

## Optimization of various steps for RP-HPLC determination of $\beta$ -carotene in milk fat

\*Dhankhar, J., Sharma, R. and Mann, B.

Dairy Chemistry Division, National Dairy Research Institute, Karnal, Haryana, India-132001

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### Abstract

Main objective of this study was to optimize the analytical procedures, especially extraction and saponification method for the better analysis of  $\beta$ -carotene in milk fat. Saponification method with suitable potassium hydroxide (KOH) concentration, use of antioxidant, saponification time, and temperature was optimized to get best extraction procedure for  $\beta$ -carotene. Analysis of  $\beta$ -carotene was carried out by using reversed phase high performance liquid chromatography (RP-HPLC) using C18 column. Chromatographic conditions best tested for the study involves methanol: tetrahydrofuran: water (MeOH: THF: H<sub>2</sub>O). The effect of time temperature combination at 45°C/30 min with saturated ascorbic acid had a pronounced effect on the  $\beta$ -carotene extraction from milk fat. Study also reveals that a concentration level of 10M KOH was best suited for the extraction of unsaponifiable.

### Keywords

$\beta$ -carotene  
Milk  
Saponification  
RP-HPLC  
Unsaponifiable

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### **Introduction**

$\beta$ -carotene, the most predominant of all carotenoids in fat globule of milk, is present in highly bio-available form in milk (Castenmiller and West, 1998; Chauveau-Duriot *et al.*, 2010).  $\beta$ -carotene, known mainly for its pro vitamin A activity, possesses one-sixth of the activity of the all-trans-isomeric forms of vitamin A (Ishida and Bartley, 2005).  $\beta$ -carotene is a potent natural antioxidant present in milk and nowadays, there has been high interest in its research due to the beneficial and positive effects it exerts on human health (Pais and Dumitrascu, 2013). Numerous epidemiological studies have shown strong association between carotenoid intake and lowering of the risk of chronic diseases, such as carcinogenesis, cardiovascular diseases, inflammation, eye degeneration and neuronal damages (Ferguson, 1997; Cantuti-Castelvetri *et al.*, 2000; Yamaguchi and Uchiyama, 2003).

Considering its beneficial effects, the accurate qualitative and quantitative analysis becomes necessary to ascertain its exact amount in milk or other food matrices. In general, carotenoid analysis reported by many authors include spectrophotometric method, various colour evaluation methods (Schoefs, 2002), as well as different chromatographic techniques like column chromatography (Almeida and Pentead, 1988), thin-layer chromatography

(TLC), gas chromatography and HPLC (Lin and Chen, 2003; Pichini *et al.*, 2002). Analysis involving spectrophotometric and colorimetric method involve cumbersome sample preparation and have higher limit of detection that lies in the higher range thereby leading to requirement of methods with lower LODs and more reproducibility. Similarly amongst various chromatographic techniques used classical column chromatography and thin-layer chromatography (TLC) are time consuming and require large amounts of samples. In addition, their separation efficiency and reproducibility are poor with low recoveries of the analytes. Gas chromatography (GC) is not normally used because of low volatility and thermolability of  $\beta$ -carotene.

Recent analytical advances in high performance liquid chromatography allows for the detection of the said carotenoid in milk fat with increased sensitivity. However, the lack of standardized fat extraction and saponification methods could lead to the major limitations in the  $\beta$ -carotene analysis since it is present in small amounts (Granado *et al.*, 2001; Schierle *et al.*, 2004). For the analysis of carotenoid from the milk fat it is necessary to choose better sample preparation and extraction procedure to ensure the minimum loss of the compound.

The present methodology was planned to contribute to the optimization of the extraction and saponification steps i.e. extraction method, extraction

time, temperature, choice of extraction solvents, concentration of the saponification reagent, as well as times and temperatures of saponification procedure to make the qualitative and quantitative analysis of  $\beta$ -carotene better. The aim of current study was to obtain a rapid, reliable, and effective method to evaluate  $\beta$ -carotene in milk fat.

## Materials and Methods

### Chemicals and reagents

All chemicals used for sample preparation were of analytical grade. Solvents used for HPLC analysis were of HPLC grade.  $\beta$ -carotene standard, hexane, tetrahydrofuran were purchased from Sigma Aldrich India. Methanol, potassium hydroxide was purchased from Merck Specialties Pvt. Ltd., Mumbai. Ascorbic acid used was of purity grade and purchased from S D Fine-chem. Ltd. Water used was purified with a Milli-Q system (Pall Corporation USA; BIO WATER).

