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Simultaneous quantifications of four purine derivatives biomarkers in cow milk by SPE HPLC-DAD

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Abstract: In this study, simultaneous quantification of allantoin, uric acid, xanthine, and hypoxanthine in cow milk by solid phase extraction (SPE) and high performance liquid chromatography-diode array detection (HPLC-DAD) method was perform. Five different SPE cartridges were tested in order to evaluate the isolation of purine derivatives (PD) from cow milk. Chromatography was carried out on ODS-2 Hypersil column and 0.05 M $(NH_4)_2HPO_4$ buffer solution (pH = 7.76) as mobile phase. The HPLC-DAD validated method showed a linearity with regression coefficients higher than 0.999 and the limits of detection and quantification with values in the range 0.09–0.74 μg mL^{-1} and 0.27–2.24 μg mL^{-1}, respectively. The method showed good precision with a relative standard deviation (RSD) below 4.48%, while the accuracy ranged from 95.34 to 104.47% for all analytes. The best recovery degree of PD by SPE were obtained on Strata SCX cartridge for xanthine (87.79%) and hypoxanthine (89.02%); on Strata NH_2 for allantoin (35.09%) and on Strata C8 for uric acid (101.08%). Finally, the HPLC-DAD method with SPE on SCX cartridges was applied to quantify the PD in a batch of thirty cow milk samples.

Keywords: solid phase extraction; high performance liquid chromatography-diode array detection; ruminant milk; PD; method validation

Purine derivatives (PD) in urine or milk are biomarkers of the microbial protein synthesis in rumen, an important factor of the feeding efficiency in ruminants (Kazemi-Bonchenari et al. 2011). PD, including allantoin, uric acid, xanthine, and hypoxanthine, are found in urine and milk to varying extents (Tiemeyer et al. 1984). Many studies have suggested that urinary and mammary excretion of PD by ruminants could be used as an index of rumen microbial protein yield in dairy cows (Tas and Susenbeth 2007; Larsen and Moyes 2010). According to Timmermans Jr. et al. (2000), the excretion of allantoin and uric acid in milk ranged from 15.7 to 2.1 mmol day^{-1}. Contribution of PD in urine and milk to total PD excretion is approximatively constant in lactating Holstein cows (Kazemi-Bonchenari et al. 2011). Beside the use of PD as markers of microbial protein synthesis in rumen, uric acid in milk is believed to increase the oxidative stability of milk and dairy products (Østdal et al. 2000).

Reversed-phase high performance liquid chromatography (HPLC) is currently the most commonly used technique for the analysis of PD in various biological fluids (e.g. milk, urine, human serum) (Indyk and Woollard 2004; Cooper et al. 2006; Zuo et al. 2008, 2015).

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HPLC-diode array detection (HPLC-DAD) methods were used for the estimation of ruminal microbial protein supply in sheep based on the purine concentrations in urine and blood (Czauderna and Kowalczyk 2004; Remane et al. 2015). Recently, ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method was used to monitoring allantoin as a biomarker of oxidative stress in humans (Kozlik et al. 2020) and for analysis of 12 endogenous purine in rat plasma (Lu et al. 2016).

It is well known that monitoring of compounds that are present in trace levels, such as PD in milk, usually requires a preliminary step of isolation and/or enrichment of analytes. Thus, solid phase extraction (SPE) technique plays a crucial role in samples pre-treatment for the purification and enrichment of analytes of interest from complex matrices (Płotka-Wasylka et al. 2016).

For example, the hypercrosslinked strong cation-exchange polymer (HXLPP-SCX) sorbents enabled the selective extraction of three major purine metabolites in human serum followed by HPLC quantification (Xu et al. 2016). Another SPE sorbent based on microspheres of poly(glycidyl methacrylate-divinylbenzene) was used in analysis HPLC-MS/MS of three alkylated-purine adducts in human urine (Hu et al. 2018). Also, simultaneous determination of 23 purines and pyrimidines in biological samples was developed on SPE-UHPLC-MS/MS (Liu et al. 2019). Cu^{2+} functionalized Fe_3O_4@polydopamine core-shell microspheres used as adsorbent in magnetic SPE-HPLC is applicable for rapid extraction and determination of purines from biological samples (Zhou et al. 2019).

The aim of this paper was to simultaneous quantification of allantoin, uric acid, xanthine, and hypoxanthine (Figure 1) in cow milk by using a SPE procedure followed the HPLC-DAD analysis. This implies testing of five different SPE cartridges in order to evaluate their applicability for the isolation of PD from cow milk. Then, a batch of thirty milk samples was HPLC analysed in order to evaluate the PD biomarkers. The PD quantification in cow milk allows their use as reliable biomarkers of the microbial protein synthesis in rumen, an important factor of the feeding efficiency in ruminants, with strong implications on farms profitability and their environmental fingerprint.

