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DETERMINATION OF TOTAL PHENOLIC AND FLAVONOID CONTENTS, ANTIOXIDANT ACTIVITY AND B VITAMINS OF DIFFERENT IRANIAN NON-ALCOHOLIC BEERS

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Abstract

This paper seeks to evaluate the quantity of B1, B2 and B6 vitamins using HPLC method and investigate the amount of total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity (antioxidant activity), pH, Brix and color of Iranian non-alcoholic beers. For doing so, four bottles of non-alcoholic beer (named from A to D) purchased from Iranian markets were investigated over a three-month storage period. Results demonstrated that after the storage time, TPC, TFC and antioxidant activity of all the samples reduced noticeably. Sample B had the highest level of total phenolic content, which was 10.17 mg of GAE/mL, but it had the lowest antioxidant activity showing the higher responsibility of the type of phenolic compounds in antioxidant activity than their quantity. Moreover, the results of HPLC method, conducted in the third month, showed that sample B had the highest level of vitamin B1 and B6, which were 68.47 and 43.11 mg/kg, respectively. In addition, vitamin B1 had the highest amounts in both samples. According to the results, it can be said that Iranian non-alcoholic beers contain a significant amount of total phenolic, flavonoid compounds with high antioxidant activity and B vitamins.

Keywords; Non-alcoholic beer, B vitamins, Total phenolic content, Antioxidant activity, Total flavonoid content, HPLC.

Introduction

It is widely acknowledged that the consumers' information about what they eat has increased over the past few years (Karelakis et al. 2020). According to the market demand, foods and beverages which are rich in nutrient and health-promoting compounds are becoming more popular, and It is frequently alleged that cereals and its products have very rich nutritious substances, such as proteins and bioactive compounds (Schwan & Ramos 2019). As a cereal fermented beverage, beer is one of the most consumed drinks (Castro-Muñoz 2019). A large percentage of this advantageous drink is formed from water, while the rest components of beer consist of carbohydrates and alcohol. This product not only has B-complex vitamins but also It has plenty of minerals, including potassium, calcium and magnesium (De Gaetano et al. 2016). Besides, owing to the presence of some polyphenols such as flavonoids and phenolic acids in malt drinks, it has a protective effect against cardiovascular diseases (Chiva-Blanch et al. 2015; Costanzo et al. 2011; Lutz et al. 2019). However, because of its high carbohydrates and alcohol contents, its consumption is usually circumscribed in type 2 diabetes mellitus patients (American Diabet Association, 2018; Wheeler et al., 2012). Nevertheless, moderate consumption of this nutritious beverage, especially its non-alcoholic type, is followed by positive health effects on consumers (Brányik et al. 2012). Even though the non-alcoholic beer has a lower concentration of phenol compounds and antioxidant activity than regular beers, its moderate amount of calorie content gives rise to the growing consumption of this beverage in recent years (Müller et al. 2016). On the whole,

