Determination of purine derivatives in bovine urine using rapid chromatographic techniques

Mihaela Vlassa[1], Miuța Filip[1], Violeta Paşcalău[1], Virginia Coman[1], C. Dragomir[2]

[1]*Babeş-Bolyai University - Raluca Ripan Institute for Research in Chemistry, [†]30 Fântânele Street, RO-400294 Cluj-Napoca, Romania,*
[2]*National Research & Development Institute for Animal Biology and Nutrition (IBNA Balotesti), Calea Bucuresti nr. 1, Balotesti, Ilfov, 077015, Romania*

SUMMARY

In this study, two techniques (HPLC-UV and TLC) were assessed as alternatives to the analysis of purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) using classical spectrometry method, still largely used in South-Eastern Europe. With HPLC technique, very good calibration curves were obtained for all four purine derivatives ($r>0.999$). Also, detection limits were satisfactory: ≥ 7.5 µg/mL for allantoin, ≥ 2.7 µg/mL for uric acid, ≥ 5.5 µg/mL for xanthine and ≥ 10.02 µg/mL for hypoxanthine. The analysis of urine samples taken from dairy cows gave results which were in the range of literature data for allantoin and uric acid, but not for hypoxanthine (whose concentration was much higher than values reported in literature). TLC technique allowed good separation of standards and elaboration of good calibration curves ($r>0.984$). Detection limits were too high, if the whole range of values reported in literature for the concentrations purine derivatives in ruminants' urine is taken into account. Moreover, on urine samples the spots of creatinie overlapped the spots of uric acids, impairing the quantitative estimation of purine derivatives. It is concluded that HPLC technique can be used to assess the concentration of allantoin and uric acid in the urine of cows. The TLC technique can be used qualitatively, further studies on samples preparation and procedure being needed in order to use it for quantitative determinations.

Keywords: rumen, microbial protein, purine derivatives, HPLC, TLC

INTRODUCTION

In ruminants, estimation of the daily production of rumen microbial protein is essential in order to assess the protein value of a diet. It has to be underlined that tabular nutritive values of feeds (Burlacu, 2002) rely on rough

[†] Corresponding author address: mihaela-cecilia@yahoo.com

approximations of the coefficients describing efficiency of microbial proteosynthesis: 145 g microbial protein synthesized from 1 kg of FOM and 90% capture of ruminally degradable nitrogen, disregarding the feeding situations. One of the obstacles in elaboration of more detailed coefficients is the fact that determination of rumen microbial yield is a laborious work, requiring fistulated animals and specialized personnel.

A commonly used alternative is the estimation of the microbial proteosynthesis on the base of the urinary excretion of purine derivatives (allantoin, uric acid, xanthine, and hypoxanthine), which mostly originate from rumen microbes (Chen et al., 1990). This technique, although requiring facilities for digestibility studies – for complete collection of urine during the day, is non-invasive and simple to use. Moreover, one of its variants ("spot sampling") can be used also in farm conditions.

The technique requires quantitative determination of the concentration of purine derivatives in urine, which in South-Eastern Europe is usually done by spectrometry. This implies long and critical times of operation and limits the number of samples that can be analyzed daily (Pentz, 1969; Resines et. al, 1992). Alternative techniques using high performance liquid chromatography for the determination of purine derivative from urine (Le, 1993, Garcia del Moral et al. 2003, George et al., 2006) or blood (Kock et. al, 1993; Czauderna et al., 1997) have been described in the literature. Also purine derivatives have been separated by TLC techniques (Klaus et. al, 1991; Baranowska et. al., 2000).

The objective of this study is to evaluate the potential of adapted HPLC and TLC techniques to assess the concentration of purine derivatives in urine.

MATERIAL AND METHODS

Chemicals and solutions

All chemicals were of analytical reagent grade. The standards (allantoin, uric acid, xanthine, hypoxanthine) were purchased from Acros. The stock solutions were 1 mg/mL for HPLC technique. For TLC technique, allantoin solution was 20 mg/mL. The ethanol and $(NH_4)_2HPO_4$ were purchased from Chimopar Bucharest. Deionized water Millipore (18,3 MΩ cm) was used for preparation of all solution.

