LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BETA-CAROTENE FROM MILK AND CHEESE

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Abstract. The beta-carotene content in milk and cheese is an accurate indicator for the feeding method used for cows. It can also be sued as an indicator of adulteration of goat and other types of milk with cow milk. A high accuracy HPLC-UV method for the determination of beta-carotene from milk and cheese has been developed. After saponification with an alcoholic solution of potassium hydroxide the samples where extracted with a mixture of hexane: ethyl acetate (70:30) was used. The concentrated extract was analyzed using Perkin Elmer 200 Series High Performance Liquid Chromatograph (HPLC) with UV detector for the determination of beta-carotene content. The calibration was done in 4 points from 1-20 ng/ml. The recovery yield was between 73,8-89,7 %. The method was tested on 3 milk samples and 5 cheese samples. The highest concentration of beta-carotene was found in cheddar cheese produced with milk from pasture raised cows.

Keywords: beta-carotene, HPLC-UV, cheese, milk

INTRODUCTION

Raw milk production on EU farms was 172.2 million tons in 2018, representing an annual increase of 1.6 million tons. The vast majority of raw milk is delivered to dairy factories; only 12.2 million tons were used on farms: consumed by the farmer and his family, sold directly to consumers, used as feed or processed directly. Of the 160.0 million tons of milk delivered to dairy factories, 156.0 million tons were cow's milk, the rest being a combination of sheep's milk, goat's milk and buffalo. [1]

Traditionally, hygiene rules have required that milk collection be frequent and at short distance between farms and dairy factories. The development of on-farm cooling tanks and larger milk tanks has made this health problem less critical. Meanwhile, the elimination of national milk quotas has helped increase cross-border milk flows between farms and dairy factories. However, this is relatively limited. In 2018, one-fifth of EU cow's milk was produced by German farms, and a similar proportion (20.8%) was processed by German dairy factories. Germany, France, the United Kingdom, the Netherlands, Poland and Italy together supplied just over two-thirds (68.5%) of the raw cow's milk produced, and also in these countries a little over two-thirds (69.7%) were processed from cow's milk collected in dairy factories.[1-2]

While cow's milk is the main milk used by dairy factories in the EU, in several Member States, other milk contributes significantly to milk production. In 2018, Spain produced 1.0 million tons of milk from sheep and goats, Greece and France both producing 0.8 million tons. Italy has also produced 0.7 million tonnes of milk from animals other than cows, including almost all EU buffalo milk production. A majority (57.1%) of milk delivered to dairy factories in Greece came from sheep and goats in 2018. Just over one-fifth (22.5%) of milk delivered to dairy factories in Cyprus comes from sheep and goats. [2,3]

The average price, in real terms, of milk has fluctuated sharply in recent years. The significant decreases in 2015 and 2016 were followed by a strong recovery in 2017. Compared to these changes, the decrease in price in real terms in 2018 was moderate (-3.7% on average). Among the main milk-producing Member States, there were, in particular, average decreases in real milk prices in 2018, for example: ● Germany(-6.3%), ● Netherlands (-7.8%) and ● Ireland (-6.6%), but lower than the average decrease in ● France (-1.1%), ● United Kingdom (- 0.4%) and ● Italy (-1.8%). [1]

The milk delivered to the dairy factories is processed into a series of products. Dairy products are registered according to their weight. Therefore, it is difficult to compare the quantities of different products (for example, tons of fresh milk and powdered milk). The volume of whole or skimmed milk used in dairy processes is shown in Table 1. In 2018, 156.8 million tons (98.9%) of whole milk available for the EU dairy sector was processed. This was 0.2 million tons lower than in 2017. The production of 2.4 million tons of butter and the so-called "yellow products" in 2018 required 46.1 million tons of whole milk. The production of butter and yellow products also generated 43.0 million tons of skim milk. Together with the 15.4 million tons generated by the manufacture of sour cream, this skim milk was used for processing other dairy products. [1-3]

