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Analytical Methods

Fatty acids and fat-soluble vitamins in ewe's milk predicted by near infrared reflectance spectroscopy. Determination of seasonality

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ABSTRACT

The aim of the present work was to determine the fatty acid and fat-soluble vitamin composition and the season of ewe's milk production using NIR spectroscopy. 219 ewe's milk samples from different breeds and feeding regimes were taken each month over one year. Fatty acids were analyzed by gas chromatography, and retinol and α-, and γ-tocopherol by liquid chromatography. The results showed that the quantification was more accurate for the milk dried on paper, except for vitamins. Calibration statistical descriptors on milk dried on paper were good for capric, lauric, myristic, palmitoleic, stearic and oleic acids, and acceptable for caprilic, undecanoic, 9c, 11tCLA, ΣCLA, PUFA, ω3, ω6, retinol and α-tocopherol. The equations for the discrimination of seasonality was obtained using the partial least squares discriminant analysis (PLSDA) algorithm. 93% of winter samples and 89% of summer samples were correctly classified using the NIR spectra of milk dried on paper.

1. Introduction

Ewe's milk is used almost exclusively for cheese-making although its relevance for the manufacture of other dairy products is increasing (Albenzio et al., 2015; Balthazar et al., 2016). The composition of milk, especially the fatty acid profile, plays an essential role in the characteristics of cheeses due to the lack of an impact of the cheese-making process on the fatty acid profile (Buchin, Duboz, Le Quéré, & Grappin, 1998; Gnädig et al., 2004). Thus, a higher unsaturation degree of fatty acids is correlated with a softer texture (Bugaud et al., 2001), affects the cheese body colour (Rohm & Jaros, 1997) and flavour, because higher amounts of short-chain fatty acids are associated with a more pungent flavour (Gobetti et al., 1999). From a nutritional point of view, ewe's milk fat is characterized by a high proportion of saturated fatty acids (SFA). Nevertheless, ewe's milk fat also contains short- and medium-chain fatty acids (C6-C10), vaccenic acid, and conjugated linoleic acid (CLA), which have been shown to elicit health benefits in *in vitro* experiments (Raynal-Ljutovac, Lagriffoul, Paccard, Guillet, & Chilliard, 2008).

Apart from the foregoing, ewe's milk fat is a good dietary source of vitamin A and vitamin E, but the data relating to this are very scarce. Vitamin A can be found in ewe's milk only as retinol because dietary β-carotene is converted entirely into this form Raynal-Ljutovac et al. (2008). Vitamin E is found in milk in three forms, α-, β-, and γ-tocopherols but α-tocopherol is the most abundant form Revilla, Palacios, Hidalgo, Alvarez, and Rodriguez (2014).

Fatty acids and fat-soluble vitamin contents are strongly affected by the diet. Several reports show that the percentages of CLA and vaccenic acid in milk fat seem to be strongly linked to the linoleic acid content of grassland vegetation (Addis et al., 2005). Besides this, the contents of α-tocopherol and retinol are usually higher in cow's and goat's milk reared in pasture systems than indoors (Fedele et al., 2004), while 3R isomers from green forage are the main contributors to the total tocopherol content (Butler et al., 2008). The use of vitamin supplements with concentrates should be taken into account (Ellis et al., 2007), mainly for vitamin A, because previous works have reported that vitamin supplements have a relatively minor effect on the α-tocopherol contents of cow's milk (Butler et al., 2008).

The methods used for fatty acid and vitamin determinations involve extraction, alkaline hydrolysis and analysis by chromatography (GC, HPLC or UPLC) (Chauveau-Duriot, Doreau, Nozière, & Graulet, 2010; Herrero-Barbudo, Granado-Lorencio, Blanco-Navarro, & Olmedilla-Alonso, 2005; Lurueña-Martínez, Palacios, Vivar-Quintana, & Revilla 2010). These analytical determinations are time-consuming and require the use of pollutant solvents

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and sophisticated equipment. In contrast, Near Infrared Spectroscopy (NIRS) has the advantage of being a rapid, cheap, non-destructive, non-contaminating, and multiparametric method. Some studies have shown that it is possible to predict the fatty acid composition of cow's and goat's milk using NIRS technology (Coppa et al., 2010, 2014; Núñez-Sanchez et al., 2016), but no reports about fatty acid prediction of ewe's milk or vitamin determination in any type of milk have been found.