Standards and sample preparation

The urine samples were acidified with 15% HCl and frozen at -20ºC. The acidic sample of cow urine (pH=1-2) were bring to the room temperature and neutralized with a NaOH 4N solution until pH 7.78 (similar to the pH of the mobile phase). The samples were then filtered by PTFE 0.45μm (Teknokroma) and diluted. Samples from five Romanian Black Spotted cows were used for assessments. Cows were grazing natural pasture and fed a supplemented consisting of wheat bran.

HPLC-UV technique

The chromatographic system consisted of a High Performance Liquid Chromatograph Jasco 980 (Japan) equipped with an intelligent HPLC pump (Model PU-980), a low pressure gradient unit (Model LG-980-02), an in-line degasser (Model DG-980-50), an intelligent column thermostat (CO-2060 Plus), and UV-Vis detector (Model UV-970/975). Samples were injected manual Hamilton Rheodyne Syringe (50 µL) through a Rheodyne valve of 20 µL loop. The system was collected and data analyzed were performed with the ChromPass software. The calibration curves were obtained from peak area *versus* known injected amount of compounds.

The maximums of absorbance of purine derivatives were determined with an UV-Vis spectrometer (UNICAM UV-4) equipped with photomultiplicator detection in the 190–900 nm range using VISION software. The HPLC-UV separation was carried out at 25ºC temperature using a Nucleosil 120 C18 column (5 µm, 25 x 0.46 cm). The elution was made with a buffer solution of $(NH_4)_2HPO_4$, 0.05 M with pH 7.78. The flow rate of the mobile phase was 1 mL/min. The compounds were registered at wavelength 218 nm.

TLC technique

The TLC equipment used for this experiment contained: the applicator AS 30 HPTLC DESAGA with PC software; the densitometer CD-60 DESAGA with wavelengths of 190–900 nm; monochromator - holographic reflectance grating with 1200 lines/mm; band - width 10 nm; the lamps - deuterium lamp (190–339 nm), tungsten-halogen lamp (340–900 nm), mercury vapour lamp for fluorescence measurement, the software ProQuant (compatible WIN 9X şi NT); the double chamber DESAGA (200 x 100 cm) and the sprayer SG 1B DESAGA. The TLC separation was carried out on the Silica gel $–NH_2$, F_{254nm} plates (100 x 100 mm, Mackerey-Nagel) using a double chamber DESAGA (200 x 100 cm), the mobile phase was ethanol-water (80: 20, v/v) and visualization at the 254 nm

RESULTS AND DISCUSSION

HPLC-UV assessment

For the HPLC recording it was necessary to establish the optimum range of the UV detection. Maximum absorbances of the studied purine derivatives were determined on the basis on UV-Vis spectra recorded in the range of 190-900 nm. These spectra were processed with the Vision soft.

The UV-Vis spectra of studied purine derivatives are presented in Fig. 1. For HPLC recording of the common wavelength was selected at 218 nm. Using the HPLC chromatographic conditions described above, a linear relationship between the concentration of the purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) and the UV detector's response (peak area) was found.

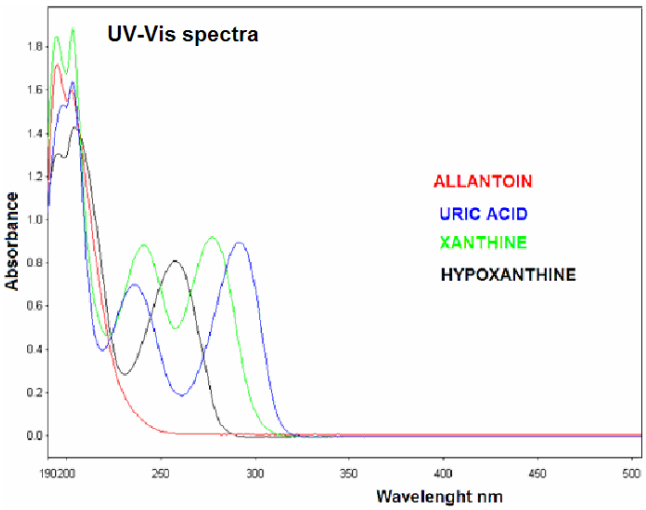


Figure 1. UV-Vis spectra of studied purine derivatives: allantoin (203 nm), uric acid (266, 292 nm), xanthine (241, 276 nm), hypoxanthine (255 nm).

