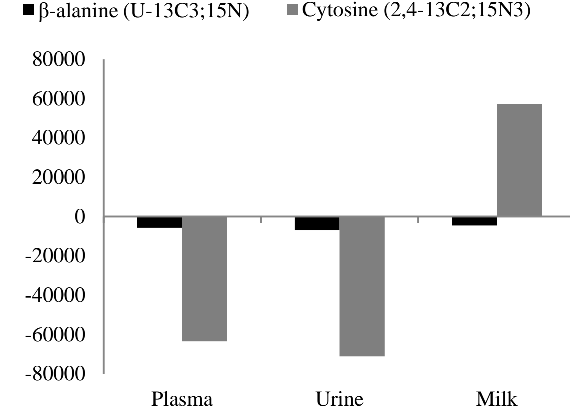
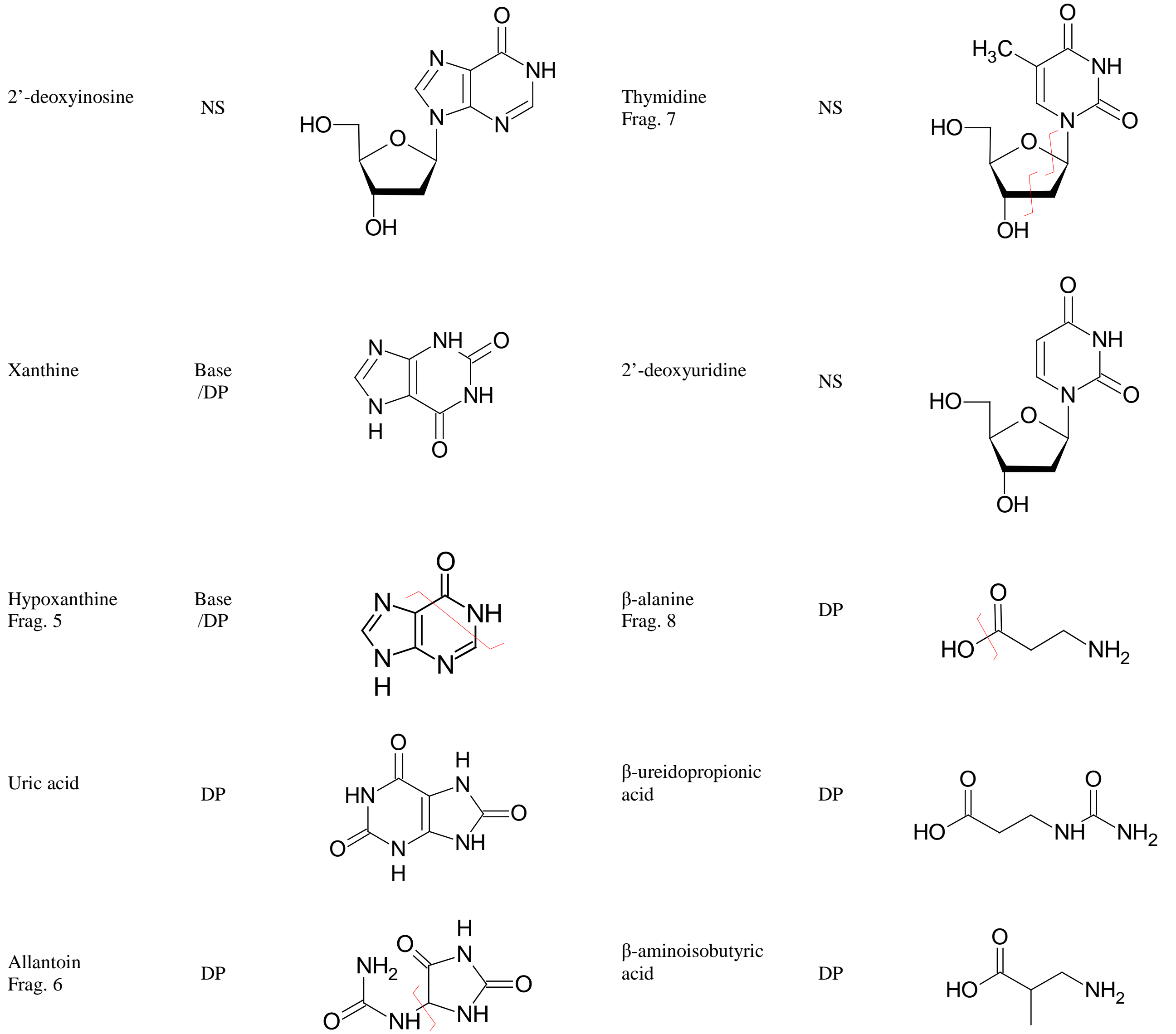


