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SPECTROPHOTOMETRIC ANALYSIS OF RETINOL AND BETA-CAROTENE IN MILK

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Abstract: *Milk is an essential part of the daily foods. Natural milks contain certain amounts of micronutrients such as vitamins and carotenoids. Vitamins are crucial for the proper development of the baby, as well as for adolescents and adults. These substances cannot be synthesized de novo in the human body and therefore they are provided through food. The concentration of the carotenoids in cow milk is critical point for the implementation of complete nutrition. In this study, spectrophotometric analyzes are persented for retinol (vitamin A) and β-carotene for UHT milk samples. Different mixtures of organic solvents were used and compared. Linear ranges of retinol and β-carotene in standard solutions were obtained by using hexane as solvent. The equations obtained for these curves were used to determine the target substances in real cow milk samples.*

Keywords: *retinol, beta-carotene, carotenoids, spectral analysis, milk, vitamin A.*

INTRODUCTION

Milk and dairy products are always a hot topic in the scientific community. Nutritionists examine all components of milk to include or exclude them from a possible diet. Milk is a mixture of proteins, fats, carbohydrates and vitamins (Ullah et al., 2017). Vitamins are essential organic micronutrients, grouped in two types – fat-soluble and water-soluble. The fat-soluable vitamin A, presented as retinyl fatty acid esters and as free retinol, has the highest concentration of all other vitamins in milk (Ugarković et al., 2020). Vitamin A has proven effect on the quality of human vision. It also plays a key role in reproductive capacity, embryonic growth and development (Meléndez-Martínez, 2019). This vitamin is essential also for the immune system and therefore it is important to take it in optimal amounts in a pandemic, as it is the case today with the COVID-19 pandemic. Li and colleagues (2020) discussed that in their study about the potential of vitamin A uses in the treatment of COVID-19.

Milk also contains fat-soluble organic color compounds named carotenoids. Carotenoids are divided into carotenes and xanthophylls. Great amount (~90%) of the carotenoids in milk is β-carotene, also called provitamin A (Ugarković et al., 2020). It is synthesized only in plants (fruits and vegetables) and some invertebrates but not in animals (not in humans) (Ullah et al., 2017). Nevertheless, β-carotene is found in cow milk (Ugarković et al., 2020) and it can easily be provided to a human when consuming milk. Ugarković and coauthors (2020) describe carotenoids as important antioxidants with preventative effects for cardiovascular and eye diseases, and certain cancers.

Given the benefits of carotenoids on human health, it is good to monitor their concentrations in the milk that is served to consumers. In the present study, a spectrophotometric method was proposed for the determination of vitamin A (retinol) and beta-carotene in cow's milk.

EXPOSITION

Materials

The standard solution was from PhytoPharma “Vitamin A”, each soft jelly capsule contained 1450 μg RE and 1 000 μg β-carotene. All of the organic solutions were purchased from Sigma-

Aldrich. The used water was distilled water. The absorbance was read by Jenway 7205 UV/Vis spectrophotometer (Cole-Parmer, UK). All samples were analyzed in duplicate.

Choice of solvent of the standard solution

Stock standard solution was with concentration 9 mg/mL retinol and 6.25 mg/mL β-carotene. Five different solvent mixtures were used and compared for determination of standard solution absorbance. The used solvent mixtures were: 1) hexane/i-propanol/water – 6/8/1 *v/v/v*; 2) petroleum ether/acetone – 7/3 *v/v*; 3) petroleum ether/diethyl ether – 8/2 *v/v*; 4) acetone/hexane – 2/3 *v/v*; 5) hexane (Braniša et al., 2021; Aremu and Nweze, 2017). The initial solutions for measurement contained 2 μL standard and 2 mL solvent mixture. Analyzes were made rapid in a controlled environment. If the resulting standard scan was out of range of the apparatus, appropriate dilution of the sample with the same solvent mixture was made.

Standard curve obtaining

Standard solutions of the initial standard (retinol and β-carotene) were made in hexane. Stepwise two-fold dilutions of the solution were prepared. Therefore, retinol was with concentration from 0.047 to 9 mg/mL, and β-carotene was with concentrations from 0.032 to 6.25 mg/mL. Absorbance was read of each sample at 325 nm for retinol and 448 nm for β-carotene. Graduated tubes with caps were used from all of the solutions.