For all four purine derivatives, the *r* (correlation coefficient) values were >0,999 at the eight levels, ranging from 1.00 to 0.10 mg/mL. The detection limits (DL) which were calculated considering a signal-of-noise ratio (S/N) of 3: allantoin DL ≥ 7,5 µg/mL; uric acid DL ≥ 2,7 µg/mL; xanthine DL ≥ 5,5 µg/mL and hypoxanthine DL ≥ 10,02 µg/mL. HPLC chromatogram and calibration curves of purine derivatives standards are shown in Fig. 2 and Fig. 3.

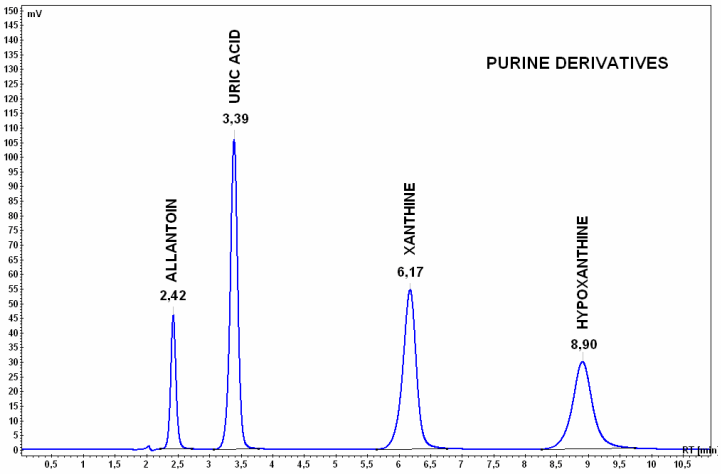


Figure 2. HPLC chromatogram of purine derivative standards

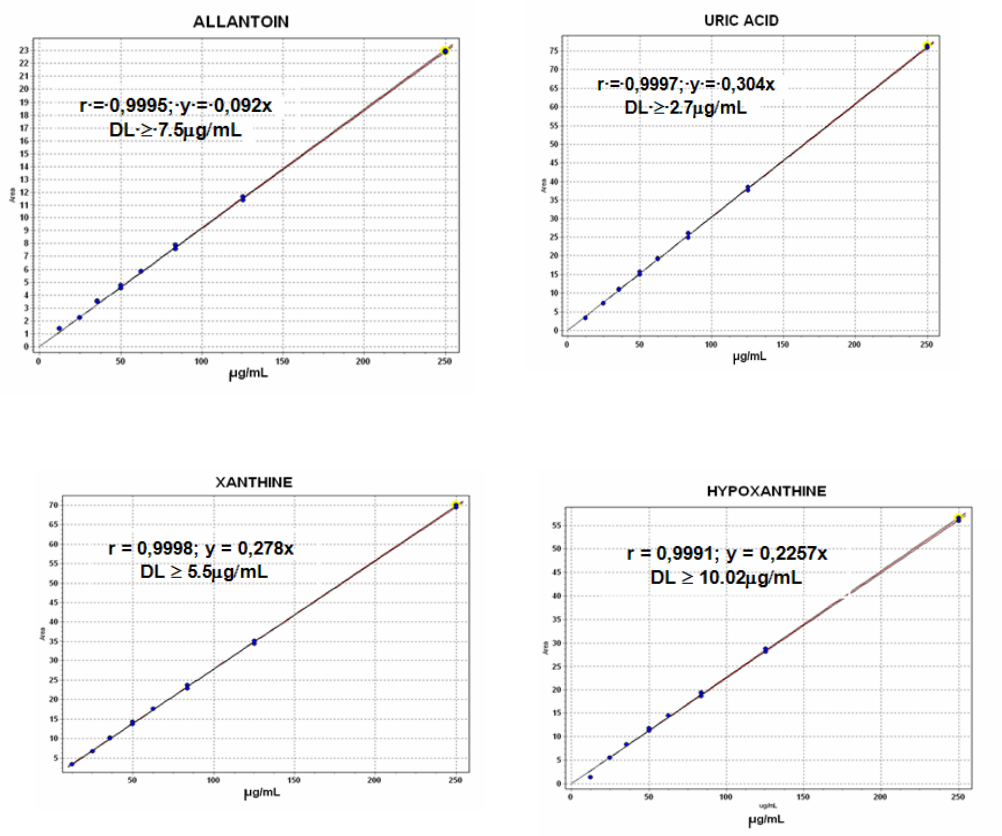


Figure 3. The calibration curves of studied purine derivatives

Recovery study was carried out in order to determine the accuracy of the technique. Samples were analyzed before and after the addition of known amounts of allantoin, uric acid, xanthine and hypoxanthine and