Table 1

Names, types, empirical formulae and suggestions for fragmentations of the compounds analyzed by the LC-MS/MS method

Purines			Pyrimidines		
Name	Type	Empirical formula	Name	Type	Empirical formula
Adenine Frag. 1	Base		Cytosine	Base	
Guanine Frag. 2	Base		Thymine	Base	
Guanosine Frag. 3	NS		Uracil	Base	
Inosine	NS		Cytidine	NS	
2'-deoxyguanosine Frag. 4	NS		Uridine	NS	



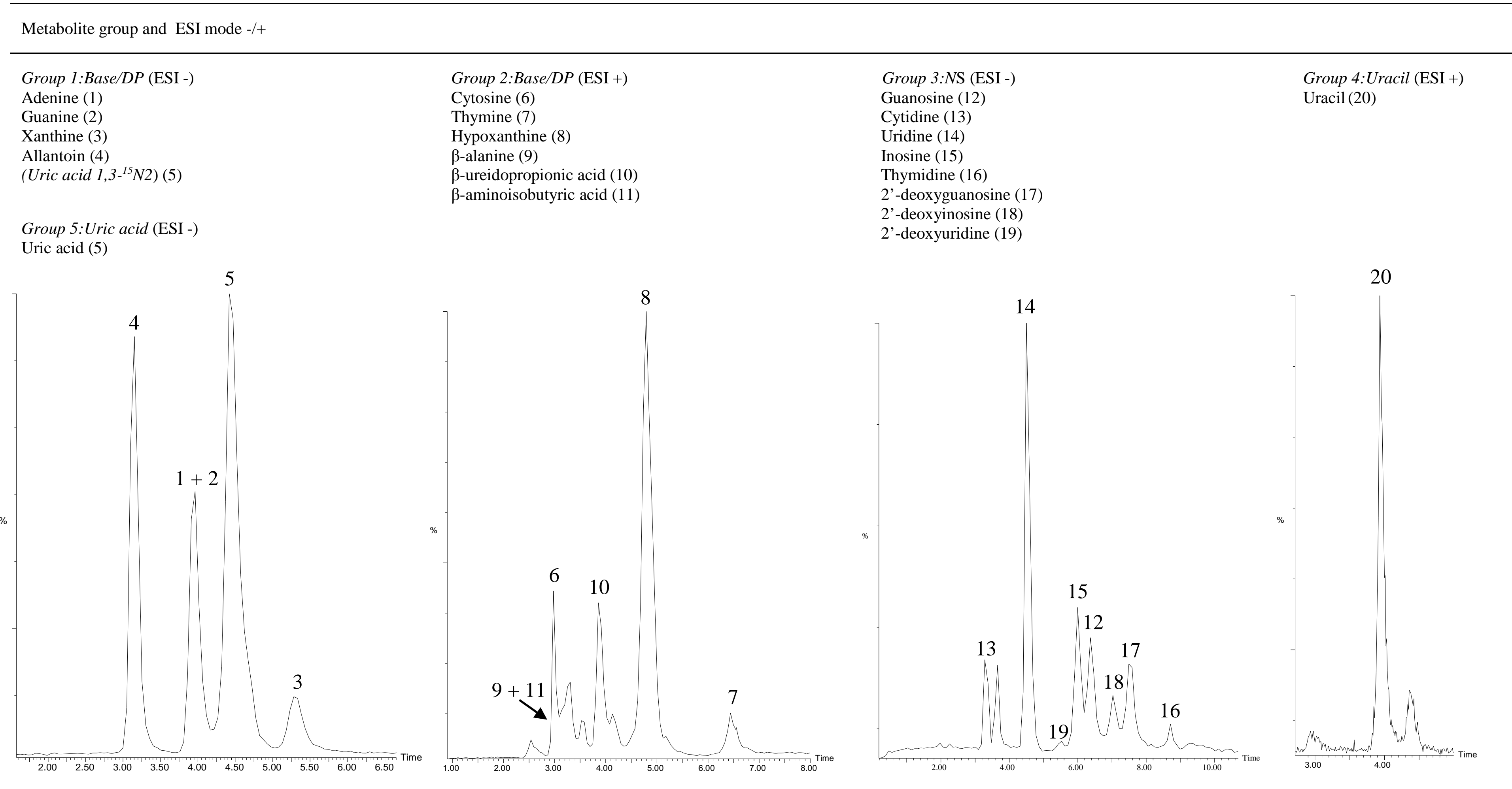
Metabolite	Type	Metabolite	Type
2’-deoxyinosine	NS	Thymidine Frag. 7	NS
Xanthine	Base /DP	2’-deoxyuridine	NS
Hypoxanthine Frag. 5	Base /DP	β-alanine Frag. 8	DP
Uric acid	DP	β-ureidopropionic acid	DP
Allantoin Frag. 6	DP	β-aminoisobutyric acid	DP