MATERIAL AND METHODS

Chemicals and materials. The PD standards (allantoin, uric acid, xanthine, and hypoxanthine) were purchased from Acros Organics (Thermo Fisher Scientific Inc., USA). Acetonitrile (ACN, HPLC-grade) was purchased from Merck (Germany) and $(NH_4)_2HPO_4$ was purchased from Chimopar (Romania). Deionised HPLC grade water (18.2 MΩ cm) was prepared by Simplicity UV (Millipore, USA). The SPE Strata cartridges, SCX, Strata-X; NH_2, C18-E, and C8, were purchased from Phenomenex (Muso, Romania).

Milk samples collection. The milk samples were collected from multiparous dairy cows, with a milk yield of ~25 L day^{-1}, using an autosampler included in the milking parlour equipment. The milk aliquots were kept on ice (in refrigerator) during the handling, and then stored at –20 °C until analyses.

Preparation of standard solution. Stock solution of standard mixture of PD (1 mg mL^{-1} each) was prepared in water at pH = 7.76, adjusted with 1 N NaOH solution. The solution was kept in the refrigerator at 4 °C, and were stable for three weeks. Working standard solutions were prepared as needed by appropriate dilution of the concentrated stock solution in water.

SPE Method. The extraction procedure was performed on SPE System (Phenomenex, Muso, Romania). The SPE cartridges were priorly conditioned with 2 × 1 mL of ACN and equilibrated with 2 × 1 mL of water. Prior to being applied to the extraction cartridge, equal volumes of the milk sample, buffer solution and ACN (for protein precipitation) were mixed thorou-

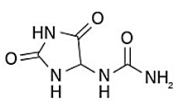
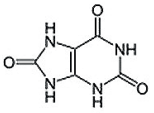
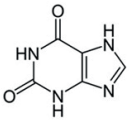
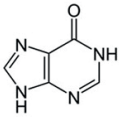
Parameters	Allantoin	Uric acid	Xanthine	Hypoxanthine
Structure				
Chemical formula	$C_4H_6N_4O_3$	$C_5H_4N_4O_3$	$C_5H_4N_4O_2$	$C_5H_4N_4O$
Molar mass (g mol^{-1})	158.117	168.112	152.110	136.112
Acidity (pKa)	8.48	5.60	7.53	8.70
Log*P*	–3.14	–1.107	–0.73	–1.10

Figure 1. Structure and properties of studied purine derivatives (PD)

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$$\text{SPE recovery} = \frac{\text{Amount of analyte after SPE extraction}}{\text{Initial amount of analyte}} \times 100\ (\%) \quad (1)$$

$$\text{HPLC recovery} = \frac{\text{Detected amount} - \text{original amount}}{\text{Added amount}} \times 100\ (\%) \quad (2)$$

ghly in a centrifuge tube, and left still for 5 min and centrifuged for 20 min at 4 400 rpm. The supernatant (2 mL) was loaded onto a SPE cartridge and drawn through on SPE System. Then the SPE cartridge with samples was washed with 2 × 1 mL of water and dried in air flow for two minutes. PD retained on cartridges were eluted with 2 × 500 µL of 0.05 M $(NH_4)_2HPO_4$ buffer solution, filtered and injected in HPLC.

The recoveries of SPE extraction of the PD (Equation 1) were determined by the analysis of the standards mixture of PD at concentrations of 3.125 and 12.5 µg mL^{-1}, due to it is not known the initial amount of PD in milk.

HPLC-DAD method. Chromatography was performed on a HPLC (Agilent Technologies 1200 Series, USA), equipped with quaternary gradient pump, autosampler, degasser, column thermostat, DAD detector. The system and data were controlled by ChemStation software (B.03.01, 2007).

Separation of PD were carried out on ODS-2 Hypersil column (250 × 4.6 mm, I.D., 5 µm) (Thermo Scientific, USA) at 25 °C. Mobile phase was 0.05 M $(NH_4)_2HPO_4$ buffer solution, pH = 7.76. Flow rate was 1 mL min^{-1} and injection volume was 20 µL. The UV detection wavelengths for allantoin, uric acid, xanthine, and hypoxanthine were 218, 292, 276, and 255 nm, respectively.

The HPLC-DAD method was validated for selectivity/specificity, sensitivity, linearity, precision accuracy, and robustness according to the ICH (2005).

Selectivity/specificity was assessed by evaluating the potential interference with endogenous compounds. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the ratio of standard deviation (SD) of response and slope of calibration curve, of three and ten times respectively. Linearity calibration curves was assessed based on plot of analyte peak area against analyte concentration. Calibration range was between 3.125–100 µg mL^{-1} from each PD. The precision and accuracy of method were evaluated on milk samples after SPE extraction and spiked with three concentration levels of PD. Intra-day precision was estimated on six replicates of same sample, spiked with 3.125, 12.5, and 50 µg mL^{-1} concentration during the same day. Inter-day precision was determined by analysis of three replicates spiked with three different concentrations levels for three consecutive days. Both parameters were evaluated by the relative standard deviations (RSDs). The accuracy of the HPLC method was determined by recovery test (Equation 2) (Marson et al. 2020). The milk sample (after SPE extraction) were spiked with standard mixture of 3.125, 12.5, and 50 µg mL^{-1} of PD.

Robustness of the method was determined by observing the changes in different experimental conditions, in the pH of mobile phase (± 0.1), flow rate (± 0.1 mL min^{-1}), and column temperature (± 2 °C) for each PD and evaluated from the RSD of the peak area for each PD.