it was found that recoveries ranged between 91,58- 99,50%. In Table 1 and Fig. 4 are presented the recovery studies, using diluted samples.

Table 1. The recovery data of studied purine derivatives

Purine derivatives	Amount, µg/mL		Amount, µg/mL		Recovery, %
	Sample	Added in sample	Expected	Obtained	
Allantoin	34.48	31.25	65.73	62.40	94.93
Uric acid	2.79	31.25	34.04	33.90	99.50
Xanthine	-	31.25	31.25	29.75	97.02
Hypoxanthine	3.1	31.25	34.25	31.46	91.50

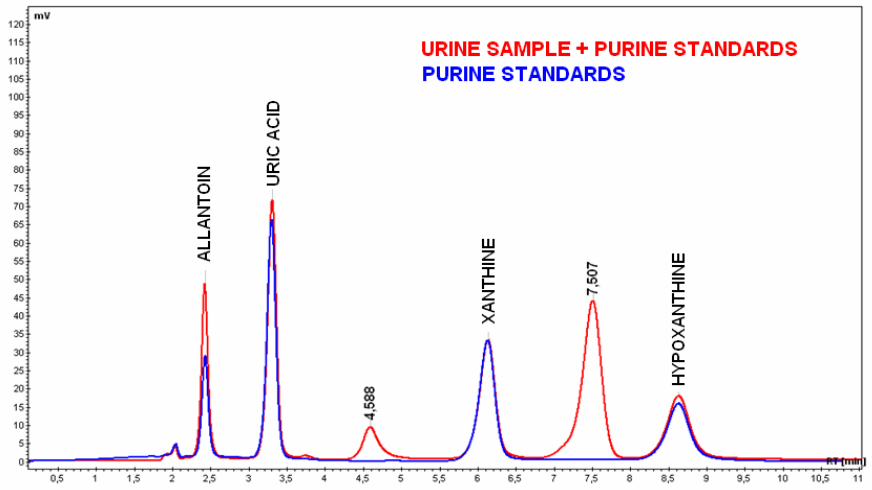


Figure 4. The HPLC chromatograms of sample 1+ standards and a standards

A HPLC chromatogram of a urine sample is presented in Fig. 5 and the results obtained for urine samples are presented in Table 2. The chromatograms of cow urine samples indicate the presence of the purine derivatives as allantoin, uric acid and hypoxanthine. The xanthine has not been detected.