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



Metabolite group and ESI mode -/+			
Group 1:Base/DP (ESI -) Adenine (1) Guanine (2) Xanthine (3) Allantoin (4) *(Uric acid 1,3-^{15}N2)* (5) *Group 5:Uric acid* (ESI -) Uric acid (5)	*Group 2:Base/DP* (ESI +) Cytosine (6) Thymine (7) Hypoxanthine (8) β-alanine (9) β-ureidopropionic acid (10) β-aminoisobutyric acid (11)	*Group 3:NS* (ESI -) Guanosine (12) Cytidine (13) Uridine (14) Inosine (15) Thymidine (16) 2'-deoxyguanosine (17) 2'-deoxyinosine (18) 2'-deoxyuridine (19)	*Group 4:Uracil* (ESI +) Uracil (20)

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-^{15}N2) 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

Metabolite/SIL	Mw (g/mol)	Retention time (min)	Precursor ion (*m/z*)		Cone voltage (V)	Product ion (*m/z*)	Collision energy (eV)	Neutral loss (NL)	Fragmentation 1-8	
Purines										
Adenine/	135.13	3.81	134	-	35	107	16	27	- HCN	1
Adenine (8-^{13}C)	136.12		135		36	108	17			
Guanine/	151.13	3.86	150	-	28	133	13	17	- NH_3	2
Guanine (8-^{13}C,7,9-^{15}N2)	154.11		153		30	136	13			
Guanosine/	283.24	6.18	282	-	33	150	19	132	- deoxyribose	3
Guanosine (U-^{13}C10;U-^{15}N5)	298.13		297		33	160	20	137		
Inosine/	268.23	5.81	267	-	26	135	20	132	- deoxyribose	3
Inosine (U-^{15}N4)	272.20		271		26	139	20			
2'-deoxyguanosine/	267.24	7.31	266	-	26	150	19	116	- ribose	4
2'-deoxyguanosine (U-^{15}N5)	272.17		271		28	155	20			
2'-deoxyinosine/	252.23	6.74	251	-	27	135	20	116	- ribose	4
2'-deoxyguanosine (U-^{15}N5)[a]	-	-	-		-	-	-			
Xanthine/	152.11	5.18	151	-	29	108	16	43	- HNCO	5
Xanthine (1,3-^{15}N2)	154.10		153		31	109	16	44		
Hypoxanthine/	136.11	4.56	135	+	34	92	16	43	- HNCO	5
Hypoxanthine (^{15}N4)	140.09		141		34	113	19	27	- HCN	1
Uric acid/	168.11	4.28	167	-	26	124	16	43	- HNCO	5
Uric acid (1,3-^{15}N2)	170.10		169		29	125	14	44		
Allantoin/	158.12	3.05	157	-	16	97	16	60	- $HNCONH_2$	6
Uric acid (1,3-^{15}N2)[a]	-	-	-		-	-	-			
Pyrimidines										
Cytosine/	111.95	2.91	112	+	29	95	20	17	- NH_3	2
Cytosine (2,4-^{13}C2;^{15}N3)	116.08		117		30	99	19	18		
Thymine/	126.11	6.21	127	+	27	110	7	17	- NH_3	2
Thymine (^{15}N2)	128.10		129		27	111	16	18		
Uracil/	112.09	3.97	113	+	26	96	7	17	- NH_3	2
Uracil (U-^{13}C4;U-^{15}N2)	118.04		119		27	101	16	18		
Cytidine/	243.22	3.19	242	-	23	109	14	133	- deoxyribose	3
