HPLC ANALYSIS OF VITAMIN B_{12} IN FODDER AND GOAT MILK

— communication —

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Abstract: Filling the assessment of fodder value by specifying the content of vitamins which has to meet the multilateral requirements of the body is one of the main issues in the study of improving the goat milk with vitamin B_{12}. In this research raw goat milk enhanced vitamin B_{12} supplements is analysed and the content in vitamin B_{12} is measured by HPLC with diode-array detection by using a Shimadzu HPLC system. Two methods for milk samples processing for proteins precipitation and vitamin B_{12} extraction are presented, using trichloroacetic acid and sulphuric acid. The results indicate that the chromatographic method is adequate for determining vitamin B_{12} in goat milk enhanced with vitamin B_{12} after the protein precipitation by acid hydrolysis with trichloroacetic acid or sulphuric acid, both acid treatments giving good results. The addition of the the yeast *Saccharomyces carlsbergensis* in goat forage give an increased content of vitamin B_{12} in milk.

Key words: milk, goat, supplements, vitamin B_{12}

INTRODUCTION

With the current rapid growth of world population and prolongation of human life, of raising the living level and targeting food towards growing extent agro-food products with high nutritional and biological value, the need for food, mainly of animal origin has increased more than ever (Banu et al., 2000). The category of biologically active substances also includes vitamins, substances widely distributed in nature and necessary for the proper functioning of animals and human bodies. Vitamins are organic

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substances which in small quantities are essential for normal growth and development of living organisms, participating, along enzymes and hormones to the regulation and stimulation of metabolic processes (Donaldson, 2000).
In recent years the term probiotic is used to define the living microorganisms which, administered in adequate doses, improve the health of the host. Probiotics are substances or food products which make part of our digestive ecosystem and contain live microorganisms: lacto acid producing bacteria, bifido - and lactic bacteria, yeast which are found in the normal intestinal microflora of a healthy person (Banu, 2000).
Existing microorganisms in probiotics are not pathogenic or toxic substances and their passage through the gastrointestinal tract does not modify their viability. Probiotics are not seen as drugs, but as health beneficial substances.
In this paper, the determination of the vitamin B_{12} in animal food was made. For the supplement of their nutritional value in order to meet the requirements of the animal organism and to increase the amount of vitamin B_{12} in raw milk, the nutritional quality of milk was improved by the administration of the yeast *Saccharomyces carlsbergensis* in forage (Bahcivangi, 1999) (Pascal, 2007) (Zamfirescu, 2009).

MATERIAL AND METHODS

The research was realised in an animal farm in Sibiu, and the institutional and organizational framework in which the laboratory research were conducted were the Laboratory within the Faculty of Agricultural Sciences, Food Industry and Environmental Protection, University of „Lucian Blaga" from Sibiu and National Research Institute for Biological Sciences, Bucharest.
Research has been conducted and performed by following the next steps:
First step to improve milk quality is the analysis of various dietary supplements rich in vitamin B_{12}. Goats's fodder ration consisting of alfalfa hay, wheat and oat bran, was improved with progressive yeast concentrations, from 1,50% of total fodder, to 3,00%, considered the *second stage.* The yeast obtained as a by-product of brewing, dry and inactivated, can be used as a dietary supplement by virtue of its content of vitamin B (Burlascu, 1983).
Chromatographic measurements were performed using a Shimadzu HPLC system with the following components: pumps LC-20AD sp Pump, column Kromasil 100-5C18, 250x4, 6mm (E26265), degas DGU-20As Degasser,