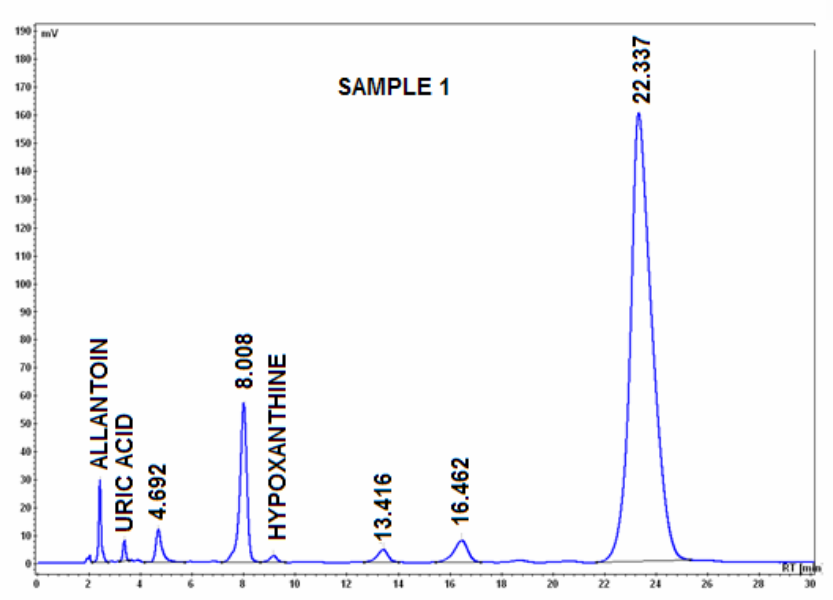


Figure 5. HPLC chromatogram of sample 1

The allantoin concentrations obtained in the present study varied from 11.39 to 16.58 mmol/L are higher than concentrations reported by Peterson, 2006 (7.4 – 8.5 mmol/L) and slightly higher than those reported by Gonzalez-Ronquillo et al., 2003 and 2004 (ranging from 188 to 354 mmol/d, equivalent to 7.52 – 14.16 mmol/L, assuming an average urine volume of 25 L/d).

On the other hand, the results are in range of those obtained by Gonda and Lindberg, 1997, who obtained in two experiments values ranging from 12.84 to 22.55 mmol/L. It has to be mentioned that variability of allantoin concentration in urine is very high e.g. from 1 to 6 times in sheep (IAEA, 1997).

Table 2. The HPLC experimental data obtained for urine samples

Urine sample	Sample dilution [v/v]	allantoin [mg/mL]	uric acid [mg/mL]	hypoxanthine [mg/mL]
1	1:50	1.80	0.15	0.18
2	1:50	2.61	0.12	0.15
3	1:50	2.28	0.24	0.11
4	1:50	2.50	0.26	0.28
5	1:50	2.62	0.24	0.17

The concentration of the uric acid was 0.05 - 10.4 % of the concentration of the allantoin (Table 4). This is lower than usual ratio allantoin:uric acid, which is 85:15 for cows. On the other hand, the observed ratio is similar to those reported by Peterson, 2006 (5.7 – 8.1%) and Gonzalez-Ronquillo, 2003 and 2004 (4.8 – 13.7%).

Usually, xanthine and hypoxanthine are virtually absent from the urine of bovines (IAEA, 1997). There are reports on the presence of xanthine and hypoxanthine in urine samples from cows (Pimpa, 2001), but the concentrations are very low. In the mentioned paper, ratio hypoxanthine : allantoin was 2%, much lower than the values obtained in the present study: 4.8 – 10%.

Whereas assessment of allantoin and uric acid seems reliable, further studies are needed in order to take hypoxanthine into account when calculating total purine derivatives, the value which is to be used in estimation of microbial proteosynthesis.

TLC assessment

This technique allowed the separation on the thin layer plate of the purinic derivatives of interest. Because of the poor molar absorbance of the allantoin the work concentration of the solution of that substance had to be increased to 20 mg/mL. The chromatographic plate of the uric acid, xanthine and hypoxanthine, the TLC densitograms at different concentrations and the calibration curves obtained with standards are shown in Fig. 6, 7 and 8.

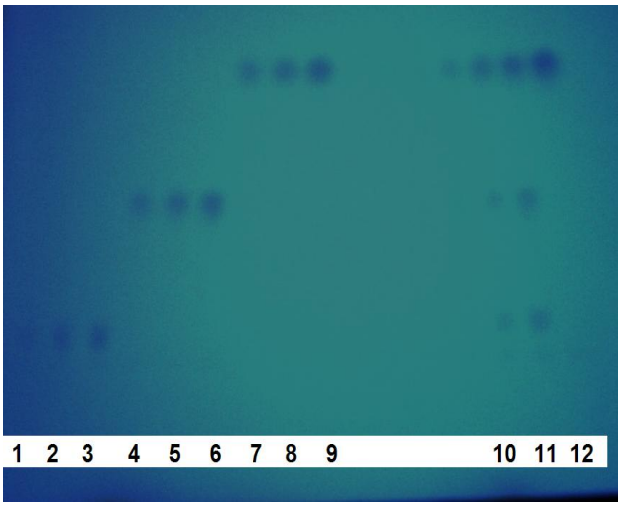


Figure 6. TLC plate for calibration curves (UV 254 nm). Standard solutions: 1 mg/mL. Items 1 to 12 on the horizontal band express the spot number (1, 2, 3 = uric acid; 4, 5, 6 = xanthine, 7, 8, 9 = hypoxanthine, 10, 11, 12 = mixture, at concentrations of 0.2, 0.4, and 0.6 µg, respectively)