Cytidine (U-^{13}C9;U-^{15}N3)	255.13		254		21	116	15	138		
Uridine/	244.20	4.50	243	-	23	110	15	133	- deoxyribose	3
Uridine (U-^{13}C9;U-^{15}N2)	255.12		254		28	116	16	138		
Thymidine/	242.23	8.52	241	-	25	151	12	90	- rearrangement	7
Thymidine (U-^{15}N2)	244.22		243		26	153	11			
2'-deoxyuridine/	228.20	5.34	227	-	22	184	12	43	- HNCO	5
2'-deoxyguanosine (U-^{15}N5)[a]	-	-	-		-	-	-			
β-alanine/	89.09	2.91	90	+	13	72	10	18	- H_2O	8
β-alanine (U-^{13}C3;^{15}N)	93.07		94		14	76	7			

β-ureidopropionic acid/	132.12	3.77	133	+	11	115	10	18	- H_2O	8
β-alanine (U-^{13}C3;^{15}N) [a]	-	-	-		-	-	-			
β-aminoisobutyric acid/	103.12	2.98	104	+	13	86	10	18	- H_2O	8
β-alanine (U-^{13}C3;^{15}N) [a]	-	-	-		-	-	-			

SIL, stable isotopically-labelled reference compound.

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

[a] 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

Metabolite		Range[a]		Linearity		Precision (test-day)[d]	
	Type	Concentration levels	Calibration range (µmol/L)	Lack of fit[b] *P*-value	Quantification range[c] (µmol/L)	Intercept *P*-value	Slope *P*-value
Purines							
Adenine	Base	7	0-5.0	0.84	0.08-5.0	0.096	0.059
Guanine	Base	7	0-5.0	0.15	0.08-5.0	0.041	0.994
Guanosine	NS	7	0-5.0	0.79	0.16-5.0	0.071	0.003
Inosine	NS	7	0-5.0	0.23	0.08-5.0	0.013	0.004
2'-deoxyguanosine	NS	7	0-5.0	0.06	0.08-5.0	0.029	0.294
2'-deoxyinosine	NS	7	0-5.0	0.92	0.16-5.0	<0.001	0.021
Xanthine	Base/DP	7	0-5.0	0.67	0.16-5.0	0.087	0.006
Hypoxanthine	Base/DP	7	0-5.0	0.40	0.08-5.0	0.009	<.001
Uric acid	DP	7	0-200	0.99	3.15-200	<.001	0.003
Allantoin	DP	4	15-500	0.64	124-500	0.427	0.897
Pyrimidines							
Cytosine	Base	7	0-7.5	0.84	1.92-7.5	0.566	0.274
Thymine	Base	7	0-5.0	0.68	1.27-5.0	0.035	0.030
Uracil	Base	7	0-5.0	0.88	0.66-5.0	<0.001	0.042
Cytidine	NS	7	0-5.0	0.70	5.15-5.0	0.086	0.670
Uridine	NS	7	0-7.5	0.02	1.91-7.5	0.286	0.480
Thymidine	NS	7	0-5.0	0.48	- [e]	0.741	0.599
2'-deoxyuridine	NS	7	0-5.0	0.35	- [e]	0.151	0.309
β-alanine	DP	7	0.25-13	0.59	13-13	<0.001	0.070
β-ureidopropionic acid	DP	7	0-75	0.87	4.67-75	0.003	0.283
β-aminoisobutyric acid	DP	7	0-5.0	0.29	0.31-5.0	0.026	0.571

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

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

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.