17.0 million tons of skim milk, along with 59.1 million tons of whole milk were used to produce 10.3 million tons of cheese in 2018. Together, cheese and butter production used two-thirds (67.1%) of the total milk available. The EU also produced 30.1 million tons of drinking milk, of which 12.6 million tons came from skim milk and another 17.3 million tons from whole milk. Drinking milk accounted for about one-tenth (11.0%) of the total milk used by dairies in 2018. Another 21.3 million tons of raw milk were used to produce 3.0 million tons of milk powder. [1-3]

Carotenoids are widely distributed in nature. They are considered to be potentially beneficial in prevention of a variety of diseases, including cardiovascular disease, certain types of cancer and eye diseases. The concentration of carotenoids in cow's milk varies very much due to the feeding and the season. [4] Based on their chemical structure, carotenoids are classified under two groups: carotenes (hydrocarbons) and xanthophylls (oxygenated derivatives of the hydrocarbons). Included in the carotene group are P-carotene, a-carotene, and lycopene, and among the xanthophylls are lutein, zeaxanthin, and p-cryptoxanthin. [4-6]

There are many parameters of milk quality and authenticity that can be quantified by liquid chromatography, some common, some less common. The chromatographic method was developed for the determination of beta-carotene in milk and dairy products. [7] Beta carotene is found only in milk and dairy products that come from cows raised on pastures. The lack of this compound in the product indicates that the cows were not bred in the free system and concentrates or other types of feed were used. If the product is yellow and beta-carotene is not present, this means that synthetic dyes were used to obtain the color. Its presence in products that are 100% goat and sheep indicate that the product is not made from 100% goat or sheep milk but from a mixture with cow's milk.[5-6]

The purpose of the study is to develop a method for the determination of beta-carotene from milk and cheese samples.

MATERIAL AND METHODS

Samples

Milk and cheese samples were collected from marketplaces around Cluj county area.

Reagents and standards

Methanol, acetonitrile, hexene, ethyl acetate, ascorbic acid and ammonium acetate where all high purity or HPLC grade from Merck. β-Carotene synthetic standard, ≥93%, from Sigma-Aldrich was used for the calibration curve.

Equipment

The extraction was done using a Hettich centrifuge. The concentration of the samples was done a rotavapor for Hiedolph which allowed the control of the temperature and the pressure. Perkin Elmer 200 Series High Performance Liquid Chromatograph (HPLC) with fluorescence detector was used to determine the beta-carotene content.

Determination of beta-carotene

The extraction steps applied for sample extraction are:

1. Weighing: 5 g of sample, with an accuracy of 0.01 g;
2. Saponification: with an alcoholic solution of KOH 25% for 30 minutes on an ultrasonic bath, at 40 ºC in the presence of ascorbic acid as an antioxidant;
3. Extraction of the saponified solution: with 5 ml of hexane: ethyl acetate (70:30);
4. Centrifugation and organic phase collection;
5. Extraction no. 2 of the saponified solution: with 5 ml of hexane: ethyl acetate (70:30);
6. Centrifugation and organic phase collection;
7. Evaporation of the collected organic phase to dry rotavapor and return to the mobile phase.

The parameters and characteristics of the chromatographic method used to determine the β-carotene content are presented in Table 1.

Table 1

Liquid chromatographic parameters

Crt. No	Parameter name		Value / features
1.	Mobile phase:	Methanol + ammonium acetate 3.2 g/l	70 %
		Acetonitrile	30 %
2.	Flow		1.7 ml/min
3.	Injection volume		8 µl
4.	Column temperature		40 ºC
5.	Chromatographic column		Thermo ScientificTM AcclaimTM C30, 3 µm, 3.0 × 150 mm
6.	Wavelength		460 nm

RESULTS AND DISCUSSION

The chromatogram of beta-carotene is presented in figure 1.