Statistical analyses. The overall statistical differences among recovery values corresponding to the five cartridges were tested using the General Linear Model (GLM) procedure of Minitab (Minitab 16, UK), with the analysed concentration (3.125 and 12.5 µg mL^{-1}) and the analysed individual PD as controlled factors. Tuckey post-hoc test was used in order to compare the recovery value of each cartridge with every other cartridge. Statistical significance was declared at $P < 0.05$; when P ranged between 0.05 and 0.1 the differences were declared as tendencies.

RESULTS AND DISCUSSION

SPE procedure of milk samples. SPE is a powerful and effective technique for the removal the interfering matrix components, the isolation of the analytes of interest, and the concentration of samples, prior to HPLC or gas chromatography (GC) analysis. With the well-selected sorbent, the octanol/water coefficient (log$P$) and acidity constants (pKa) of studied analytes, must be taken into account for selection of an appropriate extraction solvent (Mitra et al. 2018).

In this study, ACN, buffer solution, and its mixtures were tested as extraction solvents. It has been found that by using ACN (solvent stronger than the mobile phase) the peaks of uric acid and allantoin were splits. Thus, the elution with buffer on the cartridges was chosen.

Five types of SPE cartridges were tested for PD extraction from milk. Thus, for two standard solutions of 3.125 and 12.5 µg mL^{-1} were calculated the recovery

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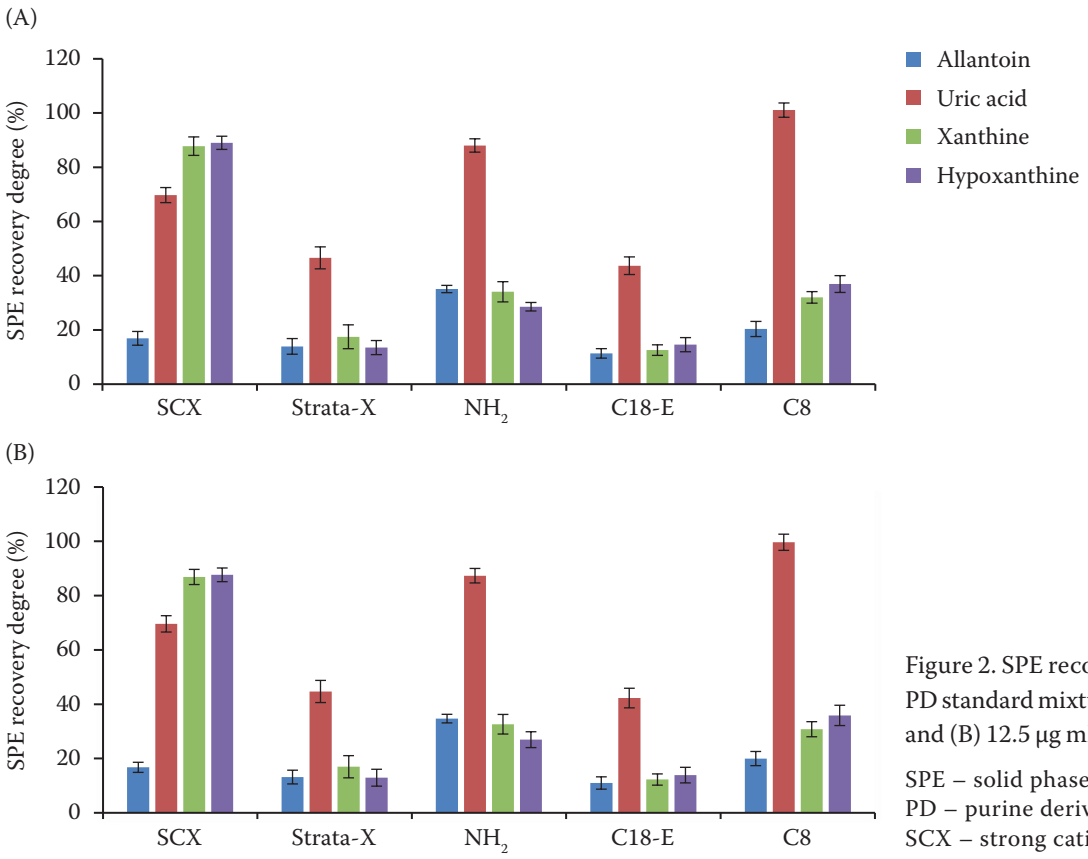

Figure 2. SPE recovery degrees of PD standard mixtures of (A) 3.125 and (B) 12.5 μg mL^{-1} (mean ± SD)

SPE – solid phase extraction; PD – purine derivatives; SCX – strong cation exchange

degrees (Figure 2). Because it is not known the initial amount of PD in milk, we chose to study the degree of recovery by SPE using two standard mixtures of PD of different concentrations.