### Sample

Raw milk samples were obtained from the National Dairy Research Institute (NDRI) Cattle yard based at Karnal, Haryana, India. The samples were collected in amber colour sample bottles and were stored at frozen temperature until the fat extraction. Pooled cow milk of more than 30 cows was selected for the proposed experiments to avoid any major difference in the results. Stored samples were shaken thoroughly for proper mixing of fat with milk before mixing the solvents. All experiments were conducted under dark conditions and amber glass wares were used where appropriate.

### Sample extraction procedure

Three sample extraction procedures were performed prior to saponification procedure. a) traditional Folch method (Folch *et al.*, 1957), b) method by Iverson (Iverson *et al.*, 2001) and c) method proposed by Capuano (Capuano *et al.*, 2014).

### Saponification procedure

Saponification was performed with different concentrations of KOH viz., (1, 3, 5, 7, 10, 12, 15, 20M). For sample analysis, 200 mg of fat sample was weighed and treated with 500  $\mu$ l of different aforesaid concentrations of alkali for 30 minutes in all the treatments. All the sample preparation was performed in an environment with subdued lighting. After saponification treatment, a mixture consisting of water and hexane in the ratio of 1:5 was added to the saponified sample and vortexed thoroughly for

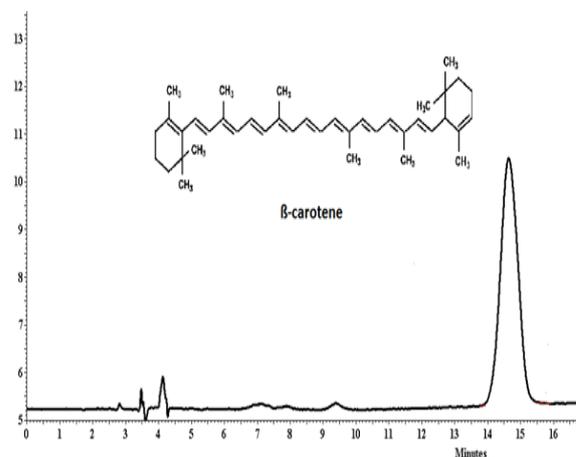


Figure 1. Representative chromatogram of  $\beta$ -carotene standard solution at  $\lambda=453$  nm. Chromatographic conditions: C18 column (ZORBAX 300 SB-C18 (4.6 x 250 mm x 5 $\mu$ )). Methanol/THF/Water; DAD detector (1260 DAD VL+)

2-3 minutes. Hexane layer was separated carefully and residue was mixed with same ratio of hexane and water for repeated extraction. The hexane phases were pooled and subjected to dryness in incubator at 45°C. The dried sample was then reconstituted with hexane and filtered through 0.45  $\mu$ m syringe filters before being injected in to the HPLC system.

### Instrumentation and chromatographic conditions

HPLC analysis was carried out by method optimized by Andres *et al.*, 2014 using a Agilent 1260 infinity (Agilent Technologies, USA) equipped with binary pump system chromatograph coupled with a Diode Array Detector (DAD) detector (1260 DAD VL+) and reversed phase C18 column (ZORBAX 300 SB-C18 (4.6 x 250mm x 5 $\mu$ )). The wavelength was 453 nm. Methanol/THF/Water solution was used as a mobile phase and the flow rate was adjusted at 0.8 ml min<sup>-1</sup>. Solvent mixture was filtered through 0.22  $\mu$ m filter membrane and sonicated at the rate of 40 Hz for 10 minutes.

## Results and Discussion

### Extraction procedure optimization

Efficiency of milk fat extraction and  $\beta$ -carotene extraction from unsaponifiable is dependent on various factors viz., selection of extraction solvents, composition of extraction solvents, extraction time, extraction temperature, repeated extraction etc. As previously stated pooled cow milk was used for the optimization of extraction study.

