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Technical note: Simultaneous carotenoid and vitamin analysis of milk from total mixed ration-fed cows optimized for xanthophyll detection

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ABSTRACT

Concentrations of retinol, α-tocopherol, and major carotenoids in dairy products are often determined simultaneously by liquid chromatography. These compounds have different polarity and solubility; thus, extracting them simultaneously can be difficult and inefficient. In milks with low carotenoid concentrations, the xanthophylls lutein and zeaxanthin may not be completely resolved using common extraction techniques. A simplified method was developed to optimize extraction efficiency and the limit of detection and limit of quantification (LoQ) of lutein and zeaxanthin in bovine milk without decreasing sensitivity to other vitamins or carotenoids. The developed method evaluates lutein, zeaxanthin, β-carotene, retinol, and α-tocopherol simultaneously by ultra-high performance liquid chromatography–photodiode array detection. Common saponification temperatures (40–60°C) and concentrations of KOH in water (10–50% KOH wt/vol) were evaluated. Multiple solvents were evaluated for optimal xanthophyll extraction (diethyl ether, dichloromethane, hexane, and tetrahydrofuran) following saponification. The limit of detection and LoQ were defined as 3:1 and 10:1 signal-to-noise ratio, respectively. All experiments were performed in triplicate. The optimal saponification procedure was a concentration of 25% KOH at either 40 or 50°C. Saponified extracts solubilized in solutions containing diethyl ether had greater concentrations of lutein- than hexane- or tetrahydrofuran-based solutions, with peak areas above LoQ values. The solution containing diethyl ether solubilized similar concentrations of retinol, α-tocopherol, and β-carotene when compared with other solutions. The proposed optimized method allows for the simultaneous determination of carotenoids from milk with increased lutein and zeaxanthin sensitivity without sacrificing recovery of retinol, α-tocopherol, and β-carotene.

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Technical Note

The carotenoids, β-carotene and xanthophylls, such as lutein and zeaxanthin, are among the most common carotenoids found in milk (Nozière et al., 2006; Plozza et al., 2012; Gill et al., 2016). Changes in carotenoid concentration in bovine feed affect carotenoid content in bovine milk (Calderón et al., 2007a). Thus, accurate detection of β-carotene, lutein, and zeaxanthin in bovine milk can serve as a marker for diet quality of dairy cows (Prache et al., 2002; Martin et al., 2005; Butler et al., 2008) and traceability of feed management in dairy cows (Calderón et al., 2007a) and can also play a role in human health (Kohlmeier and Hastings, 1995; Rapp et al., 2000; Macias and Schweigert, 2001; Jewell et al., 2004; Schweigert et al., 2004).

Retinol, α-tocopherol, and carotenoids in bovine milk are often determined simultaneously by solvent extraction followed by ultra-high performance liquid chromatography–photodiode array detection (**UPLC-PDA**) or MS (Indyk, 1987; Ollilainen et al., 1989; Giuliano et al., 1992; Jinno and Lin, 1995; Oliver and Palou, 2000; Turne et al., 2001; Blake, 2007; Calderón et al., 2007b; Kamao et al., 2007; Chauveau-Duriot et al., 2010; Plozza et al., 2012; Gentili et al., 2013). However, unlike β-carotene, the xanthophylls lutein and zeaxanthin are difficult to quantify in milks with low carotenoid content using UPLC-PDA detection (Nozière et al., 2006). Chauveau-Duriot et al. (2010) reported that lutein in whole milks was below the limit of quantification (**LoQ**) and occasionally below the limit of detection (**LoD**) using a UPLC-PDA method, although they were detected using other instrumental techniques such as MS (Gentili et al., 2013). Percent recovery for lutein was below 70% in Chauveau-Duriot et al. (2010). Gill and Indyk (2008) designed a method to determine lutein in milk with a percent recovery of lutein >95%; however, this method was not optimized for fat-soluble vitamins or carotenes. Lutein concentration in bovine milk is dependent on feed and total

fat content (Havemose et al., 2004). Milk of cows fed a TMR contains approximately 20 to 60% less lutein than pasture-fed cows (Nozière et al., 2006; Havemose et al., 2004). Lower concentrations of total lutein in TMR milks may prevent accurate lutein determination.

Several factors that may limit lutein recovery rates are saponification parameters and lutein solvent affinity. Saponification is often necessary to remove carotenoids from lipids in samples containing fat (Giuliano et al., 1992; Calderón et al., 2007b; Gill et al., 2016); however, heat and caustic solutions used in saponification can result in lutein degradation (Khachik et al., 1997; Salo-Väänänena et al., 2000; Gill and Indyk, 2008). Granelli and Helmersson (1996) demonstrated 30°C to be sufficient for complete recovery of β-carotene, despite the claim by Indyk (1987) that high-temperature saponification was needed for complete extraction of carotenoids and retinol from milk fat. Thus, determining the temperature and KOH concentration during saponification may contribute to increased lutein and zeaxanthin recovery rates while preserving β-carotene and retinol recovery rates.