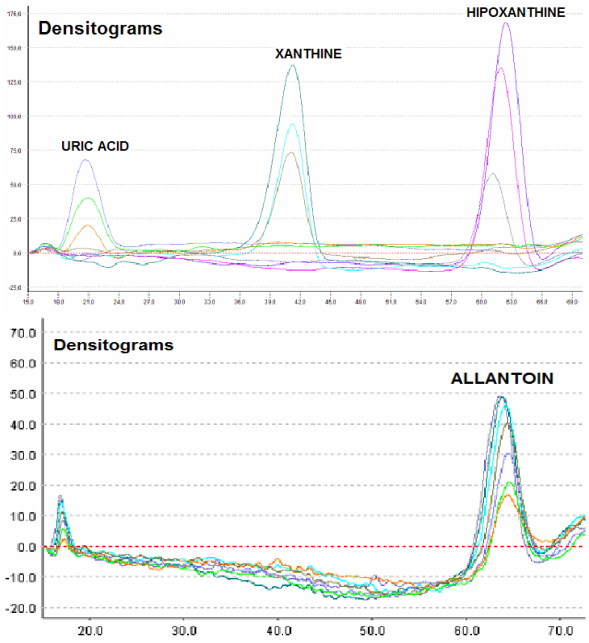
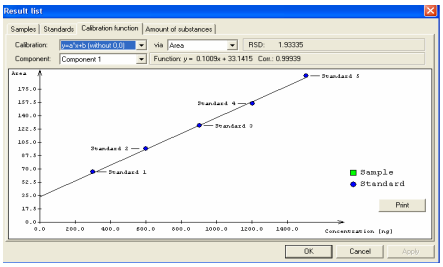
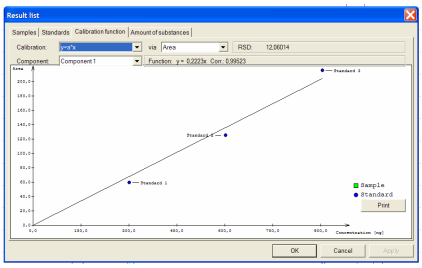


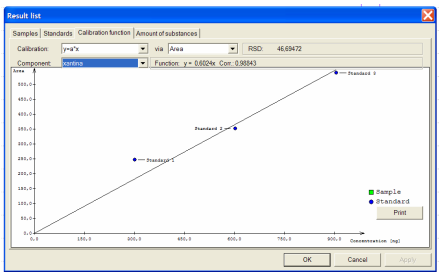
Figure 7. The TLC densitograms of studied purine derivatives



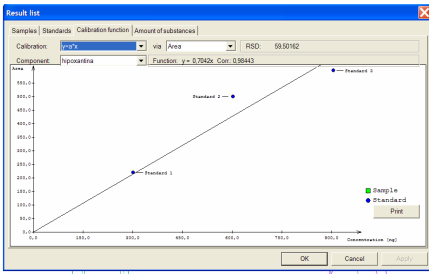
Allantoin, r=0.9844 DL ≥ 20 µg/µL



Uric acid, r=0.9952 DL ≥ 1 µg/µL



Xanthine, r=0.9884 DL ≥ 1 µg/µL



Hypoxanthine, r=0.9844 DL ≥ 1 µg/µ L

Figure 8. The TLC calibration curves of purine derivatives

The spots of uric acid, xanthine and hypoxanthine standards (Fig 7) and allantoin standard (not shown) separated at distinct and reproducible Rf. The values of correlation coefficients (r) of the calibration curves calculated from three concentration levels (ranging from 0.2 to 0.6 µg/spot) were 0.9952 for uric acid, 0.9884 for xanthine and 0.9844 for hypoxanthine. For allantoin, r was 0.9844 (calibration curve based on five concentration levels, ranging from 4 to 12 µg/spot). Detection limits were 1 mg / ml, with the exception of allantoin, where poor absorbance occurred – in this case detection limit was 20 mg / ml. Anyway, in all cases these detection limits were not only much higher, comparing to HPLC technique, but also unsatisfactory from the point of view of the range of purine derivatives concentrations that were reported in literature.