### Extraction Solvents

The choice of extraction solvents plays an

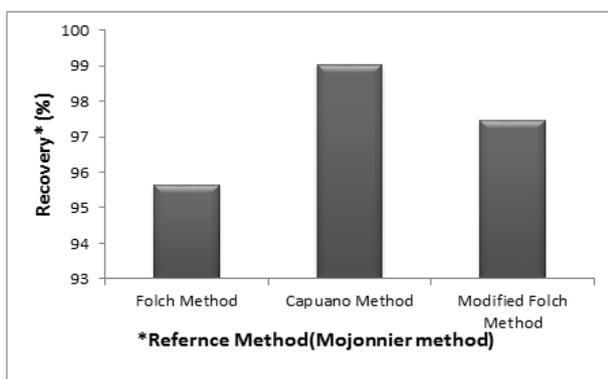


Figure 2. Comparison of recovery of existing fat extraction methods

important role in the analysis. A number of solvents and their combinations therefore were tested for their efficacy to extract the from milk fat from pooled milk samples of cows in cattleyard. Different combinations of solvents (chloroform: methanol, 20:1 v/v; Dichloromethane: ethanol; 2:1) were analysed for milk fat. The results of the effect of extraction solvents are summarized in the Figure 2. Different procedures of solvent extraction were analyzed. For instance, Folch method (Folch *et al.*, 1957) employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the sample. While method developed by Iverson (Iverson *et al.*, 2001) was found to yield recovery of ~95% despite the solvent reduction. In contrast method developed by Capuano (Capuano *et al.*, 2014) involved usage of solvents like dichloromethane which are safer as compared to chloroform and also recovery attained upto level of 99.2%. The efficiency of milk extraction (% yield) from pooled cow milk from different extraction procedure shows a significant change in the resulting milk fat yield. Solvent composition of dichloromethane and ethanol (Capuano *et al.*, 2014) gives better result as compared to the other solvents combinations and hence was optimized for the rest of the study.

### Saponification

$\beta$ - carotene is predominant carotenoids in milk; comprising approximately 80-90% total carotenoids content present in milk (Paul *et al.*, 1992). Due to the sensitive nature of carotenoids optimization of safe, better and efficient saponification method was required. It is the fundamental step involved in dissociation of fatty acids matrix and there by maximizing the release of carotenoids present in oil matrix in to the solvent phase (Bhatnagar *et al.*, 2015). Alkaline hydrolysis of fat using KOH plays a vital role in the saponification process. Different conditions were optimized for the saponification process considering the labile nature of carotenoids

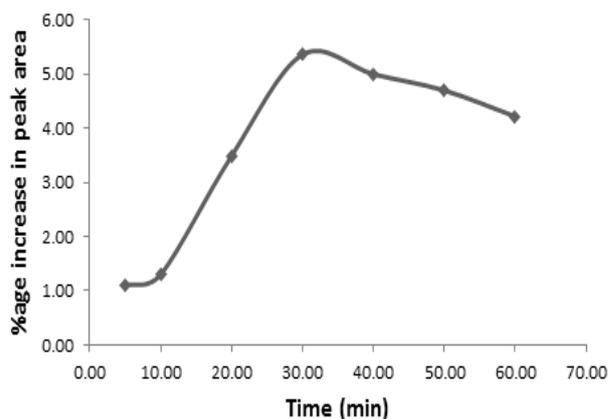


Figure 3. Effect of varying concentrations of KOH on the peak area

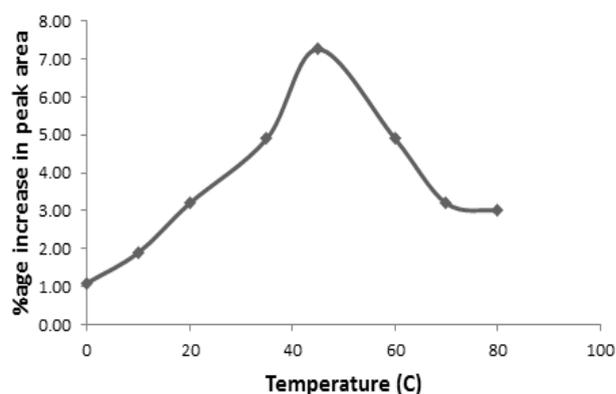


Figure 4. Effect of varying temperature on percentage peak area of  $\beta$ -carotene

present in the milk fat with respect to, concentration of KOH, temperature, antioxidant content and time.

### Effect of KOH concentrations

Different concentrations of alcoholic KOH (1, 3, 5, 7, 10, 12, 15, 20M) were used in the saponification process during the  $\beta$ -carotene extraction from milk fat. 200  $\mu$ l of 20% Ascorbic acid was used in order to prevent the oxidation of fat. From the study conducted the best figures appears at 10M KOH conc. for 30 minutes (Figure 3). Research shows that higher concentration of alkali is detrimental to carotenoids stability and is prone to destruction due to degradation of polyene backbone (Khachik *et al.*, 1997; Oliver *et al.*, 1998; Rodriguez-Amaya *et al.*, 2004).