Measurements with milk

UHT cow milk from the local market was used. The milk had 3.7% fat, 4.7% carbohydrates, 3.3% proteins. The carotene extraction method was according to Hulshof and co-authors (2006) with some modifications. First, 1 mL milk was loaded in a glass centrifugal tube with a cap, also 0.25 mL 25% ammonia water and 1 mL 96% ethanol containing 0.1% (w/v) ascorbic acid were added. The mixture was shacked and the extraction was performed by adding 2 mL diethyl ether and 2 mL petroleum ether. It was mixed on a magnetic stirrer for 5 min and the obtained mixture was centrifuged at 5 000 rpm for 5 min. The extracted carotenoids were in the top layer and they were gently separated in a glass (inner diameter 38 mm). The liquid phase was evaporated (maximum temperature 32°C). The dry residue was saponified by 1.5 mL of fresh prepared 20% (w/v) KOH in 96% ethanol. The glass was placed in dark on magnetic stirrer for 90 min. After that, 1 mL water was added to dissolve the obtained salts. Final extraction was performed twice in a small separatory funnel with hexane. The final volume (2.5 mL) was collected in a graduated tube with a cap. Absorbance was read at 325 nm and 448 nm wavelength. The same procedure was performed with milk sample with added 2 μL standard solution.

RESULTS AND DISCUSSION

Carotenoids are two groups according to their structure – hydrocarbon carotenoids (also called carotenes) and xanthophylls. Carotenes are non-polar compounds, unlike the xanthophylls that are more polar, due to O-containing (Merhan, 2017). The choice of a suitable organic solvent is crucial for obtaining good spectral results. The absorption maxima of retinol and beta-carotene were studied in combinations of different solvents. The results are presented in Figure 1. A key point is the different polarity they provide. The lowest results were obtained by using acetone/hexane – 2/3, and petroleum ether/diethyl ether – 8/2. It was also noticed that in three of the cases the peaks were clearly written. Those solutions were: petroleum ether/acetone – 7/3; hexane; hexane/i-propanol/water – 6/8/1. The other part of the analyses was performed using hexane as organic solvent for absorbance measurements. The absorbance maximum of retinol was at 325 nm and β-carotene was at 448 nm, using hexane.

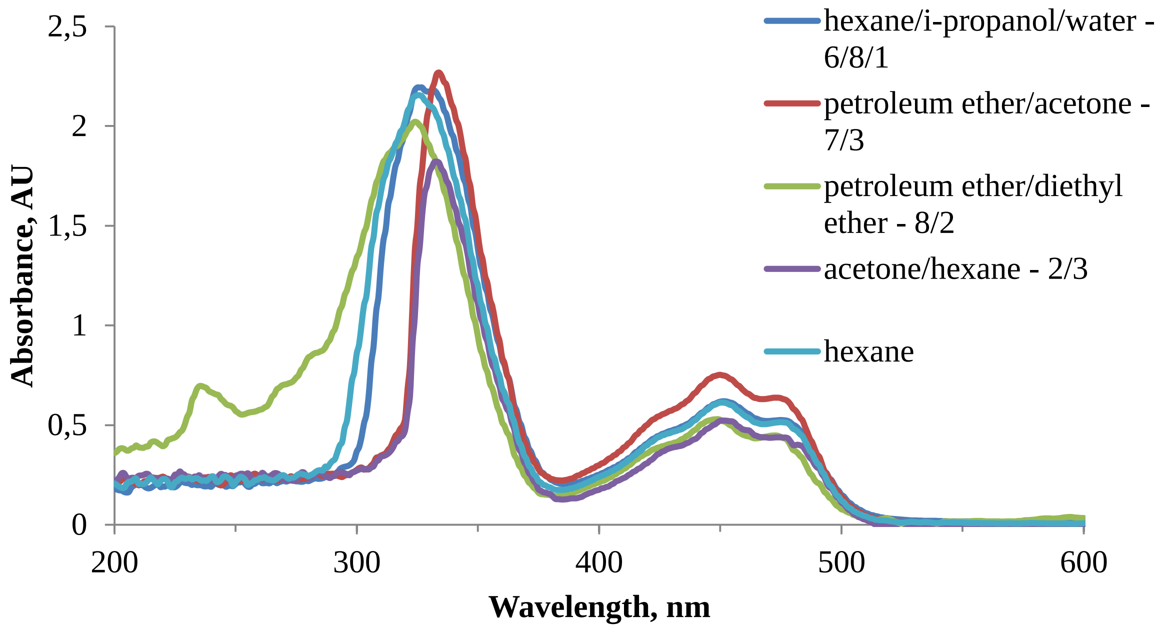

Fig. 1 Absorbance maximums for retinol (first peak) and β-carotene (second peak) in different solutions

Standard curves and equations were obtained by serial dilutions of the standard and using hexane for solvent. Retinol had large linear range from 0.47 to 3 μg/mL and equation: $y = 0.1906x + 0.0039$, $R^2 = 0.9998$ (Fig. 2).

