HPLC Analysis of Vitamin B₃ and Vitamin C from A Dairy Product Containing Brewer’s Yeast

Adriana Păucean¹*, Raluca Maria Pârlog², Dan Cristian Vodnar², Carmen Socaciu², Elena Mudura¹

¹Dept. of Food Science and Technology, ²Department of Chemistry and Biochemistry, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine, 3-5 Mănăștur str., 400372 Cluj-Napoca, Romania,

Received: 15 April 2010; Accepted: 30 May 2010

Abstract

Two vitamins, nicotinic acid (B₃) and ascorbic acid (C), from a functional dairy product (FDP) containing an optimized mixture of Lactococcus bacteria and brewer’s yeast were identified by HPLC –PDA and quantified by UV-VIS spectrometry. The water-soluble vitamins were analyzed by HPLC on a Restek Ultra Aqueous C18 column (250×4.6 mm, 5 µm). Functional dairy product content in these two vitamins was monitored during the shelf-life (1-21 days) of the product. During this period the content of vitamin B₃ increased and the vitamin C content reached its maximum peak on the first day of the shelf-life, and then it fell to almost half of the original content on day 7, only to climb again during the next timeframe.

The presence of the two vitamins in the product proves the bioactive potential of the brewer’s yeast and contributes to the functional features of the product.

Keywords: functional dairy product, HPLC, vitamin B₃, vitamin C, brewer’s yeast, Lactococcus

1. Introduction

The area of functional foods has attracted a great deal of interest since it is now recognized that many foods contain bioactive ingredients which offer health benefits or disease resistance. Functional dairy products provide several possible sources of bioactive ingredients: the microorganisms themselves (dead or alive) and metabolites of the microorganisms formed during fermentation [1]. In addition to beneficial microorganisms, functional dairy products contains vitamin, minerals and essential amino acids that help the body with healing and maintenance functions [2].

While the nutritional value of brewer’s yeast was recognized early, the identification of the nutritional compounds did not take place until the early 20th century. That was the period when several vitamins were first extracted and characterized from yeast, including biotin, niacin, pantothenic acid and thiamine. Nowadays, brewer’s yeast is recognized as a rich source of natural B-vitamins and provides also minerals, protein, amino acids, nucleic acids [3]. Brewer’s yeast is a by-product of the brewing industry and due to its functional value is available as a nutraceutical supplement in powder, tablet and flake forms. But in these supplements, yeasts cells are dried and inactive [4,5].

Brewer’s yeast is one of the best dietary sources of vitamin B₃ (niacin, nicotinic acid, nicotinamide). Niacin is a water-soluble vitamin that participates in more than 50 metabolic functions, all of which are important in the release of energy from carbohydrates.

Corresponding author: e-mail: apaucean@yahoo.com
Because of its pivotal role in so many metabolic functions, niacin is vital in supplying energy to, and maintaining the integrity of all body cells. Niacin also assists in antioxidant and detoxification functions, and the production of sex and adrenal hormones. Vitamin B₃ lowers cholesterol by preventing its build-up in the liver and arteries. Niacin moves fat from tissues for fat metabolism, burning it for energy. It promotes healthy skin, the health of the myelin sheath and good digestion, where it is also vital for the production of hydrochloric acid in stomach. It is an aid in protecting the pancreas, and is necessary for the health of all tissue cells [6].

Vitamin C is considered as L-ascorbic acid, dehydroascorbic acid, and other forms that are inactive. L-ascorbic acid is produced from glucose in the animal and vegetable cell. To this synthesis, in the last stage, the presence of gulonolakton oxydase is required. Humans and some animals do not posses this enzyme so that they are not capable of producing L-ascorbic acid [7]. L-ascorbic acid is a powerful water-soluble antioxidant that is vital for growth and maintenance of all tissue types in humans. One important role of ascorbic acid is its involvement in the production of collagen, an essential cellular component for connective tissues, muscles, tendons, bones, teeth and skin. Ascorbic acid helps regulate blood pressure, contributes to reduced cholesterol levels, and aids in the removal of cholesterol deposits from arterial walls. Ascorbic acid also aids in the metabolism of folic acid, regulates the uptake of iron, and is required for the conversion of the amino acids L-tyrosine and L-phenylalanine into noradrenalin. Although intestines easily absorb ascorbic acid, it is excreted to the urine within two to four hours of ingestion. Therefore, humans must have access to sufficient amounts of ascorbic acid from adequate dietary sources or supplements in order to maintain optimal health [8].

Milk and milk products are not good sources of this vitamin, although its content in milk varies, depending on the effect of various factors. Some authors [7-9] indicate that a certain increase in the vitamin C content in fermented milk products may be associated with its synthesis by some lactic acid bacteria (Lactococcus sp.). Accurate quantitative measurements of vitamins in food are useful as necessary requirement of sufficient input of vitamins.

