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Using front-face fluorescence spectroscopy for prediction of retinol loss in milk during thermal processing

Jinfang Liu [a], Anna Zamora [a], Manuel Castillo [a, *], Jordi Saldo [a, b]

[a] Centre d'Innovació, Recerca i Transferència en Tecnologia dels Aliments (CIRTTA), CERPTA-UAB, Department of Animal and Food Science, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[b] Centro de Desarrollo de Producto Bióticos (CEPROBI), Instituto Politécnico Nacional (IPN), Yautepec, Morelos, Mexico

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ABSTRACT

The objectives of the present study were to model the kinetics of retinol loss and assess the potential of predicting retinol loss during thermal processing using front-face fluorescence spectroscopy (*FFFS*). A factorial design with three different temperatures (70, 80 and 90 °C) and five heating times (0, 5, 10, 15 and 30 min) was used in this study. The kinetics of the retinol degradation process fitted well a first-order reaction. Further, several milk native fluorophores were selected as fluorescent markers for development of retinol prediction models such as tryptophan, dityrosine, Maillard intermediate compounds and riboflavin. The maximum emission intensities of the markers were used to evaluate the correlation of retinol concentration with fluorescent markers and to establish the corresponding prediction models of retinol concentration. Retinol concentration was significantly correlated ($P < 0.05$) with all the fluorescent markers. The optimum prediction model was obtained from 45 samples using three fluorescent predictors, with an R^2 of 0.87. The results obtained are encouraging and suggest that *FFFS*, as a low-cost, rapid, direct and non-destructive technique, has the potential to replace existing conventional analytical techniques practiced for retinol evaluation in heat treated milk and be adopted by dairy industry.

1. Introduction

Vitamin A, also known as retinol, is one of the most important micronutrients for human health. It plays an essential role in vision, bone and tooth development, and the health of skin and mucous membranes (Ball, 2006). Synthesis of retinol only occurs naturally in animals (Wilkinson, Earle, & Cleland, 1981). Generally speaking, dietary vitamin A is obtained from animal-derived foods, while plant foods provide carotenoid precursors (Ball, 2006). Good sources of retinol include eggs, dairy products (milk, cheese, yogurt, etc.) and, particularly, liver.

Being milk a widely consumed rich source of retinol, its loss originated by thermal processing should not be neglected. Heat treatment is essential to maintain and extend the shelf-life and ensure milk is free from potentially pathogenic organisms. But the prevention treatment affects the sensory, biophysical and nutritional properties of milk too. The main undesired events occurring upon heating are protein denaturation, Maillard reaction and vitamins loss depending on the temperature and duration of heating (Anema & McKenna, 1996; Birlouez-Aragon et al., 1998; Henle, Walter, & Klostermeyer, 1991). To minimize the loss of retinol during processing, the key points are controlling treating time, temperature, light and oxygen exposure because retinol is susceptible to these factors (Manzi & Durazzo, 2017; Tamime, 2009).

The most common method to quantify the retinol loss during thermal treatments is high performance liquid chromatography (HPLC). The whole procedure is laborious and very time consuming. Furthermore, there is an increasing demand of the consumers and

Abbreviations used: F_{Trp}, F_{MC}, F_{Rb370}, F_{Rb450}, Maximum fluorescence intensity of tryptophan, Maillard compounds and riboflavin (excited at 370 nm and 450 nm); [*Re*], Concentration of retinol; $[Re]_0$, Initial concentration of retinol.

* Corresponding author. Department of Animal and Food Science, Universitat Autònoma de Barcelona, Edifici V, Campus de la UAB, 08193 Bellaterra, Barcelona, Spain.
E-mail address: manuel.castillo@uab.es (M. Castillo).

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stakeholders of the food industry sector to have means of measurement allowing the simple, non-destructive, and quick characterization of raw materials (Karoui & Debaerdemaeker, 2007).

Thus, optical methods such as front-face fluorescence spectroscopy (*FFFS*) have shown potential as alternative, rapid method for quantification of fluorophores naturally occurring in milk that might be affected by thermal treatments (Andersen & Mortensen, 2008; Hougaard, Lawaetz, & Ipsen, 2013; Schamberger & Labuza, 2006). Tryptophan (*Trp*), dityrosine (*Dt*), Maillard compounds (*MC*) and riboflavin (*Rb*) are among those naturally occurring fluorophores (Liu & Metzger, 2007), so in present study those fluorophores were selected to be used as fluorescent markers. Although retinol is a self-fluorescent substance, the excitation and emission wavelengths are too close to tryptophan and the fluorescence of the later in milk is much stronger. Without extraction, it is difficult to remove the influence of tryptophan fluorescence. Therefore, retinol fluorescence was not used as a predictor in the present study.