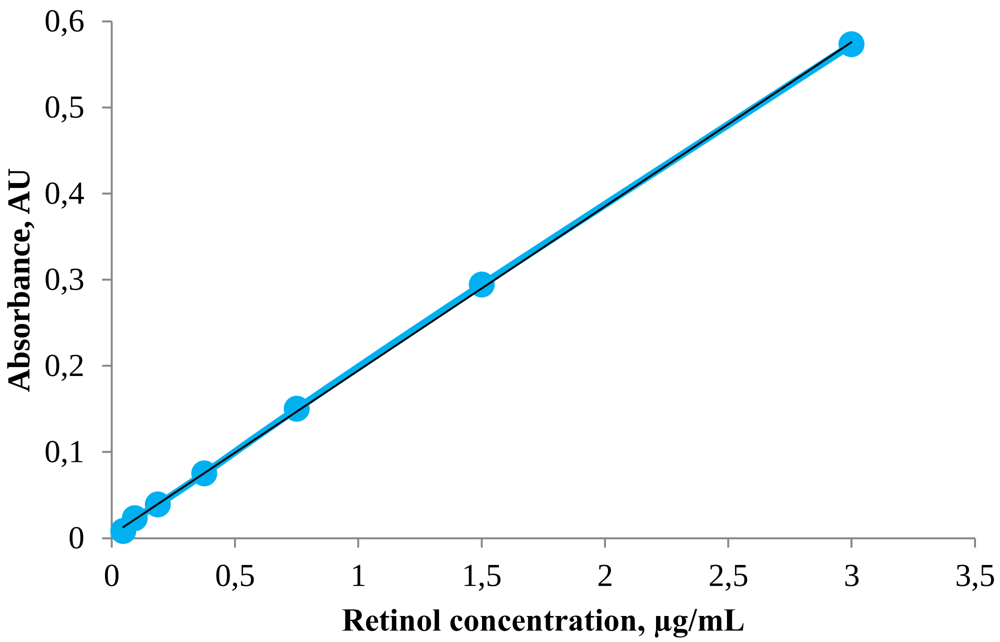

Fig. 2 Standard curve of retinol in hexane, at wavelength 325 nm

Beta-carotene, measured at the same conditions and 448 nm, had two separate linear ranges (Fig. 3 and Fig. 4). Linear range was observed at concentrations from 0.032 to 1.042 μg/mL. The equation with those concentrations was: $y = 0.0721x + 0.0006$, $R^2 = 0.9991$ (Fig. 3). The other linear range with β-carotene in hexane was at concentrations from 1.56 to 6.25 μg/mL. The equation was: $y = 0.1172x + 0.0285$, $R^2 = 0.997$ (Fig. 4).