Taking into account the lability of such vitamins, their content in food preparations needs to be checked, in order to ensure correct intake [10].

New techniques like high performance liquid chromatography (HPLC), connected to different detection are leading analytical tools for the identification, separation and quantification of vitamins.

The aim of this study was to quantify nicotinic acid (vitamin B₃) and ascorbic acid (vitamin C) contents in a dairy product which was manufactured using an optimized mixture of Lactococcus bacteria, kefir and brewer’s yeasts. For this purpose we used high performance liquid chromatography (HPLC) with Photodiode Array (PDA) detector. The presence of these vitamins in the product proves the bioactive potential of the brewer’s yeast and contributes to the functional features of the product.

2. Materials and methods
2a. Instrumentation and chemicals
A High Performance Liquid Chromatograph (HPLC, Szimatsu) coupled with a Photo Diode Array (PDA) detector was used. Organic solvents of HPLC grade, such as methanol and ammonium acetate were obtained from Merck (Darmstadt, Germany). Water used as eluent for analysis was filtered by a Millipore system. All water soluble vitamins: nicotinic acid and ascorbic acid were purchased as pure standarts from Sigma Aldrich (Germany).

2b. Preparation of standard solutions and curve
The aqueous stock solutions (1.2 mg/mL) of each vitamin were prepared every week, kept in refrigerator, in an aluminium foil, protected from light and working standards (in the range of 0.15 – 1.2 mg/mL), were used daily by appropriate dilution of the stocks. The standard curves were made by running min. 4 different concentrations of each vitamin on HPLC system.

2c. Chromatographic HPLC protocol
For the separation of the vitamins B₃ and vitamin C, a Restek Ultra Aqueous C18 column (250×4.6 mm, 5 µm) was used at ambient temperature. The mobile phase consisted of 0.05 M CH₃COONH₄/ CH₃OH (99/1) (A) and H₂O/CH₃OH (50/50) (B) at a flow rate of 0.8 mL/min. The injection volume was 20 µL. A multi step gradient was used, as we can see in table 1.
The PDA detection was monitored at 260 nm for both vitamins. Identification of resolved peaks in real sample was made by comparing their spectra with those derived from standard solution.

**Table 1.** Chromatographic HPLC gradient protocol used for the separation of the Vitamins B\(_3\) and C

<table>
<thead>
<tr>
<th>Time / min</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>4-18</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>19-25</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>26-30</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>31-43</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>43.01</td>
<td></td>
<td>STOP</td>
</tr>
</tbody>
</table>

2d. Dairy product manufacturing

The dairy product was manufactured in the pilot station of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. It was used 1.8% fat (w/w) skimmed, pasteurized milk, cooled at 30°C and inoculated with starter cultures and brewer’s yeast. The starter cultures were freeze-dried powders and Direct Vat Set (DVS) and consisted on:

1. a mesophilic bacterial culture FD-DVS CHN-22 (provided by Chr. Hansen) of *Lactococcus lactis* (ssp.cremoris, ssp. lactis and ssp lactis biovar diacetylactis), *Leuconostoc mesenteroides* subsp. cremoris, containing \(10^{10}\) colony forming units per mL (cfu/mL).

2. a yeast culture consisted on \(10^{10}\) cfu/ml *Debaryomyces Hansenii* kefir yeasts LAF 3, provided by Chr. Hansen.

3. the brewer’s yeast (*Saccharomyces cerevisiae*) represented the second yeast culture (\(10^{10}\)cfu/mL), separated from the secondary fermentation of beer, provided by a local brewer, having a cellular viability of 96%.

The volumetric ratio *Milk: bacterial culture: kefir yeast culture: brewer’s yeast* was 2000:2:4:1. After inoculation, the milk was incubated at 29-30°C for 12 hr, pre-cooled at 18-20°C for 1 hr, cooled again at 4-6°C for 10 hr and stored up to 21 days at 0-4°C. Two replications of all batches and samples were performed. In the final dairy product the density of *Lactococcus* bacteria fell during the first seven days of shelf life from a value of \(10^6\) cfu/mL to a value of \(10^5\) cfu/mL, and then remained constant at that level until the expiry date.

2e. Product sample preparation

The samples were analyzed after alkaline hydrolysis using sodium hydroxide (NaOH) 1N for 1 hour, at 50°C. After hydrolysis the sample pH was adjusted to the one of the mobile phase with hydrochloric acid (HCl). Prior to HPLC injection, the samples were centrifuged and then filtered through Teknokroma syringe filters (0.2 µm).