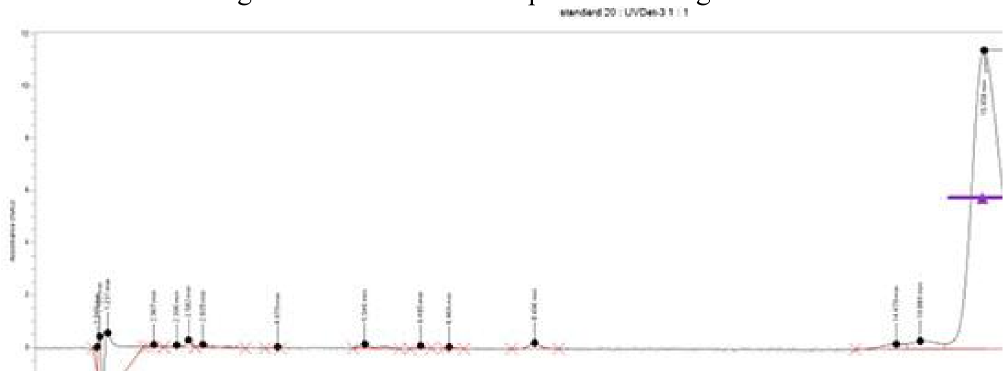

Fig. 1. Beta-carotene chromatogram

The calibration curve was performed in 4 points with a concentration of 1; 5; 10 and 20 ng / ml. (figure 2)

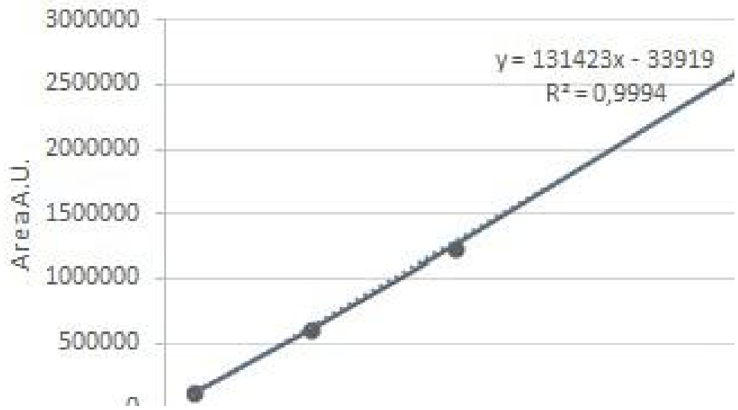

Fig. 2. Beta-carotene calibration curve

3 milk samples and 3 cheeses were fortified with 10 ng / ml beta-carotene solution, extracted according to the established protocol and analyzed by HPLC-UV. The degrees of recovery obtained, depending on the matrix, are presented in table 2.

Table 2

Recovery yield

Crt. No.	Sample type	Recovery yield %
1.	Cow milk	86,5
2.	Buffalo milk	85,7
3.	Goat milk	89,7
4.	Cheddar cheese	73,8
5.	Low fat Mozzarella cheese	86,9
6.	Brie cheese	88,5

To determine the efficiency of the method, 3 types of milk and 5 types of cheeses were analyzed. The results obtained are presented in table 3.

Table 3

Values of beta carotene content in milk and dairy products

No.	Sample type	Value obtained for beta carotene, µg/100 g product
1	Cow milk	5,7
2	Buffalo milk	< LD
3	Goat milk	< LD
4	Cheddar cheese	60,5
5	Cheddar cheese	74,3
6	Low fat Mozzarella cheese	34,5
7	Mozzarella	54,1
8	Brie cheese	< LD

CONCLUSIONS

Beta-carotene was not found in the sample of goat's milk and buffalo, which shows that: (1) these animals have the ability to convert carotenoids into vitamin A; (2) the milk is not counterfeit / adulterated;

- The highest amount of beta-carotene was determined in chedar cheese with high fat content, 74.3 µg / 100g, which shows that the cows were raised freely;
- The best degree of recovery was obtained for goat's milk, of 89.7% and the weakest for chedar cheese, 73.8% - which shows the good quality of the developed method;
- Beta-carotene can be a marker for the detection of adulterations of milk and dairy products.

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