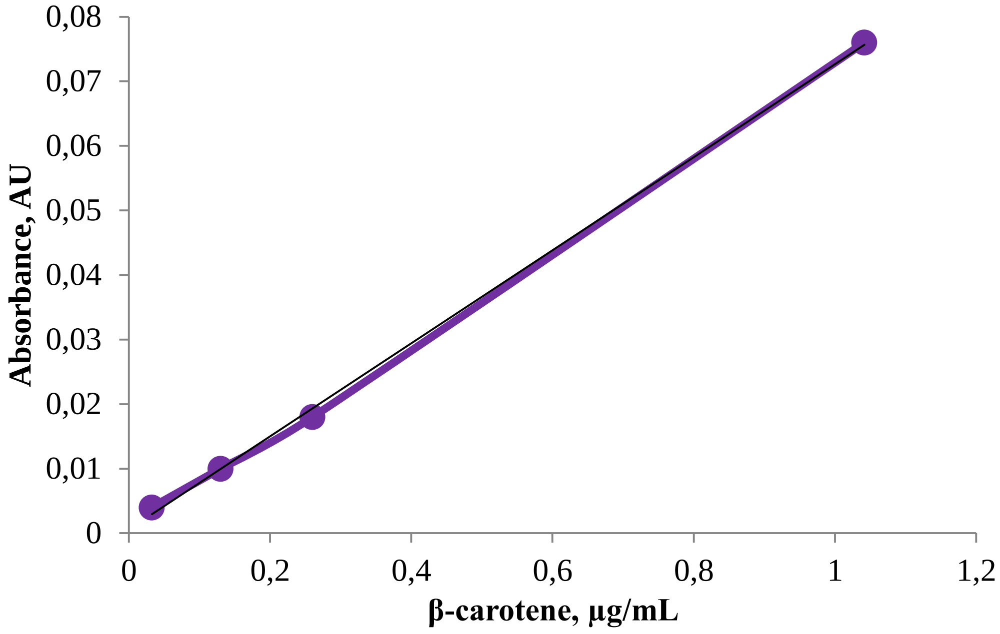

Fig. 3 Standard curve of β-carotene (≤ 1 µg/mL) in hexane, at wavelength 325nm

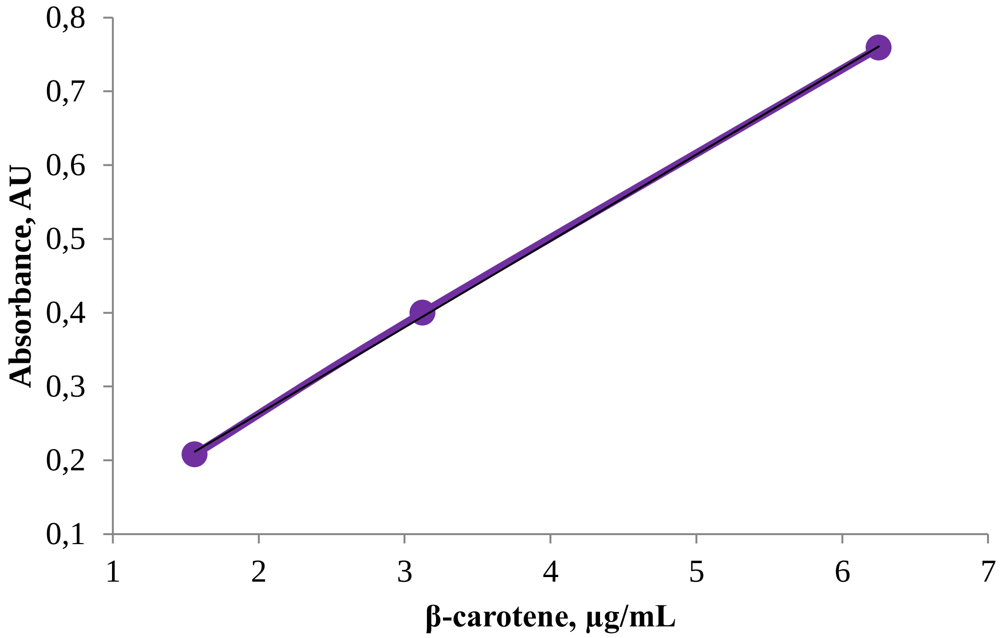

Fig. 4 Standard curve of β-carotene (> 1 µg/mL) in hexane, at wavelength 325 nm

The obtained equations were used for milk samples. The results with UHT cow milk were compared with literature data and also with milk with added known analytes concentrations. The obtained results are shown in Table 1. As can be seen from the results, the presented method offers a good way to determine the concentration of carotenoids in milk. Also, by adding a known concentration of analytes, similar results can be obtained. The described method for retinol and β-carotene determination in milk can be applied in research laboratories, but also in university laboratory exercises. It is a relatively fast and inexpensive method, in which the only equipment is a spectrophotometer, and the work with organic solvents teaches the laboratory assistant the application of good laboratory practic**e**.

Table 1 Vitamin A and β-carotene in cow milk

	Cow milk	Cow milk with added carotenoids
Retinol (Vitamin A) expected concentration, μg/100 mL	10 – 100[a] 40[b] 33[c]	≥514[d]
Retinol (Vitamin A) obtained concentration, μg/100 mL	33	598
β-carotene expected concentration, μg/100 mL	3 – 50[a] 20[b] 26[c]	≥357[d]
β-carotene obtained concentration, μg/100 mL	13	325

[a] Ugarković et al., 2020; [b] Hulshof et al., 2006; [c] Strusińska et al., 2010; [d] Calculated concentration, if the milk has no carotenoids before adding.

CONCLUSION

Vitamin A and β-carotene are essential for human health, especially in COVID-19 pandemic. Milk could be a good source of important nutrients, including carotenoids. Routine testing (like the presented analyses) of the concentrations of vitamin A and β-carotene in milk on the market would ensure guaranteed quality of the product offered, and hence a healthy population.

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