The results show that the best recoveries of PD from milk on Strata SCX cartridge were of 86.87 and 86.87% for xanthine and 87.66 and 89.02% for hypoxanthine. Similar results were obtained by Xu et al. (2016) on HXLPP-SCX cartridges. Instead, the allantoin shows a lowest recoveries comparative with the other purines, with values of 34.7% and 35.09% on Strata NH_2. At the same time on the others cartridges the recoveries obtained is much smaller. The low recoveries of allantoin indicated that this was not retained on SPE because of its high water solubility (log*P* = –3.14). Allantoin is not well retained on most SPE due to polar character, but by using strongly alkaline conditions can be facilitate retention of allantoin on anion-exchange matrices (Gruber et al. 2009). In our case, using buffer solution (slightly alkaline) for the allantoin elution on Strata NH_2 anion-exchange cartridge, causes a higher recovery than on the other cartridges. For uric acid the best recoveries of 99.65% and 101.08% were obtained on Strata C8 cartridge, that is specific to extraction of compounds with moderately polarity like uric acid (log*P* = –1.107; pKa = 5.6) and stable in basic solutions (Xinhua 2006).

Overall, for the simultaneous analysis of PD in milk, it was observed that higher recoveries were obtained on SCX cartridge for PD mixture compared to other cartridges (Figure 1). For this reason the SCX cartridge was chosen for further analysis of batch of milk samples. The mean of recovery degree for each PD was applied to determine the amounts from selected milk samples.

The statistical analysis using GLM showed that the recovery rates of PD on SPE cartridges varied upon the nature of the PD but also upon the type of cartridge ($P < 0.001$). Figure 3 shows the significance differences among recoveries corresponding to the five cartridges (Tuckey post-hoc test). SCX cartridge had the highest mean recovery of 65.56% followed of C8 and NH_2 cartridges with an average recovery of 47.08 and 45.93%, respectively. The lowest recoveries were obtained on Strata-X and C18-E cartridges of ≤ 22.41%. On the other hand, the concentration of the standard mixtures did not influence the recovery rates.

HPLC-DAD method validation. The HPLC-DAD method, developed by Vlassa et al. (2009), was im-

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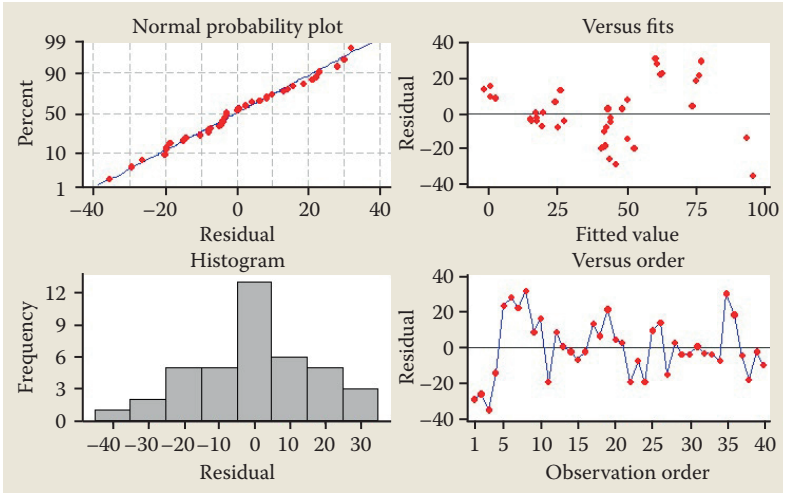

	Mean Rd ± SD
Strata SCX	65.56[a] ± 31.56
Strata-X	22.41[bc] ± 16.15
Strata NH_2	45.93[abc] ± 27.58
Strata C18-E	20.20[c] ± 14.80
Strata C8	47.08[ab] ± 34.07
SEM	6.541
P-value	$P < 0.001$

Figure 3. Statistical parameters and residual plots for SPE recovery degree (Rd) of PD

[a, b, c]Mean values which do not have a common superscript letter are significantly different ($P < 0.05$); PD – purine derivatives; SPE – solid phase extraction; SEM – standard error of the mean; SCX – strong cation exchange

proved, optimised and validated for analyses of PD in cow milk. For method validation, the parameters like selectivity/specificity, linearity, sensitivity, accuracy, precision, and robustness were assessed according to ICH (2005).

Selectivity/specificity was tested by comparing the retention times of chromatograms of PD standard solution, the milk sample (Figure 4), and standard solution added in milk sample. A good separation of each PD can be observed, whereas no interferences occurred.

Linearity and sensitivity of HPLC-DAD method are presented in Table 1. Calibration curves were constructed from peak areas of six standard solutions of PD versus their concentrations and regression coefficients (R^2) were higher than 0.9998. The obtained LOD (0.09–0.74 μg mL^{-1}) and the LOQ (0.27–2.24 μg mL^{-1}) show a good sensitivity of method.

The intra-day and inter-day precisions (Table 2) were expressed as RSD percentage. The precision data showed that RSD percentage varied between 1.01 and 2.91 for intra-day precision and between 1.57 and 4.48 for inter-day precision, respectively. This values are within the limits of acceptable variability in method analysis (ICH 2005).

The accuracy (recovery test; Table 2) of the HPLC-DAD method was assessed on three levels of PD concentration and ranged from 95.34 to 104.47%. All the above data have shown that the HPLC-DAD method has good accuracy and precision.