Although xanthophylls and carotenes are chemically similar, the electron density on the oxygen atoms of xanthophylls can influence polarity and solubility (Mortensen and Skibsted, 1997; Yan et al., 2007). Simultaneous extraction of carotenoids and fat-soluble vitamins in bovine milk may be influenced by these solubility differences (Craft and Soares, 1992; Indyk, 1987; Gill et al., 2016). Previous methods have used extraction solutions containing hexane, dichloromethane, tetrahydrofuran (**THF**), acetonitrile, and ethanol (Granelli and Helmersson, 1996; Giuliano et al., 1992; Chauveau-Duriot et al., 2010; Plozza et al., 2012); however, Craft and Soares (1992) demonstrated that lutein solubilized poorly in hexane and has reduced solubility in dichloromethane compared with β-carotene. Although rarely used in carotenoid extraction of milk, previous research has shown that diethyl ether could efficiently solubilize both lutein and β-carotene (Craft and Soares, 1992; Oliver and Palou, 2000). No study to our knowledge has determined if a particular solvent optimizes lutein and zeaxanthin extraction without affecting β-carotene, retinol, and α-tocopherol extraction.

Previous research for simultaneous carotenoid and fat-soluble vitamin determination in fluid milk may not consistently detect xanthophylls above the LoQ. This may be due to aggressive saponification parameters and inadequate solubility. The objective of our study was to determine an extraction and purification method that optimized lutein detection without reducing the detection of other carotenoids and vitamins.

Saponification conditions and extraction solvents following saponification were compared to determine the optimal conditions for extraction and measurement of carotenoids (including lutein) and fat-soluble vitamins by UPLC-PDA. The verification of the method through measurement of commercial bovine milks, from both pasture- and TMR-fed cows, was then conducted.

Reagent-grade solvents (ethanol, ethyl acetate, and hexane), HPLC-grade THF and acetonitrile, and >99% crystalline retinol, ≥99.5% β-carotene, ≥99.5% zeaxanthin, ≥99.5% lutein, and ≥99.0% α-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO). The HPLC-grade water, diethyl ether, hexane, ethanol, and methanol were purchased from Fluka (Seelze, Germany). Reagent-grade KOH was purchased from VWR (Randor, PA).

All milks were extracted and prepared with overhead lights off to prevent light oxidation. Three milliliters of each milk was incorporated into a container with ethyl alcohol and a saponification solution containing various concentrations of KOH and deionized water (Table 1; described below). Samples were flushed with nitrogen, capped with a polytetrafluoroethylene-lined cap, and sonicated for 5 min. Samples were placed in a water bath at varying temperatures for 30 min as a saponification step (Table 1). After saponification, 6 mL of hexane:ethyl acetate (70:30) was poured into each vial, vortexed, sonicated for 5 min, and centrifuged at 800 × *g* for 10 min at 25°C. The organic layer was then separated from the aqueous layer and the aqueous phase was treated with an additional 3 mL of hexane:ethyl acetate (70:30). This complex was then vortexed, sonicated for 5 min, and centrifuged again at 800 × *g* for 10 min at 25°C. The resulting organic phase was removed and added to the original organic phase removed. This process was repeated a third time. The pooled extract was evaporated to dryness under nitrogen. The dried sample was then dissolved in 700 μL of a solution containing HPLC-grade ethanol, acetonitrile, and water at a concentration of 58:18:4; the final 20% of that solution was varied (Table 2, described below). Samples were then filtered with a 0.20-μm nylon filter (VWR) and placed in HPLC vials (Sigma-Aldrich). Finally, 3.0 μL were injected onto the UPLC.

Lutein, β-carotene, retinol, zeaxanthin, and α-tocopherol were analyzed by UPLC (Acquity UPLC H-Class, Waters Corporation, Milford, MA) with a C18 reversed phase column (Acquity UPLC BEH C18 1.7 μm, 2.1 × 50 mm, 130 Å, Waters). The column temperature was maintained at 32°C ± 0.3°C, whereas flow rate was maintained at 0.30 mL/min (Acquity UPLC H-Class, Quaternary Solvent Manager, Waters). Aliquots of each sample were then eluted with the respective mobile phase (described below). Peaks were analyzed by photodiode array detection (Acquity UPLC H-Class, Photodiode Array Detector, Waters) with wavelengths