non-alcoholic beer, the same as the regular one, has a lot of bioactive compounds (Alvarez et al. 2009; De Gaetano et al. 2016). Indeed, depending on the production technique and the raw materials, beer has a different composition (Yalçınçıray et al. 2020).
Due to lipid oxidation during beer staling, saturated and unsaturated aldehydes (e.g. trans-2-nonenal) are formed and can impact on the flavor of beer. Thus, it can be said that the higher the DPPH radical scavenging activity, the more stable the beer flavor is. Both synthetic and natural antioxidants, including sulfites, flavonoids and ascorbates can be used in the brewing industry to improve beer flavor stability, but consumers' demand, stiffening regulations and the existence of endogenous antioxidants cause brewers to minimize employing additives in beer (Vanderhaegen et al. 2006). Among all antioxidants, phenolic compounds are frequently found in different beers, and they play a crucial role in the brewing process by delaying, retarding, or preventing oxidation processes (Lentz 2018; Wannenmacher et al. 2018). In addition to the mentioned compounds, other essential components, which are key factors in human metabolism processes, are B1, B2 and B6 vitamins, so that B1 and B6 vitamins make an important contribution to the cellular metabolism of sugars, proteins and fats. Also, vitamin B1 deficiency leads to neurological disorders (Chen et al. 2015).
In light of the mentioned critical roles of vitamins, phenolic compounds, flavonoids and other antioxidants, lots of researches have been conducted for the determination of them in food samples. Zhao *et al.*, (2010) conducted a study to assess the phenolic profiles and corresponding antioxidant activities of 34 commercial beers in Chinese markets. They concluded that there was a difference among total phenolic content and antioxidant activity of various beer brands. In addition, Vinson *et al.*, (2003) measured the free phenols in different types of beer. Eventually, they found out that the non-alcoholic beer had the lowest phenolic compounds' concentration. Lugasi (2003) investigated the total polyphenol content and antioxidant properties of 5 lagers and 3 dark beers. Finally, the outcomes showed that the antioxidant properties of beers were dependent on total polyphenol content. There are many types of experimental methods that have been carried out for determination of water-soluble vitamins (Brunetti & Desimoni 2014; Dziomba et al. 2012; Jia et al. 2007; Navarro-Pascual-Ahuir et al. 2016). Because of various chemical structures, properties and low levels of occurrence in natural products, simultaneous determination of B1, B2 and B6 vitamins in food samples causes some analytical problems (Lebiedzińska et al. 2007). Among all the analytical techniques, the HPLC technique was used lower than others for determination of these compounds in food and pharmaceutical samples (Gliszczyńska-Świgło & Rybicka 2015; Lebiedzińska et al. 2007; Márquez-Sillero et al. 2013; Nurit et al. 2015). Hucker *et al.*, (2012) used the HPLC method for the analysis of thiamine (B1) and riboflavin (B2) vitamins, in unmalted and malted grains and found that in comparison to darker malts, lighter malts contain higher concentrations (2–5 times higher) of thiamine and riboflavin vitamins.
In this paper, we aimed to evaluate the amounts of B1, B2 and B6 vitamins by means of an HPLC method and the secondary objective of this study was to measure the amounts of phenolic compounds and antioxidant activity of different brands of non-alcoholic beers produced in Iran.

Methods and Materials

Non-alcoholic beer samples and chemicals

Firstly, four bottles of non-alcoholic beer produced from malt and hop, with different brands and same production dates, were purchased from local markets in Iran. Secondly, they were named from A to D (In this paper, we did not mention the brand of samples). All the samples were analyzed within three months and during the analysis, they were stored in 24 ± 1 °C. Sodium carbonate, gallic acid, aluminium chloride, methanol, tris-HCl buffer and DPPH reagent were bought from Merck (Darmstadt, Germany). Furthermore, Folin–Ciocalteu reagent and ethanol were purchased from Sigma-Aldrich (Steinheim, Germany) and Golriz (West Azerbaijan, Iran), respectively. Double distilled water was used for all stages.

Brix, pH, and Color analysis

Determination of pH was carried out using a pH meter (PM12; Fan Azma Gostar., Tehran, Iran). A refractometer (HSR-500; Atago., Tokyo, Japan) was used for evaluating the Brix. Both pH and Brix analyses were performed after the accurate calibration of instruments. The color analysis was conducted using HunterLab method. For doing this, the Hunetrlab colorimeter was calibrated, and then, L*, a*, b* parameters were recorded.

Determination of total phenolic content (TPC)
The Folin–Ciocalteu colorimetric method, with modifications, was used for the measurement of TPC (Gorinstein et al. 2004). First, 2000 µL of 2% sodium carbonate was blended with 100 µL of non-alcoholic beer sample. After 5 minutes, 100 µL of 50% Folin–Ciocalteu reagent was added to the obtained mixture, and the solution was left at 24 ± 1 °C to react for 20 minutes. Afterwards, a spectrophotometer device (U-3900/3900H; Hitachi Ltd., Tokyo, Japan) was utilized to analyze samples at 750 nm against a blank. Finally, the amounts of TPC were obtained using a calibration curve (with a line equation of y=0.1419x-0.014, R^2=0.9846) as mg of Gallic acid equivalent (GAE)/ mL of sample.