3. Results and Discussions

The analysis was performed during the shelf-life (1 – 21 days) of the functional dairy product which was stored refrigerated. Figure 2 presents the HPLC chromatograms of the dairy product in the days 1, 7, 14 and 21 of the shelf-life after alkaline hydrolysis. Identification of the vitamins B3 and C peaks in the samples was made by comparing their retention time and their spectra with those derived from standard solution (Fig.1)
It is well known that nicotinic acid is one of the most common compounds found in brewer’s yeast. Recent studies show though that vitamin C may be produced by *Saccharomyces cerevisiae* yeasts in certain environmental conditions [11,13]. There are several debates regarding the capacity of yeasts to synthesize L-ascorbic acid.

Due to modern analytical methods, we now know that *Saccharomyces cerevisiae* yeasts produce D-erythroascorbic acid, an analogue of L-ascorbic acid with the same reductive properties and which can also be oxidized by ascorbatoxidase.

Therefore, D-erythroascorbic acid has the same anti-oxidizing properties in the yeast cell as its plant-produced homologue. Due to the fact that *Saccharomyces cerevisiae* yeasts do not possess the ability to synthesize L-ascorbic acid from D-aldoses, the driving force of this metabolic transformation seems to be L-galactono-1,4 lactone, an hypothesis which is also supported by our isolation of two L-galactono-1,4 lactono-oxidases from the yeasts’ mitochondria [11,12].

To obtain ascorbic acid in vivo, yeasts need an environment (substratum) which contains a source of carbon, which may be represented by glucose, galactose, or lactose and a source of nitrogen, which may be represented by aminoacids, purines, or pirimidines. Milk can therefore be a good environment for the synthesis of ascorbic acid or its analogues, despite its low content of vitamin C. Another facilitating factor is the oxidizing stress to which the yeast cell is subjected [13,14], in the absence of a sufficient concentration of oxygen to maintain fermentation.

After analysis of data contained in Table 2, we observed that during the refrigerated shelf life of the product, the content of vitamin B3 increased. The biggest climb took place during the first seven days of shelf life, by 2.4 times. We think this was due to the multiplication of the yeast cells which took place in the same timeframe, to a level of $10^5$ cfu/ml. During the next period, despite the fact that the level of the *Saccharomyces cerevisiae* yeast remained relatively constant, the synthesizing process of nicotinic acid continued to rise.

<table>
<thead>
<tr>
<th>Vitamin mg/100g</th>
<th>Sample 1 day</th>
<th>Sample 7 days</th>
<th>Sample 14 days</th>
<th>Sample 21 days</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>2.38</td>
<td>5.8</td>
<td>6.08</td>
<td>6.4</td>
<td>7.49</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>3.9</td>
<td>2.57</td>
<td>2.73</td>
<td>2.66</td>
<td>3.61</td>
</tr>
</tbody>
</table>

With regards to the vitamin C content, it reached its maximum peak on the first day of the shelf life, and then it fell to 65% of the original content on day 7, only to climb again during the next timeframe. We think that the variation is due to the fact that during the first stage both microbial species, *Lactococcus* and *Saccharomyces* synthesize vitamin C, possibly in a number of analogues. During the following timeframe, lactic bacteria participate less to synthesizing vitamin C, and *Saccharomyces* start
using the substratum in conditions of oxidizing stress [14].

This phenomenon is undoubtedly related to the absence of monocarbohydrates from the environment, consumed in the fermentation process, as well as to the cell development dynamic of the yeasts during the shelf life of the product.

The decrease in vitamin C content might be the result of ascorbic acid oxidation by microorganisms included in the kefir starter culture. Ascorbic acid as an antioxidant reduces substance that are formed during metabolic changes of microorganism [7]. Other authors reported lower values for these vitamins in dairy products, but we consider that our results are due to the presence of brewer’s yeast in product composition and to the microbial mixture used for the manufacturing process.

4. Conclusion

In this work we used a high performance liquid chromatography (HPLC) coupled with a Photo Diode Array (PDA) detector to identify and quantify the content of two vitamins (nicotinic acid and ascorbic acid) from a dairy product which was manufactured using an optimized mixture of Lactococcus bacteria, kefir and brewer’s yeasts. The analysis was performed during the shelf-life (1 – 21 days) of the functional dairy product which was stored refrigerated.

These two vitamins were chosen due to their origin from the brewer’s yeast. It is well known that nicotinic acid is one of the most common compounds found in brewer’s yeast.

During the refrigerated shelf-life the content of vitamin B₃ increased; the biggest climb took place during the first seven days of shelf life due to the multiplication of the yeast cells at a level of 10⁵ cfu/ml.

During the next period, despite the fact that the level of the Saccharomyces cerevisiae yeast remained relatively constant, the synthesizing process of nicotinic acid continued to rise.

The vitamin C content, it reached its maximum peak on the first day of the shelf life, and then it fell to almost half of the original content on day 7, only to climb again during the next timeframe. This variation is due to both microbial species, Lactococcus and Saccharomyces, and is correlated with their cellular development. The presence of these vitamins in the product proves the bioactive potential of the brewe’s yeast and contributes to the functional features of the product.

References