CTO-20AC Column Oven, detector SPD-M20A diode array detector, LCMS solution software.
Other equipment used to prepare stock solutions and samples were the analytical balance Mettler Toledo, Heidolph REAX vortex, Hettich UNIVERSAL320R spin. Mobile phase was filtered through a membrane ® O-20/25 CHROMAFIL, 0.20µm and treated in a ultrasonic bath TRANSSONIC 460/H in order to remove the air dissolved in the solution.
Samples were filtered before injection through the ultrafiltration cells Millex Syringa Driven Filter Unit, Millipore 0.22 mm.
The amount of injection vitamin B_{12} standard, as for the samples processed as described below, was 20 µl. The wavelength, to identify the compound of interest, was chosen according to the absorption spectrum, namely 361 nm which is the maximum specific absorption cyanocobalamin.
Both standard and samples were analyzed under identical conditions, as mobile phase being binary, with A component constituted from heptansulfonic acid (5 mm) in methanol and B component consisting of acetic acid (1%) which were filtered through a PTFE membrane. A flow rate of 1 ml / minute in isocratic way was used, the ratio between components of the mobile phase being 30:70 (A: B). The column was balanced for one hour before injecting samples.
Total analysis time was 20 minutes, therefore, between two successive samples the column was rebalanced with the mobile phase for 20 minutes. After a series of comprehensive analysis, HPLC system was cleaned with H_2O and methanol for one hour.
Analyses were performed at 25°C.
Determination of vitamin B_{12} was performed using standard, vitamin B_{12} first, Sigma Aldrich (Andrei and Pintes, 2004). From the analysis of chromatograms was observed that the retention time for vitamin B_{12} is characterictic $t_{R\ B12}$= 5,51min and was used then to identify vitamin B_{12} in samples, measurements being made at 361nm.

RESULTS AND DISCUSSIONS

In order to determine the response with the concentration variation of B_{12} vitamin, a calibration curve was drawn using injections of different concentrations of vitamin B_{12}. Measurements were performed in triplicate (Figure 1).
It is noted that the response presented an excellent linearity between the specific vitamin B_{12} peak area and concentration of the injected analyte, over

a range of concentrations, between $1.8x10^{-7}$ mol/L $-2.2x10^{-6}$ mol/L, the correlation coefficient being R2 = 0.9976.

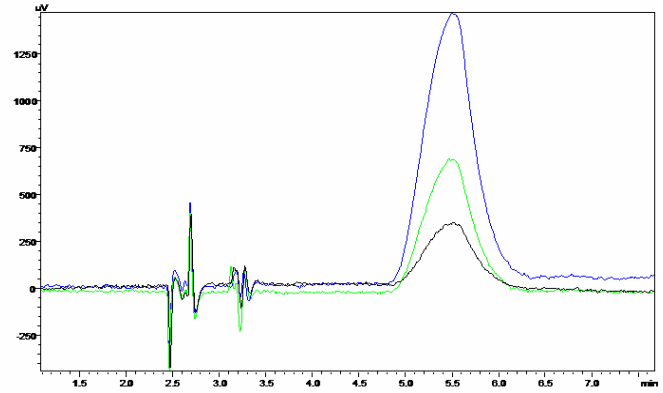

Figure 1. Chromatograms obtained for vitamin B12 to three levels of concentration from the calibration curve (0.75, 1.5 and 3 ppm)

Characteristic parameters for determining the B_{12} vitamin are presented in Table 1.

Table 1. Some performance characteristics of the developed chromatographic method for analysis of vitamin B_{12}

Analyte	Retention time	Equation of the calibration straight	R^2
Vitamin B_{12}	t_R = 5,51min	Y = aX + b a = 18554 b = -2263	R = 0,9976

Milk samples were analysed by high performance liquid chromatography (HPLC) using the method described above, after, in advance, they have undergone a treatment in order to achieve precipitation of interfering proteins and quantitative extraction of vitamin B_{12} from the sample's matrix. It should be noted that, as stated above, the identification of vitamin B_{12} was performed using the specific retention time, obtained for standard, by applying an acceptable coefficient of variance CV = 0.19 (leading to an average value of t_R = 6.134 min ± 0.19%).