Determination of total Flavonoid content (TFC)
The crystalline Aluminum Chloride assay developed by Maksimović, Malenčić, & Kovačević (2005) was used for the determination of TFC. Briefly, 1500 µL of the sample was combined with the same volume of 2% aluminum trichloride, and in order to formation of a flavonoid-aluminum complex, they were permitted to react for 15 minutes. Following that, the absorbance was recorded at 430 nm. Eventually, the amounts of TFC were stated as quercetin equivalents (QE) using a calibration curve (with a line equation of y=0.2015x-0.0203).

DPPH radical scavenging activity (antioxidant activity)
The method described by Yamaguchi et al., with some modifications, was employed to evaluate the DPPH radical scavenging activity (Yamaguchi et al. 1998). Initially, we blended 0.2 L of methanol or sample with 0.1 M Tris-HCl buffer (pH 7.4, 800 µL). In the next stage, the obtained mixture was added to 250µM of DDPH solution (1,1-diphenyl-2-picrylhydrazyl), which was created from 1000 µL of 0.5 M DPPH and ethanol. The mixtures were agitated intensively and left to react in a dark place for 20 minutes at 24 ± 1 °C. Eventually, the absorbance was read at 517nm using a spectrophotometer. Equation 1 was used for calculating the antioxidant activity of the samples:

Equation 1. $\text{Scavenging activity } \% = (1-(A_{sample}-A_{blank})/(A_{control}-A_{blank}))\times 100$

Where the A_{sample} is the absorbance of non-alcoholic beer + Tris-HCl buffer + dissolved DPPH in Ethanol, the $A_{control}$ is the absorbance of Tris-HCl buffer + dissolved DPPH in Ethanol and the A_{blank} is Ethanol (98%).

Determination of B Vitamins (B1, B2, B6)
The HPLC method, using a Waters 600 HPLC apparatus (Waters Corp., Milford, MA, USA), was used for the determination of B vitamins (B1, B2, B6) in the third month. This part of the project was performed for B and D samples. Briefly, 1000 µL of each sample was poured into a 25-mL volumetric flask and diluted with distilled water. Afterwards, they were placed into an HPLC device using vials and results were recorded using a Pentium IV PC.

Statistical analysis
To analyze the results and compare the samples, one-way ANOVA was used and the Tukey test was used to compare the means at 95% level. Experiments were performed in triplicate. Minitab 16.00 and Excel software were used to analyze the statistics and drawing charts, respectively.

Results and Discussion

Brix and pH
The results obtained from the Brix of samples are presented in figure 1. According to the data, it is clear that there is no significant difference between various months in each sample. Although sample B has the highest Brix, sample D has the lowest Brix. As stated by Azhuvalappil et al., (2010) thermally process influences the yeast's growth, and as a result, Brix of the products from various brands differ from each other. Therefore, it can be said that different process condition may affect the products' Brix.

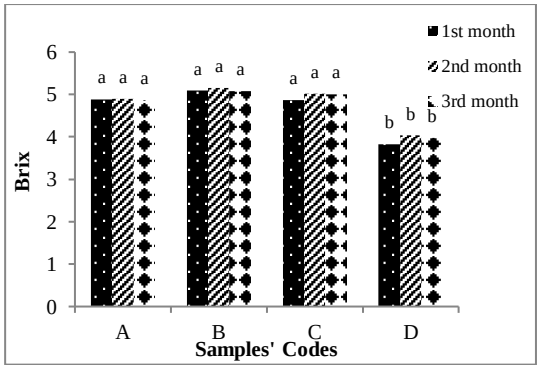

Figure 1. Brix of all the samples during different months (A-D are samples).