### Effect of temperature

Temperature had a significant impact on the saponification process. Different saponification temperatures decided for the study were 0°C, 10°C, 20°C, 35°C, 45°C, 60°C, 70°C and 80°C. Optimized concentration of alcoholic KOH (10M) was used

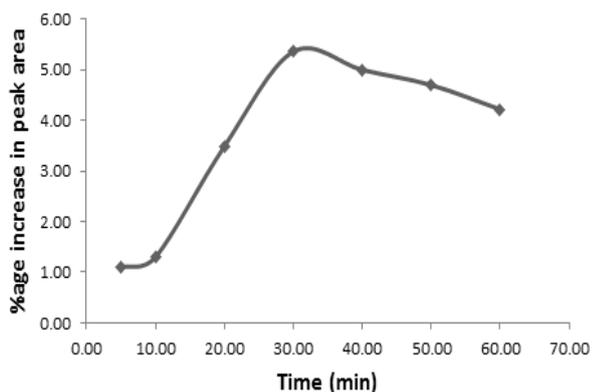


Figure 5. Effect of varying time on percentage peak area of  $\beta$ -carotene

during the temperature optimization. Percentage peak area was found maximum at 45°C for  $\beta$ -carotene (Figure 4). However more increase in temperature leads to decrease in peak area this might be due to degradation of ascorbic acid content. Moreover heat labile nature of  $\beta$ -carotene could also lead to carotenoids destruction at high temperature. Study is supported by (Kim *et al.*, 1990; Patton *et al.*, 1990; Giuliano *et al.*, 1992; Liu *et al.*, 1998) where the loss of 10%  $\beta$ -carotene at higher temperature was suspected.

#### Effect of Ascorbic acid concentration

To reduce the oxidation reactions that could impact the  $\beta$ -carotene during saponification an antioxidant such as ascorbic acid was added to the sample solution. During saponification  $\beta$ -carotene undergoes losses due to isomerization. The loss incurred due to isomerization can be minimized by the use of antioxidant. The purpose of using ascorbic acid as antioxidant in the present study is that it does not interfere with chromatographic analysis. Heat sensitive nature of ascorbic acid leads its destruction at higher temperature i.e., above 45°C as optimized previously.

#### Effect of time on saponification

Time is other crucial parameter; longer duration resulted in to the degradation of carotenoids. High alkaline conditions for the longer duration are detrimental to the integrity of  $\beta$ -carotene structure. Temperature duration of 30 min was optimized for the experiment (Figure 5). As the structure of carotenoids are made up of large conjugated hydrocarbon skeletons that undergoes degradation when left at optimized temperature for longer duration.

#### Dissolution of sample and standards

Samples were finally reconstituted with hexane because of the solubility of  $\beta$ -carotene.  $\beta$ -carotene is not soluble in water. HPLC grade hexane was used as

a diluent for sample and standard. Use of A.R. grade hexane showed distorted peaks in chromatogram as non HPLC grade hexane could induce the isomerisation of  $\beta$ -carotene and promoted artefact formation (Marsili and Callahan, 1993). Choice of solvent is made carefully so as to avoid such type of problems.

#### Mobile phase

Different mobile phase solvents were studied for the optimization of  $\beta$ -carotene on HPLC system. Methanol and acetonitrile are most widely studied for the carotenoid analysis by HPLC. However use of acetonitrile was avoided because of the low recovery than methanol. Tetrahydrofuran was added to the mobile phase as a strong organic modifier and did not lead to peak distortion.

#### Qualitative analysis of $\beta$ -carotene

The developed HPLC method is specific for  $\beta$ -carotene detection. The chromatogram shows a clear individual elution of the said compound. Sample  $\beta$ -carotene shows a retention time of 14.6 minute which coincided with the standard retention time. The better elution of  $\beta$ -carotene reveals the accurate choice of solvents, HPLC method standardisation and optimized extraction parameters.

#### Conclusion

$\beta$ -carotene is fat soluble carotenoid, out of the various methods for maximum fat extraction compared, method using dichloromethane and ethanol (2:1) was best suited.  $\beta$ -carotene content which is highly dependable on sample preparation step due to its heat labile and photo sensitive nature was extracted and estimated accurately by saponification at 10M KOH at 45°C for 30 minutes. This study could yield a short runtime detection method for  $\beta$ -carotene as compared to previously reported HPLC methods using DAD detector.

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