The TLC plate and densitograms of purine derivatives from cow urine samples are shown in Fig. 9. Standards (allantoin alone – colum 1 and mixture of 4 urine derivatives – column 7) were also added on the plate for identification of the spots corresponding to the urine samples. From the TLC plate and the densitograme (recorded at 254 nm), presence of significant concentrations of allantoin and uric acid in the cow urine samples could be observed. No xanthine or hypoxanthine was detected in the urine samples. As expected for urine samples from bovines, the substances are probably present in very low concentrations. This is also in opposition with the results of HPLC technique for hypoxanthine (which are unreliable for this substance, as shown before).

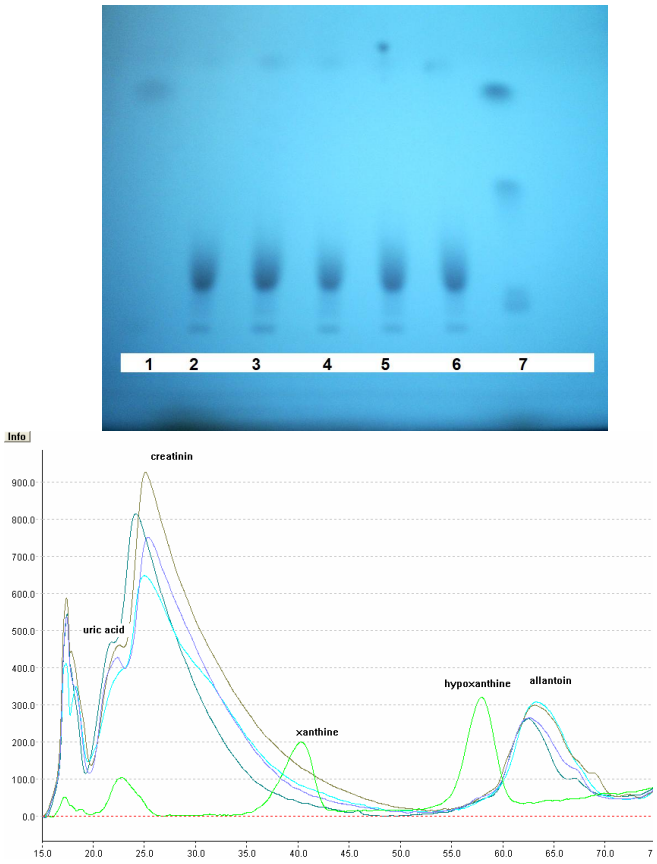


Figure 9. TLC plate and densitograms of purine derivatives from cow urine samples and standards. Spot 1: 1 µL allantoin (20 mg/mL); Spots 2-6: 1 µL cow urine samples; Spot 7: 1 µL mixture of standards.

The TLC technique is simple, fast and cheap, allowing also simultaneous analysis of many samples. On the other hand, for urine samples, the spots of creatinine overlaps the spots of uric acid and, when present, the spots of xanthine, impairing the quantitative use of TLC (together with the problem of the too high detection limits). Therefore TLC technique is suitable for qualitative analysis but further work (on sample preparation and TLC procedure itself) is to be done in order to use this technique quantitatively.

CONCLUSIONS

By HPLC technique the purine derivatives were well separated at distinct elution times. Recovery study led to recovery values higher than 95% for allantoin and uric acid, whose concentrations in urine sampled from dairy cows were also in the range of literature data. Recovery values were also high for

xanthine (97%) and good for hypoxanthine (91.5%). However, results for hypoxanthine in urine samples were inconsistent with the literature data and cannot be taken into consideration without further studies.

TLC technique is suitable for the identification of the purine derivatives from standard solution mixtures and from the samples of urine. The calibration curves were good ($r > 0.98$) but detection limits were too high for most of the concentrations that can occur in urine samples. Also, incomplete separation of uric acid and creatinine spots prevents, beside detection limits, the quantitative use of the technique.

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