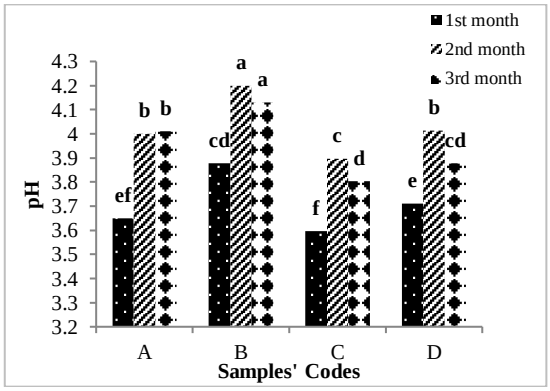

Figure 2. Trend of pH Changes of samples during different months (A-D are samples).

As it can be observed from figure 2, the results of the pH test for all the samples demonstrate a dramatic upward trend from the first month to the second month, while a decrease is observed in the third month. Approximately, in all the samples, excluding sample A, these fluctuations are observed. In the second month, sample B has the highest pH (pH=4.2). Conversely, sample C has the lowest pH in the same month. As might be noticed, sample B has the highest pH after three months (pH=4.1), whereas sample C has the lowest pH (pH=3.8). The other significant point is an overall increase in pH over the 3 months of analysis time for all the samples. Accordingly, it can be concluded that with ageing, the pH of non-alcoholic beers increases. Because yeasts produce organic acids and the dissolution of carbon dioxide forms carbonic acid. As a result of formation of these compounds, pH increases. Also, the consumption of minerals by yeasts and growth of them result in increasing pH (Hardwick 1994).

Color

Figure 3 illustrates the amounts of L* parameters for the analyzed samples. As shown, the amounts of the L* parameter vary in different months. While there is no significant difference between all the samples in the two last months, Sample A and sample B has the highest and lowest values of the L* parameter in the first month, respectively. As far as chart gives us, L* parameter decreases in all the samples over the 3 months of analysis time.

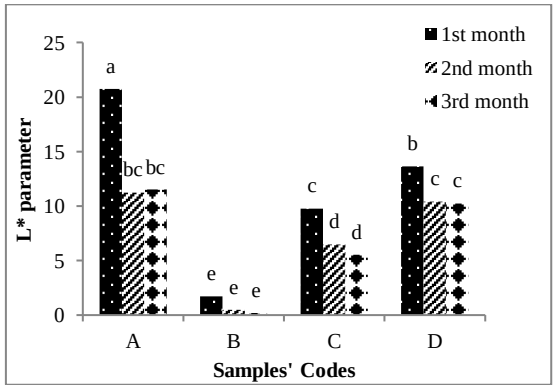

Figure 3. Trend of L* parameter changing in all the samples during 3 months (A-D are samples)

Figure 4 indicates the values of the a* parameter. Although sample A has an upward trend during the three months, sample B has a downward trend at the same time. On the other hand, this parameter has a certain fluctuation in sample C and D. All in all, among all the samples, sample B demonstrates the lowest value of the a* parameter.

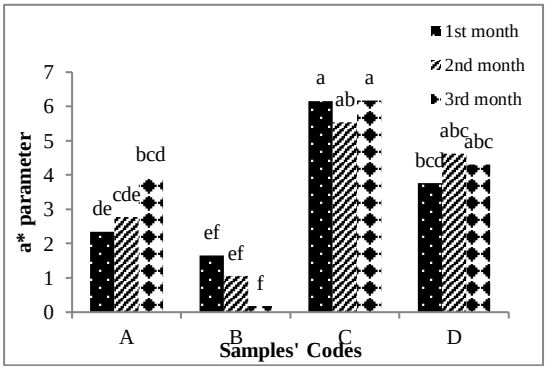

Figure 4. Trend of a* parameter changing in all the samples during 3 months (A-D are samples).

Figure 5 provides information about the b* parameter. As it can be seen, in comparison to the other samples, the highest amount of this parameter is in sample D and the lowest amount is in sample B, over the analysis time. Despite the downward trend that is observed in majority of the samples, sample A has an upward trend.