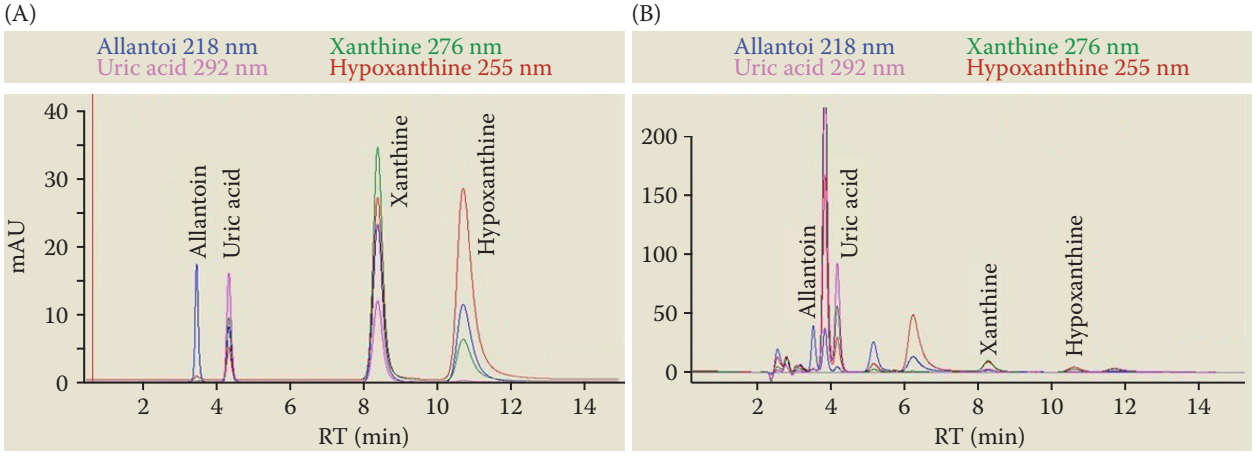

Figure 4. HPLC chromatograms of PD (A) standards mixture (12.5 μg mL^{-1} each) and (B) milk sample

HPLC – high performance liquid chromatography; PD – purine derivatives

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Table 1. HPLC-DAD calibration curve parameters for standard PD

Parameters/compound	Allantoin	Uric acid	Xanthine	Hypoxanthine
Retention time (min)	3.25	4.36	8.61	11.81
Regression equation (N = 12 points)	$y = 11.250x + 2.8052$	$y = 7.2460x + 2.1924$	$y = 5.9878x - 1.828\,84$	$y = 8.2441x - 4.3762$
Regression coefficient (R^2)	0.9998	0.9999	0.9999	0.9999
LOD (μg mL^{-1})	0.74	0.16	0.09	0.14
LOQ (μg mL^{-1})	2.24	0.47	0.27	0.44

HPLC-DAD – high performance liquid chromatography-diode array detection; PD – purine derivatives; LOD – limit of detection; LOQ – limit of quantification; y – the peak area; x – the concentration of reference compound (μg mL^{-1})

Table 2. The precision and accuracy parameters of HPLC-DAD method

Analyte	Amount spiked (μg mL^{-1})	Precision		Accuracy (recovery) (%)
		intra-day (n = 6) RSD (%)	inter-day (n = 3) RSD (%)	
Allantoin	3.125	2.43	3.89	95.34
	12.5	1.62	2.59	97.32
	50.0	2.80	4.48	96.34
Uric acid	3.125	1.40	3.78	97.88
	12.5	1.01	2.72	99.11
	50.0	1.31	3.53	100.11
Xanthine	3.125	1.57	2.22	98.42
	12.5	1.54	2.18	99.19
	50.0	2.46	3.49	101.43
Hypoxanthine	3.125	1.13	2.73	99.89
	12.5	1.45	1.57	100.54
	50.0	2.91	3.16	104.47

HPLC-DAD – high performance liquid chromatography-diode array detection; n – number of HPLC replicates; RSD – relative standard deviation

The results of robustness testing showed acceptable limits (RSD less than 2.0%) (Ravisankar et al. 2015) for a minor change of method conditions. Thus, the HPLC method is robust.

Application of SPE HPLC-DAD method in cow milk analysis. A milk sample was subjected of evaluation extraction of PD on all SPE cartridges (Figure 5).

It can be observed that on SCX cartridge all four PD were quantified, while on the others cartridges, the xanthine it was not detectable. These results and SPE standards recoveries lead to the selection of the SCX cartridge for PD analysis from thirty milk samples (Figure 6).

In order to assess the potential of the optimised method to detect the effects of various feeding situations, milk samples from two groups of 15 cows each, fed two diets were collected. The diets were designed to influence the microbial protein synthesis in rumen, which can be indirectly assessed through the proportion of purine derivatives in milk.

The results showed that differences higher than 10% could be detected as being significant. Also, the method allowed the detection of concentration as low as 0.96–2.15 mg L^{-1} (for xanthine) and 0.7–1.49 mg L^{-1} (for hypoxanthine).

