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Short communication

# Determination of mimosine and 3,4-dihydroxypyridine in milk and plasma of goats

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**Abstract**

A simple method for determination of mimosine and 3,4-dihydroxypyridine (3,4-DHP) in plasma and milk was developed. Milk and plasma, with tyrosine as internal standard, were deproteinized using 9% trichloracetic acid and extracted with diethyl ether. Metabolites were separated by isocratic high-performance liquid chromatography, with 0.02 *M* orthophosphoric acid (pH 2.5) at 0.5 ml/min and a Hypersil ODS microbore column. Mimosine, 3,4-DHP and tyrosine were detected at 275 nm. The recovery of the mimosine added to the plasma samples was 101.6±2.3% and 103.3±1.0% for milk samples. 3,4-DHP recovery for plasma samples was 101.2±0.9% and for milk samples 100.8±1.4%. The reproducibility of the method was evaluated by analyzing six plasma samples and six goat milk samples. The analyses yielded relative standard deviations of 2.65 and 2.82%, respectively.

*Keywords:* Mimosine; 3,4-dihydroxypyridine

## 1. Introduction

Mimosine is a naturally occurring toxic amino acid found in the seeds and leaves of *Leucaena leucocephala*. The relatively high protein content of *Leucaena* (224 to 334 g/kg DM) makes it an attractive feedstuff for animals, however its value is limited due to its high mimosine (10 to 40 g/kg DM) content. Mimosine ingested by ruminants is converted in the rumen to other toxic compounds, 3,4-dihydroxypyridine (3,4-DHP) and very small amounts of 2,3-dihydroxypyridine (2,3-DHP). *Leucaena* can be successfully used in diets for ruminants when the toxic effect of mimosine/DHP is diminished by the introduction of special strains of ruminal bacteria [1] or by mineral supplementation [2]. However, ruminants fed large amounts of *Leucaena* can accumulate appreciable quantities of toxins in their organs [3] and possibly in their milk. Therefore a simple analytical method is needed for determination of these toxins in ruminant organs, plasma and milk to prevent human consumption of toxic compounds.

Tangendjaja and Wills [4] reported a method using high-performance liquid chromatography (HPLC) for estimating mimosine and DHP in blood but there are no reports concerning estimation of those compounds in milk. The preparation of plasma samples for estimation of mimosine and DHP reported by Tangendjaja and Wills [4] is expensive and time consuming. Therefore, the objective of this study was to develop a reliable and fast method for

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purification and estimation of mimosine and DHP in plasma and milk samples.

## 2. Experimental

### 2.1. Reagents and standards

Mimosine, (β-[N-(3-hydroxy-4-oxopyridyl)]-α-aminopropionic acid, 99% pure) from Koa hoale seeds, was purchased from Sigma (St. Louis, MO, USA). The 3,4-DHP was a kind gift of Dr. M. Allison (USDA, Nutrition and Animal Disease Center, Ames, IA, USA). Other reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA).

### 2.2. Sample preparation

Blood samples from Alpine and Angora goats fed diets with or without *Leucaena* were collected into 7-ml tubes containing either potassium oxalate and sodium fluoride or sodium heparin (Becton Dickinson Vacutainer Systems Rutherford, NJ, USA). The tubes were immediately chilled in an ice bath and transported to the laboratory where they were centrifuged at 1500 *g* at 4°C for 20 min. Plasma aliquots were stored at −20°C until analysis. Milk from Alpine goats fed diets with or without *Leucaena* was processed immediately. A 1-ml volume of plasma or milk was mixed with 1 ml of 9% trichloroacetic acid with tyrosine (180 mg/l, internal standard). The mixture was vortex-mixed and then centrifuged at 1500 *g* at 4°C for 10 min. The supernatant was transferred to 25-ml glass culture tubes to which 3 ml (plasma samples) or 5 ml (milk samples) of diethyl ether was added. The mixture was vortex-mixed for 2 min on a SMI Multi Tube Vortexer (American Dade, Miami, FL, USA) and allowed to settle to give separation of phases. The diethyl ether was discarded using disposable glass Pasteur pipets (Fisher Scientific) connected with a suction pump. The extraction procedure with ether was repeated and the tubes were allowed to stand for 30 min under a fume hood to evaporate the remaining ether. The supernatant was transferred to small glass tubes and stored at −20°C until analysis.

### 2.3. HPLC configuration and analysis

An AminoQuant 1090 (Hewlett-Packard, San Fernando, CA, USA) was used. One pump was used to deliver 0.02 *M* orthophosphoric acid (pH 2.5) at 0.5 ml/min to the analytical column. The column used was a Hypersil ODS microbore column (5 μm, 100×2.1 mm I.D., Hewlett-Packard) in conjunction with a Hypersil ODS precolumn (10 μm, 20×2.1 mm I.D., Hewlett-Packard). A 5-μl aliquot of the milk or plasma samples was injected on the column. Initially a diode-array detector was set to monitor absorbance in the 250 to 350 nm range. The optimal separation of compounds was achieved at 275 nm. Run time for blood was 8 min and for milk 5 min. After approximately 100 runs the analytical column was cleaned with a mixture of water and acetonitrile to remove contaminants that could interfere with estimations. The recovery of the standard solution (0.1 and 0.5 nmol) added to the samples was performed and reproducibility was also tested.