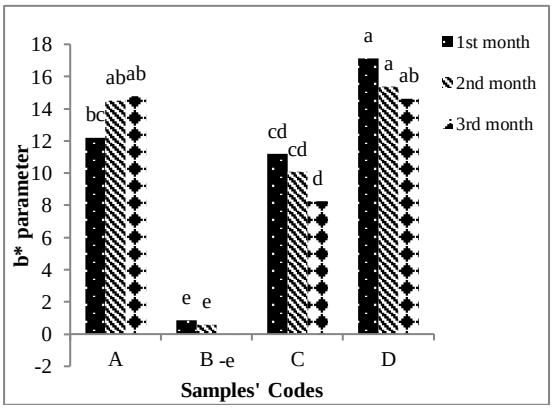

Figure 5. Trend of b* parameter changing in all the samples during 3 months (A-D are samples).

The L*, a* and b* values are often used in research conducted on color of foods. L* demonstrates the luminance or lightness component, and a*(from green to red) and b* (from blue to yellow) indicates chromatic components (Pathare et al. 2013). With due attention to figure 3, 4 and 5, it is comprehendible that sample B, on the contrary of the other samples, had the lowest values. Therefore, it can be concluded that sample B has a darker color, comparing to other samples.

TPC determination

As mentioned above, the total phenol content was measured in all the non-alcoholic beer samples using the Folin–Ciocalteu colorimetric method. The results are shown in figure 6. In the first month, the highest and lowest TPC are related to the B and C samples, respectively. Also, there is a constant trend in all the samples that is easily visible. In other words, all the samples have a downward trend over the entire analysis time. Also, relying on the results of the TPC variance analysis, all non-alcoholic beer samples has a significant difference of 1%, statistically. Overall, all the analyzed samples has a noticeable reduction after three months.

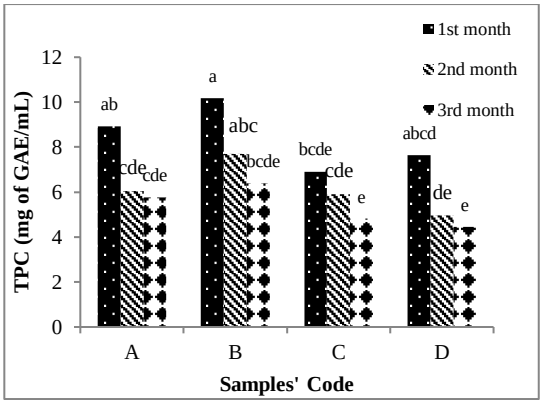

Figure 6. Total Phenolic content (TPC) of the analyzed samples, Where *GAE* is the gallic acid equivalent (A-D are samples).

What clinches our results is the research conducted by Ma *et al.,* (2019). They analyzed samples of 'Jonagold' apple and concluded that the reason for the decrease in the amount of phenolic compounds is the fact that the flavonoids and phenolic compounds were attacking by reactive oxygen species. Also, the polymerization of monomeric phenolic compounds can be the cause of this reduction (Piretti et al. 1996). Also, Wojdyło, Teleszko, & Oszmiański (2014) conducted another research which had the same result. They analyzed the storage stability of quince juice phenolic compounds and concluded that the TPC decreased successively in all quince juices after a 6-month storage period. Besides, it is proved that phenolic compounds can be bonded to the macromolecules, such as proteins, and formed a precipitate during the storage time (Guyot et al. 2002; Le Bourvellec et al. 2004). Therefore, it can be concluded that the loss of TPC is a cause of their precipitation or being attacked by reactive oxygen species or polymerization of the monomeric phenolic compounds.

Also, a key factor which can cause a profound impact on the phenolic compounds of our samples is the hop's phenolic compounds. According to the conducted research, hop has a great amount of polyphenols, so that lager beer has the highest polyphenol compound and non-alcoholic beer comes out second (Krofta et al. 2008; Monteiro et al. 2006; Piazzon et al. 2010). Accordingly, the phenolic compounds of samples are a reason for hop's antioxidant activity. Moreover, the differences in raw materials and brewing process lead to significant differences in phenolic profile and antioxidant activity of beer. Moreover, there is an argument over the relevance of phenolic compounds and antioxidant activity of beer with each other (Vanderhaegen et al. 2006).