Milk samples were processed using two methods of precipitation of proteins and extraction of vitamin B_{12}, marked in the text with P1 and P2. A first set of milk samples was submited to vitamin B_{12} extraction using trichloroacetic

acid (TCA), to precipitate proteins and to remove some interference, and a second set using acid hydrolysis (sulfuric acid, SA).
For uniformity, results are expressed in μg/100g milk for P1 (TCA), respectively μg/100ml for P2 (AS), according the stages:

1. Steps in the TCA :
   - 20g (± 0.02) milk +1.2 g milk
   - Centrifugation, 10min, 1250g, 18°C
   - Supernatant collection
   - Residue + 3 ml solution 4% TCA
   - Spin, 10min, 1250g, 18°C
   - Collection and mixing supernatant fractions brought to of 50ml volumetric flask with solution 4% TCA
   - Filter 0.22um and HPLC injection
   - Analysis HPLC

2. AS process steps
   - in 8 mL milk + 45 mL H_2SO_4 0.1N
   - Sonic in ultrasonic bath for 1 h
   - Brought to 100ml volumetric flask with methanol
   - Storage in the freezer for 1 h, -18^{0}C
   - Vortexing for 5 min and filtration through a filter 0.22um
   - HPLC analysis

Quantities of vitamin B_{12} obtained in all samples are presented in Table 2, where L **- ACT are the appropriate samples for the first processing method, while L **- AS P2 are samples treated according to the process P2.

Table 2. Concentration in B_{12} vitamin of milk samples using two methods of extraction

No.	Sample	Measurement	Retention time (min)	Concentration μg/ml	μgB_{12}/100g milk
1	L1-TCA	Vitamin B_{12}	6.134	0.1548	0.7740
2	L2-TCA	Vitamin B_{12}		0.1499	0.6850
3	L1 -AS	Vitamin B_{12}		0.1835	2.2933
4	L2 -AS	Vitamin B_{12}		0.1312	1.6398

Legend: L1 - milk with added yeast in fodder, L2 - milk without added yeast in fodder

As seen from the values presented in Table 2, taking into account the fact that they are micrograms per 100 g (100 ml), the differences between the two

extraction methods exist, but they are not dramatic. In addition, it can be said that the adequate values of vitamin B_{12} concentrations from the samples present the same type of variation, regardless the method of processing. Chromatograms corresponding to the results are presented in Figure 4 - 7.