[a] SIL used for more than one metabolite.

Table 6

The recovery and within- and across-day variation of each metabolite investigated

Metabolite	Concentration level (µmol/L)	Concentration[a] (µmol/L)	Recovery[b] (%)	Within-day variation[c] (CV%)	Across-day variation[d] (CV%)
Purines					
Adenine	4.17	4.33	104	2	5
Guanine	4.13	3.77	91	2	4
Guanosine	4.15	4.13	100	4	12
Inosine	4.18	4.10	98	2	9
2'-deoxyguanosine	4.15	4.27	103	4	7
2'-deoxyinosine	4.11	4.23	103	2	8
Xanthine	4.12	4.39	106	3	9
Hypoxanthine	4.11	4.07	99	1	6
Uric acid	4.08	4.38	78	16	55
Allantoin	41.44	45	107	34	49
Pyrimidines					
Cytosine	4.13	4,24	103	21	24
Thymine	6.86	6.72	98	4	15
Uracil	4.11	4.33	105	5	4
Cytidine	4.16	6.75	162	18	24
Uridine[c]	4.12	3.89	94	7	12
Thymidine	6.90	8.35	121	23	21
2'-deoxyuridine	6.86	10	149	33	37
β-alanine	6.86	7.21	105	12	5
β-ureidopropionic acid	6.91	6.30	91	14	13
β-aminoisobutyric acid	6.83	6.86	100	6	7

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

SIL	Four cows				Four vessels				Five species					Three matrices		
	1	2	3	4	P	H	R	A	C	P	M	H	R	W	U	M
Purines																
Adenine (8-^{13}C)	101	102	106	101	99	93	94	83	84	87	71	116	91	88	47	58
Guanine (8-^{13}C,7,9-^{15}N2)	94	91	106	96	92	88	87	79	74	86	67	106	36	94	54	54
Guanosine (U-^{13}C10;U-^{15}N5)	110	98	99	103	94	115	122	119	110	115	111	112	120	105	93	77
Inosine (U-^{15}N4)	113	97	102	101	96	116	121	114	110	114	114	115	121	107	83	68
2'-deoxyguanosine (U-^{15}N5)	110	101	103	105	95	117	121	107	109	116	117	114	123	99	62	2
Xanthine (1,3-^{15}N2)	88	86	96	93	85	76	77	67	66	76	66	117	78	97	64	67
Hypoxanthine (^{15}N4)	103	106	101	105	105	103	108	104	103	108	107	114	90	45	13	116
Uric acid (1,3-^{15}N2)	97	86	107	97	111	112	107	98	100	99	60	102	95	71	68	110
Pyrimidines																
Cytosine (2,4-^{13}C2;^{15}N3)	95	115	100	107	106	116	104	110	90	82	32	138	98	579	43	999
Thymine (^{15}N2)	97	96	98	100	100	98	101	101	93	106	98	102	97	98	79	95
Uracil (U-^{13}C4;U-^{15}N2)	104	93	106	101	101	102	103	105	94	106	97	104	106	88	81	100
Cytidine (U-^{13}C9;U-^{15}N3)	97	83	96	96	95	122	109	104	124	116	99	108	99	170	30	48
Uridine (U-^{13}C9;U-^{15}N2)	117	107	101	141	98	135	148	127	62	176	87	80	105	35	14	32
Thymidine (U-^{15}N2)	102	106	98	112	108	125	132	118	126	118	135	123	126	95	72	102
β-alanine (U-^{13}C3;^{15}N)	105	105	104	101	110	98	107	107	90	96	77	112	99	357	42	149

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.