TFC determination

Figure 7 indicates the TFC of non-alcoholic beer samples over the 3 months of our analysis. Due to the chart, the highest total flavonoid content is in the sample B. This sample has a significant difference at 1% level, statistically. Sample A and sample D relatively have the same values of flavonoid content, while sample C placed between D and B samples. The total flavonoid content in each sample does not change significantly during the three months. However, in sample B a downward trend is observable.

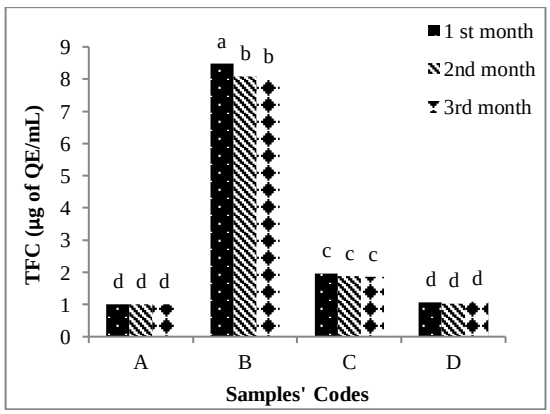

Figure 7. Total Flavonoid content (TFC) of non-alcoholic beer samples, Where QE is the

quercetin equivalents (A-D are samples).

As mentioned previously, decreasing of flavonoids may be a result of the reactive oxygen species attack (Piretti et al. 1996). Accordingly, the reason for decreasing flavonoid content in our work could be the same problem.

DPPH radical scavenging activity

According to the results, sample B has the highest radical scavenging activity (83.33%) among all the samples over the 3-months storage period. This sample has a statistically significant difference at 1% level. Sample A and other samples, in the same way, have an increase in DPPH radical scavenging activity after 3 months.

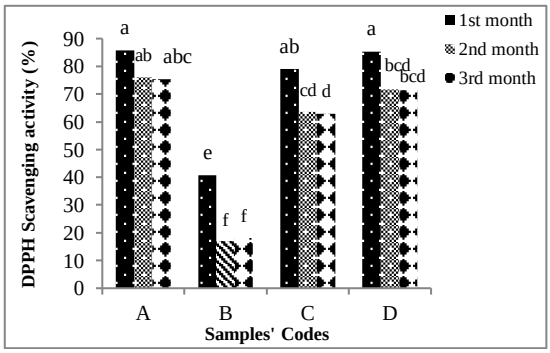

Figure 8. DPPH radical scavenging activity in the analyzed samples (A-D are samples).

The decrease of DPPH radical scavenging activity may be attributed to decrease in phenolics and flavonoids concentrations during the storage time, since antioxidant activity is closely correlated with the presence of phenolics and flavonoids (Ma & Huang 2014). Decreasing of antioxidant activity, also, was proved by Kolniak-Ostek *et al.,* (2014). As it is regularly known, the antioxidant capacity strongly depends on the polyphenolic content (Ma et al. 2019), but according to the conducted research, it is proved that the type of phenolic compounds is more responsible than their quantity for higher antioxidant activity, because the antioxidant activity of phenolic acids and their derivatives (e.g. esters) depends on the number of hydroxy groups in the molecules (Kähkönen et al. 1999; Rababah et al. 2004; Shahidi & Naczk 2003). These studies could be the reason for the accuracy of our outcomes.

Vitamin B Analysis (B1, B2, B6)

Figure 9 shows the chromatograms of the HPLC method which were recorded for evaluating B vitamins (B1, B2, and B6). Table 1 provides the values of analyzed vitamins in non-alcoholic beer. It is clear from the table that sample B has the highest level of vitamin B1 and B6. Also, vitamin B1 has the highest quantity in both samples. While the amounts of vitamin B1 and B6, in sample B, is clearly more than sample D, it has greater amount of vitamin B2.