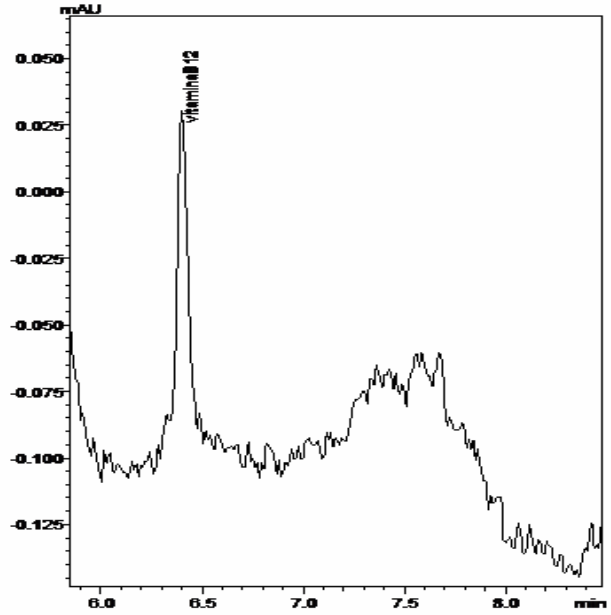

Figure 4. Chromatogram obtained for the milk sample L1-ATT

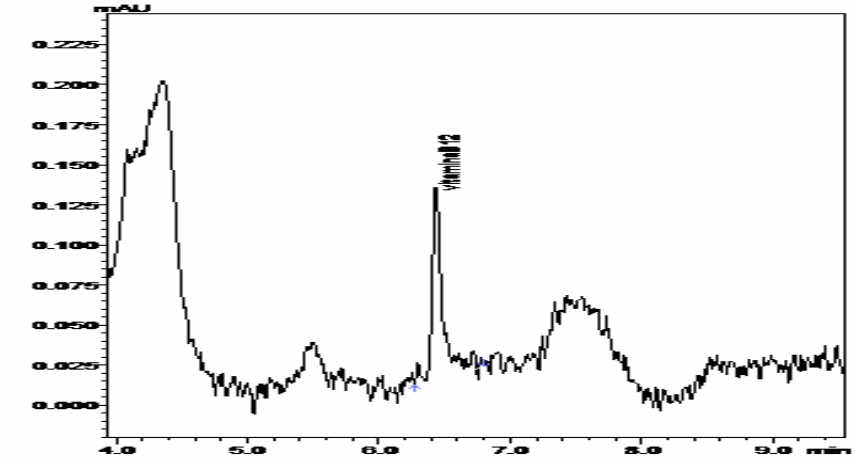

Figure 5. Chromatogram obtained from the milk sample L2-ATT

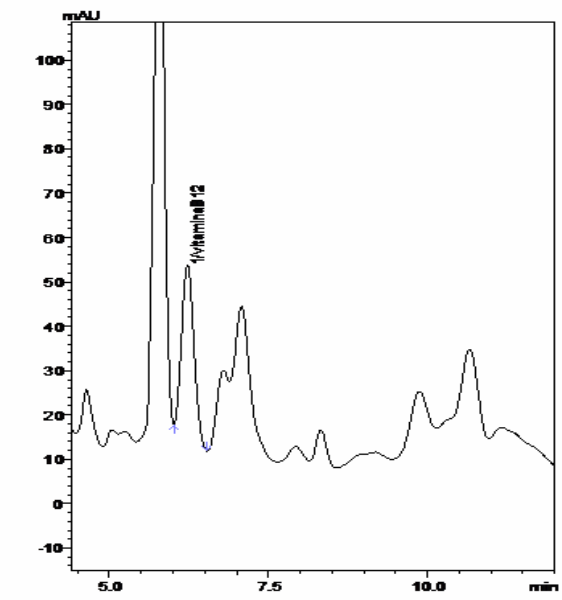

Figure 6. Chromatogram obtained for fodder sample

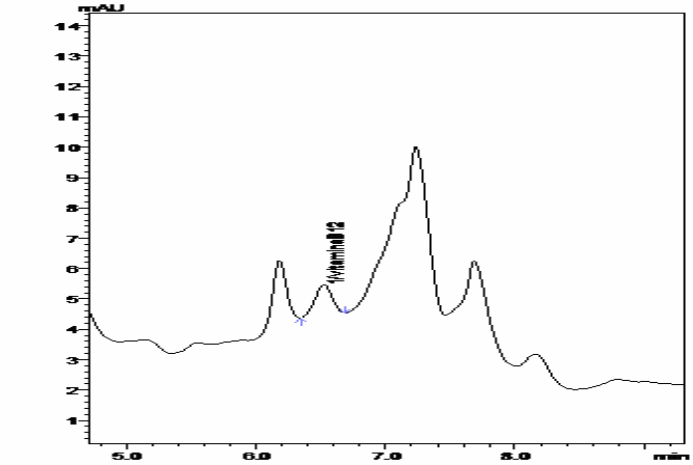

Figure 7. Chromatogram obtained for the yeast sample

Both yeast and fodder have undergone extraction processes like those of milk samples, but they were left to soak for 12 h, so the content of vitamin B12 in yeast, but also from the used forage was qualitatively noticeable, but, as amounts, below the linearity field set for the method.

CONCLUSIONS

The obtained data confirms that the developed method can be used for the determination of pure vitamin B_{12} by high performance liquid chromatography (HPLC) with diode-array (DAD) detection.
The vitamin B_{12} of milk samples can be detected after a treatment in order to achieve interfering protein precipitation and quantitative extraction of this vitamin from the fodder and yeast sample.
The identification of vitamin B_{12} was performed using the specific retention time, by applying an acceptable coefficient of variance CV = 0.19 (which leads to an average value of t_R = 6.134 min ± 0.19%), measurements being made at 361nm.

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