In some studies, the allantoin and uric acid was examined as indicator of bacterial nitrogen flow through the digestive system of ruminants, the main focus has been on for quantitative and analytical reasons. The similar quantities of allantoin and uric acid in milk were found by some authors (Sikka et al. 2001; Indyk and Woollard 2004; Gruber et al. 2009; Zuo

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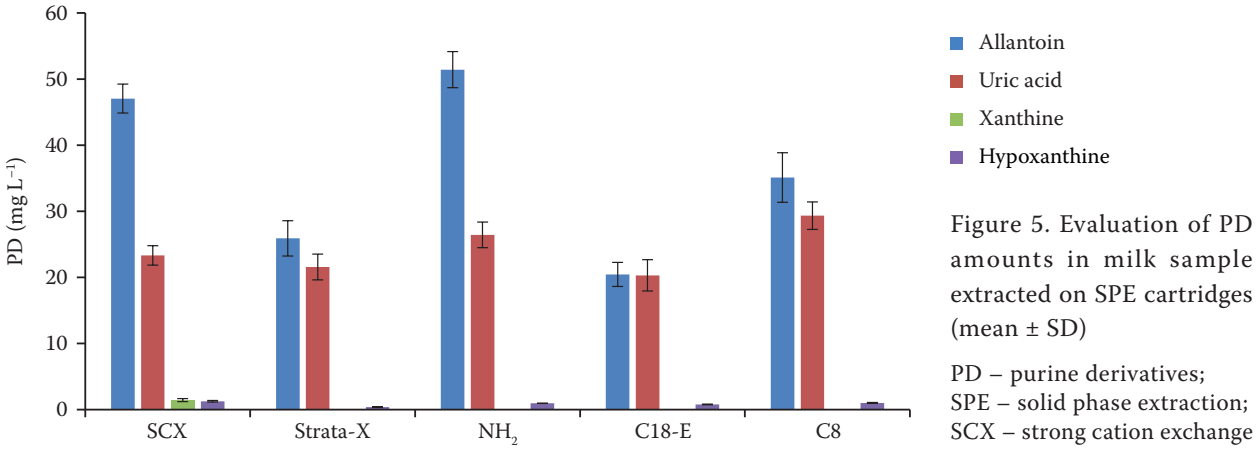
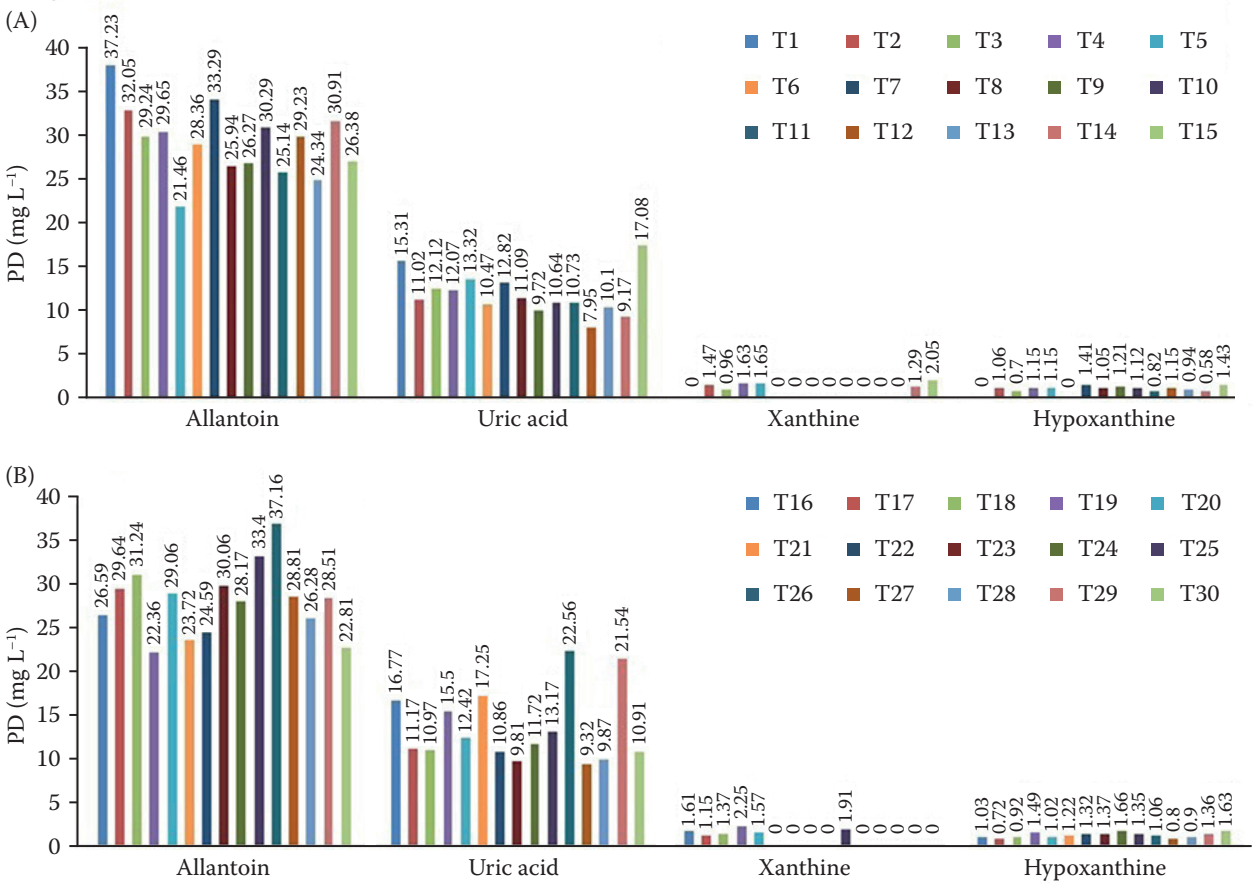