Several authors have proposed using fluorescence spectra to discriminate the technological treatments received on milk. An early research on potential use of spectroscopic methods for the characterization of raw, heated and homogenized milk by *FFFS* showed that the treatments induced changes in the fluorescence characteristics of the fluorophores (Dufour & Riaublanc, 1997). In addition, a rapid and non-destructive *FFFS* method was developed to quantify furosine and lactulose in mixtures of heat-treated and non-heated milk by several researchers (Ayala, Zamora, Liu, Saldo, & Castillo, 2015; González, Juan, Zamora, Saldo, & Castillo, 2015; Kulmyrzaev, Levieux, & Dufour, 2005). Moreover, *FFFS* was also proved quite useful for evaluating the effect on the early stages of the Maillard reaction caused by the heat processing of milk. All the above indicates that *FFFS* with no sample preparation has the potential to be used as an online instrument for monitoring and control of thermal processing of milk (Schamberger & Labuza, 2006). It could also be used for differentiating between manufacturing processes and sampling zones in ripened soft cheeses (Karoui, De Baerdemaeker, & Dufour, 2008) and differentiating sheep milk obtained from different genotypes and feeding systems (Hammami et al., 2013). Even though a lot of studies about *FFFS* of milk or dairy products have been done, retinol kinetic is still not completely characterized. The present study proposes a simple and rapid methodology to predict the retinol loss in milk with *FFFS* during thermal treatment, which has inline application potential.

2. Materials and methods

2.1. Milk supply

Three different batches of fresh raw bovine milk were obtained from a local producer (Can Badó, la Roca del Vallès, Spain) to perform this study.

2.2. Reagents and solvents

Ethyl acetate, methanol, absolute ethanol, hexane and tetrahydrofuran (HPLC grade), ascorbic acid, standards of retinol, and δ-tocopherol, pyrogallol (purity >98%), ethylenediaminetetraacetic acid (EDTA) and potassium hydroxide (KOH) were purchased from Sigma-Aldrich (Sigma-Aldrich Quimica,S.L., Madrid, Spain).

2.3. Thermal treatment

A factorial experimental design with two factors, temperature and time, and three replicates was used to heat-treat the milk samples under the following conditions: three levels of temperatures 70, 80, and 90 °C with five time levels 0, 5, 10, 15 and 30 min in a thermostatic bath (OvanTherm TC00E C, Lovango SL). One different batch of milk was used per replication and the heat treatments were applied to 45 samples in total (3 replications; 15 treatments; $N = 3 \cdot 5 \cdot 3 = 45$).

A 20 mL aliquot for each sample of raw milk was filled in a 25 mL glass test tube and capped. A digital thermometer was used to monitor the internal sample temperature reached inside the test tubes to the predetermined temperature. At the different treatment time intervals indicated by the experimental design, samples were removed from the bath and placed on iced-water immediately.

2.4. Retinol quantification

The retinol quantification method used was based on that proposed by Salo-Väänänen et al. (2000). Milk samples were held at 40 °C for 10 min in a water bath before 1 mL was transferred to a light-protected test tube. Then, 20 μL of internal standard stock solution (δ-tocopherol, 1000 $mg \cdot L^{-1}$) was added to the milk sample before saponification.

After an addition of 4 mL ethanol, 0.5 g of pyrogallol and ascorbic acid, the oxygen from headspace was removed with a nitrogen flow for 10 min. Thereafter, 0.5 mL of saturated EDTA and 0.5 mL KOH (50%) were added and sample vortexed for 30 s. Further, the sample was transferred to a boiling water bath for 20 min. Last, the sample was left on ice to cool down.

The extraction was carried out by addition of 2 mL milli-Q water and 2 mL organic extraction solution (ethyl acetate:hexane, 20:80) followed by 10 min mechanical shaking. After, the sample was centrifuged at 2000×*g* for 10 min. The supernatant organic phase was transferred to another tube and held at 40 °C. The extraction step was then repeated twice without water addition. The combined supernatant was evaporated with nitrogen and the dry residue was dissolved in 500 μL of methanol:ethanol (20:80) and filtered (Polyvinyldene fluoride, 0.2 μm) prior to HPLC analysis.

The analytical column employed was a Waters Sunfire C_{18} (Waters, Milford, Ireland) of 4.6 mm × 150 mm and particle diameter 3.5 μm. Mobile phase A was prepared by dissolution of methanol in milli-Q water (97:3). The mobile phase B was the mixture of methanol and tetrahydrofuran (90:10). The elution was achieved with a linear gradient from 0 to 100% of mobile phase B at flow rate of 1 $mL \cdot min^{-1}$ and oven temperature of 25 °C. Standards for control of absorbance and calibration were daily prepared at the concentration of 30 $mg \cdot L^{-1}$ and the spectrometry wavelengths were set at 325 and 298 nm. Retention times were around 3.70–3.90 min and 10.37–10.43 min for retinol and internal standard δ-tocopherol, respectively (recovery rate > 95%).

2.5. Fluorescence measurement

The fluorescence measurements were performed using a fluorescence spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Agilent Technologies, Madrid, Spain) equipped with 15 W lamp “press Xenon lamp” and a “front-face” geometry accessory adjusted to an angle of incidence of 35°.

The fluorescence spectrum of dityrosine (F_{Dt}) was scanned at excitation of 315 nm and emission between 350 and 500 nm. For Maillard compound (F_{MC}) excitation wavelength of 330 nm and emission range of 350–500 nm were used. For tryptophan (F_{Trp}) the wavelength used for excitation was 290 nm while for emission was 300–450 nm. Riboflavin was excited at 370 and 450 nm (F_{Rb370} and F_{Rb450}, respectively) the emission wavelength range was 470–570 nm. All determinations were performed in triplicate.