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Analytical Methods

# Quantitative profiling of retinyl esters in milk from different ruminant species by using high performance liquid chromatography-diode array detection-tandem mass spectrometry

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ABSTRACT

An effective high performance liquid chromatography-diode array detection-tandem mass spectrometry (HPLC-DAD-MS/MS) analytical approach was developed for retinoid profiling in raw milk samples (cow, buffalo, ewe, and goat). The analytes were isolated by means of liquid-liquid extraction, including a "lipid freezing" step, with yields exceeding 66%. Since the positive atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) detection is not completely selective, a reliable identification has been accomplished by fully separating the analytes on a tandem $C_{18}/C_{30}$ column system under non-aqueous reversed phase (NARP) chromatography conditions. After validation, different milk varieties obtained from pasture-fed animals were analysed, providing, for the first time, the retinoid composition of both buffalo's and ewe's milk. According to the literature, retinyl palmitate has been found to be the most abundant vitamin A vitamer, but retinyl oleate is the prevalent form in the caprine milk.


## 1. Introduction

Milk is one of the few foods consumed all over the world. Although cow's milk dominates commercial production mainly due to its abundance and lower cost, milk from other animals is important in specific regions, countries and local contexts for environmental, cultural and clinical reasons. To date, Food and Agriculture Organization of the United Nations (FAO) reports that cows provide 83% of global milk production, followed by buffaloes with 13%, goats with 2%, sheep with 1% and camels with 0.3%. The remaining share is provided by other dairy species such as equines and yaks. About one-third of milk production in developing countries comes from buffaloes, goats, camels and sheep, while in developed countries, almost all milk is produced by cattle (FAO, Dairy production and products, 2013). Buffalo's milk is also used to produce traditional cheeses in many countries: mozzarella and ricotta in Italy, gemir in Iraq, paneer in India, or domiati in Egypt, just to name a few (Ménard et al., 2010). Regarding milk from small ruminants, especially goat's milk, has become a valuable food to face malnutrition in developing countries (Haenlein, 2001), while it is mostly used for cheese production or as alternative to cow's milk by people who have digestive disorders or allergy problems in developed countries (Jandal, 1996).

Milk is an excellent source of both macro-nutrients (proteins, carbohydrates, lipids) and micronutrients (minerals and vitamins) (Belitz, Grosch, & Schieberle, 2004). It contains all the vitamins, but it is, in particular, an excellent source of vitamin A (Ball, 2006; Gentili et al., 2013) that is mainly present as retinoids (vitamin A vitamers).

Vitamin A is involved in several biological functions such as vision, embryogenesis, cell growth and differentiation, reproduction and maintenance of the immune system (Ball, 2006). In recent years, increasing attention has been paid to its chemo-preventive activity against the epithelial carcinogenesis (Freemantle, Spinella, & Dmitrovsky, 2003). Compared to carotenoids, whose main natural sources are fruits and vegetables, retinoids are absorbed by human organism with higher efficiency from foods of animal origin such as eggs, liver and milk (Harrison, 2012). Although the mechanism of absorption and active transport of the main forms of vitamin A have been explained, there is little information about the distribution of retinoids in food.