Figure 5. Evaluation of PD amounts in milk sample extracted on SPE cartridges (mean ± SD)

PD – purine derivatives; SPE – solid phase extraction; SCX – strong cation exchange



PD	T1–T15 (mean ± SD)	T16–T30 (mean ± SD)	SEM	*P*-value
Allantoin	28.65 ± 3.88	28.16 ± 3.96	0.7219	0.635
Uric acid	11.56[a] ± 2.37	13.59[b] ± 4.15	0.6218	0.025
Xanthine	0.6033 ± 0.78	0.6579 ± 0.85	0.1505	0.799
Hypoxanthine	0.9346[a] ± 0.42	1.1896[b] ± 0.29	0.0667	0.009

Figure 6. The PD content in studied milk samples (mean ± SD, mg L^{-1}): (A) samples T1-T15 and (B) samples T16-T30

[a, b]Mean values which do not have a common superscript letter are significantly different ($P < 0.05$); PD – purine derivatives; SEM – standard error of the mean; T – sample (1–30)

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et al. 2015). Allantoin output (mmol day^{-1}) increased with increased percentage of concentrate in the diet (Schager et al. 2003) and comparable results for uric acid were obtained by Larsen and Moyes (2010).

Thus, in a subsequent study, we will apply the developed method to evaluate the quantities of PD in cow milk as the effect of the feeding efficiency in ruminants. The developed analytical method allows a systematic and reliable check of the milk samples (easy to collect, store and analysed), thus offering a feed-back of the way the animals react to dietary changes, thus enabling the development of feeding strategies that lead to more efficient use of the dietary proteins and other nutrients.

CONCLUSION

The PD (allantoin, uric acid, xanthine, and hypoxanthine) were determined in cow milk by SPE HPLC-DAD method. Five different SPE cartridges (Strata: SCX, NH_2, C18-E, C8, Strata-X) were tested in order to evaluate the SPE extraction of PD from cow milk. The best SPE results for all studied PD were obtained on SCX cartridge.

HPLC-DAD method was developed and validation for simultaneous determination of PD in cow milk and was found reproductive, sensitive and robust. This HPLC-DAD validated method was applied to quantitative assay of PD in thirty cow milk samples from a selected batch. The allantoin and uric acid were found in the ranges of 21.46–37.23 mg L^{-1} and 7.95–22.56 mg L^{-1} respectively. Also, small quantities of xanthine (0.96–2.15 mg L^{-1}) and hypoxanthine (0.7–1.49 mg L^{-1}) or not at all were found in milk samples. Therefore, the SPE HPLC-DAD method has the potential to be implemented in laboratories as a routine test to monitor PD in cow milk.

REFERENCES

Cooper N., Khosravan R., Erdmann C., Fiene J., Lee J.W. (2006): Quantification of uric acid, xanthine and hypoxanthine in human serum by HPLC for pharmacodynamic studies. Journal of Chromatography B, 837: 1–10.

Czauderna M., Kowalczyk J. (2004): Simultaneous determination of purine metabolites in ovine urine and blood plasma by high-performance liquid chromatography. Journal of Animal and Feed Sciences, 13: 55–58.

Gruber J., Tang S.Y., Jenner A.M., Mudway I., Blomberg A., Behndig A., Kasiman K., Lee C.J., Seet R.C.S., Zhang W., Chen C., Kelly F.J., Halliwell B. (2009): Allantoin in human plasma, serum, and nasal-lining fluids as a biomarker of oxidative stress: Avoiding artifacts and establishing real *in vivo* concentrations. Antioxidants & Redox Signaling, 11: 1767–1776.

Hu K., Zhao G., Liu J., Jia L., Xie F., Zhang S., Liu H., Liu M. (2018): Simultaneous quantification of three alkylated-purine adducts in human urine using sulfonic acid poly(glycidyl methacrylate-divinylbenzene)-based microspheres as sorbent combined with LC-MS/MS. Journal of Chromatography B, 1081–1082: 15–24.

ICH (2005): ICH harmonised tripartite guideline. Validation of analytical procedures: Text and methodology Q2 (R1). ICH Expert Working Group. Available at https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accessed Sept, 2020).

Indyk H.E., Woollard D.C. (2004): Determination of orotic acid, uric acid, and creatinine in milk by liquid chromatography. Journal of AOAC International, 87: 116–122.

