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...
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,
bifid - 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\textsubscript{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\textsubscript{12} in raw milk, the nutritional quality of milk was improved by the
administration of the yeast \textit{Saccharomyces carlsbergensis} in forage

**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:
\textit{First step} to improve milk quality is the analysis of various dietary
supplements rich in vitamin B\textsubscript{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 \textit{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\textsubscript{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\textsubscript{2}O and methanol for one hour.

Analyses were performed at 25°C.

Determination of vitamin B\textsubscript{12} was performed using standard, vitamin B\textsubscript{12} first, Sigma Aldrich (Andrei and Pintes, 2004). From the analysis of chromatograms was observed that the retention time for vitamin B\textsubscript{12} is characteristic t\textsubscript{R B12}= 5.51min and was used then to identify vitamin B\textsubscript{12} in samples, measurements being made at 361 nm.

RESULTS AND DISCUSSIONS

In order to determine the response with the concentration variation of B\textsubscript{12} vitamin, a calibration curve was drawn using injections of different concentrations of vitamin B\textsubscript{12}. Measurements were performed in triplicate (Figure 1).

It is noted that the response presented an excellent linearity between the specific vitamin B\textsubscript{12} peak area and concentration of the injected analyte, over...
a range of concentrations, between $1.8 \times 10^{-7}$ mol/L to $2.2 \times 10^{-6}$ mol/L, the correlation coefficient being $R^2 = 0.9976$.

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

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$_{12}$

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time</th>
<th>Equation of the calibration straight</th>
<th>$R^2$</th>
</tr>
</thead>
</table>
| Vitamin B$_{12}$ | $t_R = 5.51$min | $Y = aX + b$  
$a = 18554$  
$b = -2263$ | $R = 0.9976$ |

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 submitted 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 $\mu$g/100g milk for P1 (TCA), respectively $\mu$g/100ml for P2 (AS), according the stages:

1. Steps in the TCA:
   - 20g ($\pm$ 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°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

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Measurement</th>
<th>Retention time (min)</th>
<th>Concentration $\mu$g/ml</th>
<th>$\mu$g$B_{12}$/100g milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L1-TCA</td>
<td>Vitamin $B_{12}$</td>
<td>6.134</td>
<td>0.1548</td>
<td>0.7740</td>
</tr>
<tr>
<td>2</td>
<td>L2-TCA</td>
<td>Vitamin $B_{12}$</td>
<td></td>
<td>0.1499</td>
<td>0.6850</td>
</tr>
<tr>
<td>3</td>
<td>L1-AS</td>
<td>Vitamin $B_{12}$</td>
<td></td>
<td>0.1835</td>
<td>2.2933</td>
</tr>
<tr>
<td>4</td>
<td>L2-AS</td>
<td>Vitamin $B_{12}$</td>
<td></td>
<td>0.1312</td>
<td>1.6398</td>
</tr>
</tbody>
</table>

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 B₁₂ 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
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 $t_R = 6.134 \text{ min} \pm 0.19\%$), measurements being made at 361nm.

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