## 3. Results and discussion

Preparation of the sample is a critical step in blood and milk analysis. The previously published method [4] requires several transfer steps that are time consuming and limit the number of samples processed. The developed purification method is very simple and many samples can be processed simultaneously. Use of trichloroacetic acid allows effective removal of protein and the subsequent extraction with diethyl ether removes trichloroacetic acid and other interfering substances. Ether extraction has been used previously [5] to prepare urine for determination of nucleic acid derivatives by HPLC. Addition of the internal standard to the sample adjusts for volume lost throughout the extraction procedure and tests for autosampler inaccuracy. Tyrosine is present in goat plasma in concentrations of about 50 μ*M*. When plasma and milk samples without internal standard were processed there was no detectable tyrosine peak. The detection limit for tyrosine using this method is 80 μ*M* which is much higher than the physiological concentration of tyrosine in the plasma. The amount of tyrosine added to the samples was about 10 times that found in

plasma, therefore the naturally occurring blood tyrosine portion was very small and insignificant. Recovery of the added tyrosine was 99.4±0.4% (plasma) and 101.0±0.5% (milk) when compared with tyrosine dissolved in the mobile phase.

The purification method used by Tangendjaja and Wills [4] required elution of mimosine and DHP from a cation-exchange column using a large volume of 2 *M* sodium hydroxide and rotary evaporation. This limits the number of samples that can be processed and can create errors associated with multiple sample transfer. The method was not suitable for milk samples. Our method is suitable for both milk and plasma samples.

There is no information on possible contamination of milk with mimosine or DHP from animals fed *Leucaena* diets. It was reported by Sahlu et al. [3] that significant amounts of *Leucaena* toxins could be accumulated in the liver and kidney of animals fed *Leucaena*. This suggests that *Leucaena* toxins could be transferred to milk. Increasing public awareness concerning food quality and contamination may require obligatory analysis of mimosine and DHP in milk from animals fed *Leucaena* diets.

Spectra library, collected using a diode array detector, allow us to identify optimal wavelengths for detection and quantification of metabolites. Mimosine, 3,4-DHP and tyrosine showed maximum absorbance at 275 nm (Fig. 1 and Fig. 2). The 2,3-DHP can also be detected using this method, however 2,3-DHP showed maximal absorbance at 300 nm. When animals are fed *Leucaena* only very small amounts of 2,3-DHP can be detected (1–2%). 3,4-DHP is the main metabolite produced as a result of rumen fermentation of mimosine. As 2,3-DHP is normally absent in physiological samples we do not recommend this method to analyze it. The detection limit for tyrosine was 80 $\mu M$ and for mimosine and 3,4-DHP in milk and plasma, 1.6 $\mu M$. Linear response for both mimosine and 3,4-DHP was observed up to 4 m*M*. For determination of *Leucaena* we used a microbore column which gives fast separation and allows small sample volume. Mimosine and 3,4-DHP can be separated on standard column (250×4.6 mm I.D.) but that will require a much higher flow-rate (1 ml/min) and separation time of about 16 min for plasma and 10 min for milk.

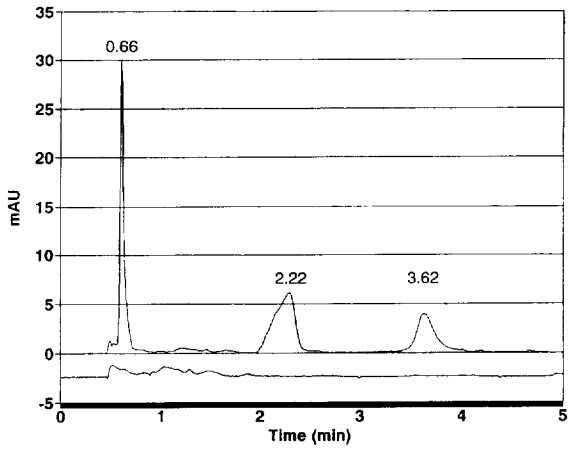


Fig. 1. Chromatogram of plasma sample. Upper-line sample with *Leucaena* toxins, lower-line sample without *Leucaena* toxins. Peak eluted at 0.66 min=mimosine (0.070 nmol), 2.22 min=3,4-dihydroxypyridine (0.063 nmol) and 3.62 min=internal standard tyrosine (2.5 nmol).

Recovery of *Leucaena* toxins was studied by adding the mimosine and DHP standards to the plasma and milk samples prior to deproteinization and extraction (0.1 and 0.5 nmol). The recovery of the mimosine added to the plasma samples was 101.6±2.3% and for milk samples 103.3±1.0%. The recovery of 3,4-DHP was 99.1.2±0.9% for plasma

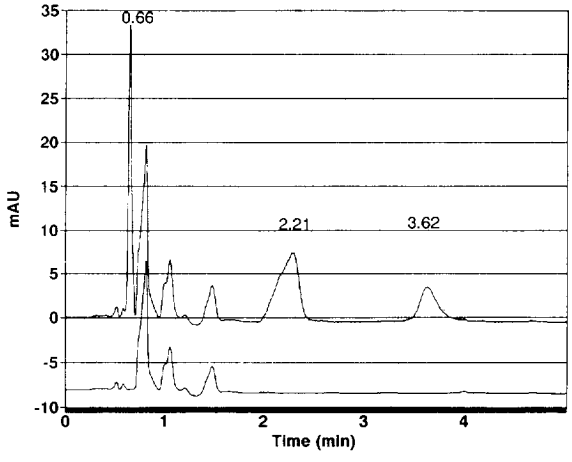


Fig. 2. Chromatogram of milk sample. Upper-line sample with *Leucaena* toxins, lower-line sample without *Leucaena* toxins. Peak eluted at 0.66 min=mimosine (0.085 nmol), 2.21 min=3,4-dihydroxypyridine (0.068 nmol) and 3.62 min=internal standard tyrosine (2.5 nmol).

samples and 100.8±1.4% for milk samples. The reproducibility of the method was evaluated by analysis of six plasma samples and six goat milk samples. Evaluation yielded relative standard deviations of 2.65% (plasma) and 2.82% (milk).

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