Kazemi-Bonchenari M., Rezayazdi K., Ghasemi H.A., Farahani A.H., Dehghan-Banadaky M., Mahdavi A. (2011): Effect of rumen degradable protein supplementation on purine derivatives excreted through urine and milk in lactating Holstein cows. Journal of Animal and Veterinary Advances, 10: 2389–2393.

Kozlik P., Hasikova L., Stiburkova B., Zavada J., Kalikova K. (2020): Rapid and reliable HILIC-MS/MS method for monitoring allantoin as a biomarker of oxidative stress. Analytical Biochemistry, 589: 113509.

Larsen T., Moyes K.M. (2010): Fluorometric determination of uric acid in bovine milk. Journal of Dairy Research 77: 438–444.

Liu C., Gu C., Huang W., Sheng X., Du J., Li Y. (2019): Targeted UPLC-MS/MS high-throughput metabolomics approach to assess the purine and pyrimidine metabolism. Journal of Chromatography B, 1113: 98–106.

Lu J.J., Jia B.J., Yang L., Zhang W., Dong X., Li P., Chen J. (2016): Ultra-high performance liquid chromatography with ultraviolet and tandem mass spectrometry for simultaneous determination of metabolites in purine pathway of rat plasma. Journal of Chromatography B, 1036–1037: 84–92.

Marson B.M., Concentino V., Junkert A.M., Fachi M.M., Vilhena R.O., Pontarolo R. (2020). Validation of analytical methods in a pharmaceutical quality system: An overview focused on HPLC methods. Química Nova, 43: 1190–1203.

Mitra S., Patnaik P., Kebbekus B.B. (2018): Environmental Chemical Analysis. 2nd Ed. Boca Raton, Florida, USA, CRC Press Taylor & Fracis Group: 191–216.

Østdal H., Andersen H.J., Nielsen J.H. (2000): Antioxidative activity of urate in bovine milk. Journal of Agricultural and Food Chemistry, 48: 5588–5592.

https://doi.org/10.17221/297/2020-CJFS

Płotka-Wasylka J., Szczepańska N., Guardia M., Namieśnik J. (2016): Modern trends in solid phase extraction: New sorbent media. Trends in Analytical Chemistry, 77: 23–43.

Ravisankar P., Navya C.N., Pravallika D., Navya Sri D. (2015): A review on step-by-step analytical method validation. IOSR Journal of Pharmacy, 5: 7–19.

Remane D., Grunwald S., Hoeke H., Mueller A., Roeder S., von Bergen M., Wissenbach D.K. (2015): Validation of a multi-analyte HPLC-DAD method for determination of uric acid, creatinine, homovanillic acid, niacinamide, hippuric acid, indole-3-acetic acid and 2-methylhippuric acid in human urine. Journal of Chromatography B, 998–999: 40–44.

Sikka P., Saxena N.K., Gupta R., Sethi R.K., Lall D. (2001): Studies on milk allantoin and uric acid in relation to feeding regimens and production performance in buffaloes. Asian-Australasian Journal of Animal Sciences, 14: 1634–1637.

Schager W.M., Harrison J.H., Gaskins C.T., Davidson D. (2003): Factors affecting application of milk allantoin as an estimator of microbial protein flow to the duodenum under commercial conditions. Journal of Dairy Science, 86: 1716–1721.

Tas B.M., Susenbeth A. (2007): Urinary purine derivatives excretion as an indicator of *in vivo* microbial N flow in cattle: A review. Livestock Science, 111: 181–192.

Tiemeyer W., Stohrer M., Tiemeyer D.G. (1984): Metabolites of nucleic acids in bovine milk. Journal Dairy Science, 67: 723–728.

Timmermans Jr. S.J., Johnson L.M., Harrison J.H., Davidson D. (2000): Estimation of the flow of microbial nitrogen to the duodenum using milk uric acid or allantoin. Journal of Dairy Science 83: 2387–2390.

Vlassa M., Filip M., Pascalau V., Coman V., Dragomir C. (2009): Determination of purine derivatives in bovine urine using rapid chromatographic techniques. Archiva Zootechnica 12: 59–70.

Xinhua D. (2006): Preparation of uric acid standard stock solution. Clinical Chemistry, 52: 2117–2118.

Xu Y., Liu J., Zhang H., Jiang M., Cao L., Zhang M., Sun W., Ruan S., Hu P. (2016): Hypercrosslinked strong cation – Exchange polymers for selective extraction of serum purine metabolites associated with gout. Talanta, 151: 172–178.

Zhou D.D., Zhang Q., Zhang H., Wang Y.Z., Yang. F.Q., Wang S.P., Wang Y.T. (2019): Cupric ion functionalized polydopamine coated magnetic microspheres as solid-phase adsorbent for the extraction of purines in plasma. Journal of Chromatography B, 1120: 95–103.

Zuo R., Zhou S., Zuo Y., Deng Y. (2015): Determination of creatinine, uric and ascorbic acid in bovine milk, and orange juice by hydrophilic interaction HPLC. Food Chemistry, 182: 242–245.

Zuo Y., Wang C., Zhou J., Sachdeva A., Ruelos V.C. (2008): Simultaneous determination of creatinine and uric acid in human urine by high-performance liquid chromatography. Analytical Sciences, 24: 1589–1592.

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