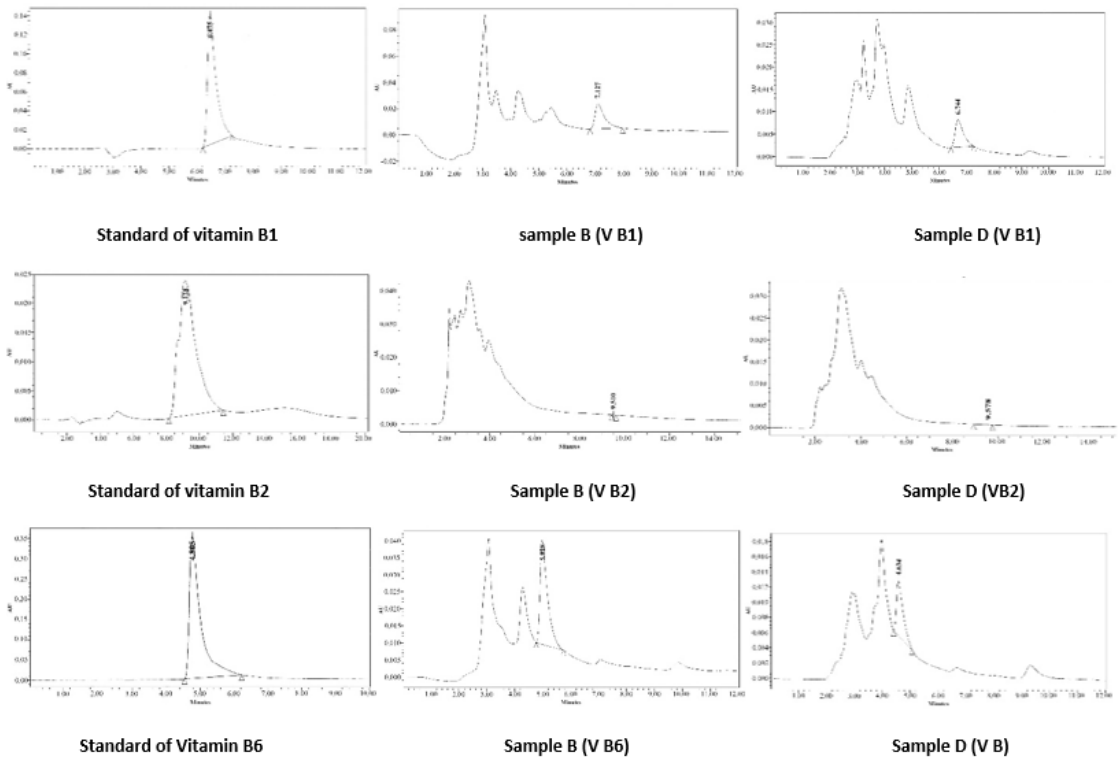

Figure 9. Chromatograms of the analyzed vitamins

Table 1- quantity of the analyzed vitamins in B and D samples

No.	Vitamins	Sample B (mg/kg)	Sample D (mg/kg)
1	Vitamin B1	68.47	19.63
2	Vitamin B2	0.02	0.08
3	Vitamin B6	43.11	9.02

According to previously conducted research by Hucker *et al.,* (2011, 2012), the existence of B1 and B2 vitamins in beer has been established. Moreover, the presence of B1 and B6 vitamins in malt drinks has been proven by Jastrzębska *et al.,* (2017). Furthermore, it has been confirmed that various types of beer may have different quantities of thiamine and riboflavin vitamins and these differences might be due to raw materials and production process (Bamforth 2002; Hucker et al. 2011, 2012). In other words, the recipe and brewing process affect the beer's chemical complex (Almeida et al. 2006).

Conclusions

In conclusion, the results of this experiment showed that Iranian non-alcoholic beers contain a significant amount of phenolic and flavonoid compounds with a high antioxidant activity. In addition, the presence of B vitamins, including high levels of vitamin B1, has been proven. Overall, among analyzed samples, sample B had the highest level of phenolic and flavonoid compounds, antioxidant activity, and B vitamins. On the other hand, it had the lowest level of L*, a* and b* parameters in the Hunterlab method. In brief, it is important to note that most of these compounds reduced in all the samples during the analysis time. However, it is fairly to say that consuming of these beverages is really recommended, because thanks to their nutritive compounds, they can play an important role in human healthiness.

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