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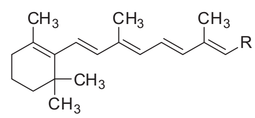
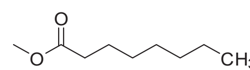
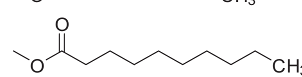
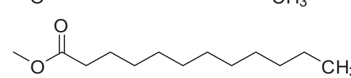
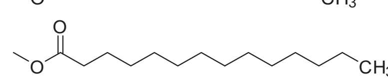
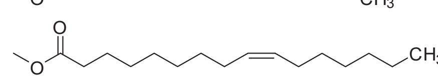
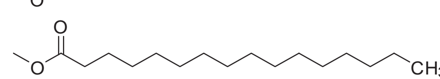
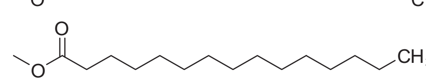
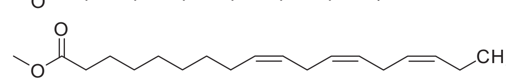
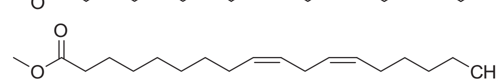
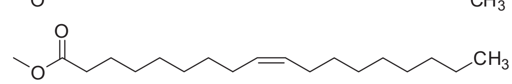
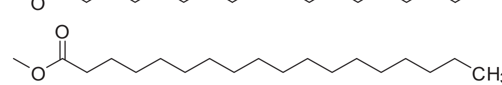
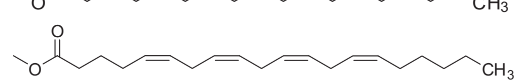
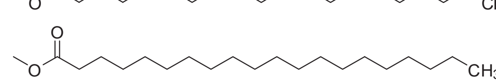
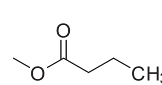
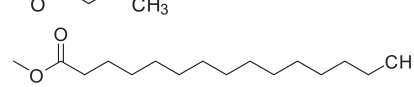
In the literature, the majority of studies concerning the determination of retinol and retinyl esters have been focused on biological samples (serum, plasma, faeces and tissues) for clinical purposes by using HPLC-UV (De Ruyter & de Leenheer, 1978; Hartmann et al., 2001; Hernandez-Alvarez et al., 2015; Kane, Folias, & Napoli, 2008; Majchrzak, Fabian, & Elmadfa, 2006; Schmidt, Brouwer, & Nau, 2003; Sowell, Huff, Yeager, Caudill, & Gunter, 1994) and, in lesser extension, by HPLC-fluorometric detection (Orth et al., 1998) and HPLC-MS (Suh, Tang, & Gudas, 2006; van Breemen et al., 1998; Wingerath, Kirsch, Spengler, Kaufmann, & Stahl, 1997). In the case of milk, most methods measure the total content of vitamin A as free retinol after hot saponification (Escrivà, Esteve, Farrè, & Frìgola, 2002; Gentili et al., 2013; Plozza, Trenerry, & Caridi, 2012; Salo-Väänänen et al., 2000). This strategy simplifies the analysis and reduces the costs, but causes a partial loss of retinol which is more prone to thermal isomerization than retinyl esters.

Only some works have performed the direct liquid extraction to quantify some few retinoids (mainly retinol, and the supplementary forms as retinyl acetate and retinyl palmitate) in cow's milk, milk-based beverages, mare's milk and infant formulae (Andrés, Villanueva, & Tenorio, 2014; Blanco, Fernandez, & Gutierrez, 2000; Gomis, Fernández, & Gutièrrez Alvarez, 2000; Herrero-Barbudo, Granado-Lorencio, Blanco-Navarro, & Olmedilla-Alonso, 2005; Stowe, 1986). Recently, Woollard, Bensch, Indyk, and McMahon (2016) have proposed a HPLC method with fluorescence

**Table 1**
Names, structures and monoisotopic masses of retinoids and ISs selected for this study.




| Systematic name<br>*Trivial name* | Monoisotopic mass | R |
|---|---|---|
| Retinal | 284.21 | –COH |
| Retinol | 286.23 | $–CH_2OH$ |
| Retinoic acid | 300.21 | –COOH |
| Retinyl octanoate<br>(Retinyl caprylate) | 412.33 |  |
| Retinyl decanoate<br>(Retinyl caprate) | 440.37 |  |
| Retinyl dodecanoate<br>(Retinyl laurate) | 468.40 |  |
| Retinyl tetradecanoate<br>(Retinyl myristate) | 496.43 |  |
| Retinyl 9-*cis*-hexadecenoate<br>(Retinyl palmitoleate) | 522.44 |  |
| Retinyl hexadecanoate<br>(Retinyl palmitate) | 524.46 |  |
| Retinyl heptadecanoate<br>(Retinyl margarate) | 538.47 |  |
| Retinyl 9,12,15- *tricis*-octadecatrieonate<br>(Retinyl linolenate) | 546.44 |  |
| Retinyl 9,12-*dicis*-octadecadieonate<br>(Retinyl linoleate) | 548.46 |  |
| Retinyl 9-*cis*-octadecenoate<br>(Retinyl oleate) | 550.47 |  |
| Retinyl octadecanoate<br>(Retinyl stearate) | 552.49 |  |
| Retinyl 5,8,11,14-*tetracis*-eicosatetraenoic acid<br>(Retinyl arachidonate) | 572.46 |  |
| Retinyl eicosanoate<br>(Retinyl arachidate) | 580.52 |  |
| *Internal standards* | | |
| Retinyl propionate | 342.25 |  |
| Retinyl pentadecanoate | 510.44 |  |