The main aim of the present study was to investigate the ability of NIRS coupled with a fibre optic probe to predict the fat-soluble vitamin and fatty acid contents of ewe's milk together with the classification of milks according to the season of milk production. The quantification was carried out both in liquid milk and after oven-drying the samples to remove the water content because this procedure has been shown to permit a more accurate quantification (Coppa et al., 2010, 2014).

2. Materials and methods

2.1. Milk samples

A total of two hundred and forty milk samples were collected from the stirred bulk of twenty commercial flocks of different sizes ranging from 280 to 1500 animals, located in different places (18 locations) of Castile and Leon (northwest Spain). The samples were taken each month during the year. The seasonal variations and the geographical origin strongly affect fatty acid composition due to the changes in the forage composition of range-fed animals (Coppa et al., 2014; Zlatanos, Laskaridis, Feist, & Sagredos, 2002). Among the flocks, 9 commercial flocks had ewes of the Churra local breed and 11 flocks had ewes of the Assaf breed because previous work has shown that the lipid profile depends on breed (Lurueña-Martínez et al., 2010). The flocks selected differed in their management and feeding practices. Thus, samples were obtained from flocks whose ewe's grazed on grass (fresh forage varying from 0% to 30% on a dry matter basis) or fed different preserved forages: alfalfa forage, corn silage, straw or other silages, with a forage-to-concentrate ratio varying from 45%:55% to 85%:15%. The concentrates were mainly cereal and protein concentrates.

Two milk subsamples were recovered at each sampling point, one for GC analysis and the other for NIRS analysis, and were stored at −30 °C until analysis. However, some samples were not correctly coded or stored and finally a total of two hundred and nineteen samples were analyzed.

2.2. Fatty acid analysis

Lipids were extracted using the International Standard Method described in ISO 14156:2001. The fatty acids of all the samples were methylated and analyzed according to the method of Lurueña-Martínez et al. (2010) using a gas chromatograph (GC 6890N, Agilent Technologies, USA) equipped with a 100 m × 0.25 mm × 0.20 μm capillary column (SP-2560, Supelco, Inc., Bellefonte, PA, USA) and an FID detector The oven temperature program started at 150 °C, increasing temperature at 1 °C/min until 165 °C was reached, followed by an increase at 0.20 °C/min up to 167 °C and finally increasing by 1.50 °C/min up to 225 °C. This temperature was held for 15 min. The injector and detector temperatures were 250 °C and 1 μL was injected into the chromatograph in split (20:1) mode. The carrier gas was helium at 1 mL/min. The different fatty acids were identified by their retention times, comparing them with the corresponding standards (Larodan Fine Chemicals AB, Malmo, Suecia) and their contents were calculated using chromatogram peak areas and were expressed as g per 100 g total fatty acid methyl esters.

2.3. Fat soluble vitamins

To estimate the fat-soluble vitamins, samples were heated (30 °C), homogenized and subjected to alkaline hydrolysis according to the method proposed by Herrero-Barbudo et al. (2005). Briefly, 1.5 mL of ascorbic acid 0.3 M and 0.1 mL of δ-tocopherol as internal standard were added to 2 mL of sample. The mixture was vortexed and methanolic KOH (40%) was added and the mixture was then vortexed again for 30 s. The mixture was heated to 70 °C and shaken (200 rpm) for 40 min. Samples were cooled for 3 min. and extracted using n-hexane:dichloromethane (5:1)/isopropanol (4/1) four times. The organic phases were pooled, washed with cooled water to remove KOH, evaporated under a nitrogen flow, reconstituted in 1 mL of acetonitrile/methanol (85/15), and filtered using a 22 μm syringe filter.

UPLC was run on a Waters Acquity system (Waters, France) equipped with a photodiode array detector, scanning at between 275 and 465 nm, and a fluorometric detector. A 150 × 2 1-mm Acquity UPLC HSS T3, 1.8-mm column (Waters, France) was used. The flow rate applied was 0.4 mL/min and the analyses were performed at 35 °C (Chauveau-Duriot et al., 2010).

Two separate isocratic chromatographic methods were employed. The mobile phase for retinol was acetonitrile:methanol (85:15)/isopropanol:water (50:50) 80/20, with λ_{exc} = 325 and λ_{em} = 475 nm for fluorometric detection. The mobile phase for the different tocopherols analyzed was acetonitrile:methanol (85:5)/isopropanol 90/10, with λ_{exc} = 295 and λ_{em} = 390 nm for fluorometric detection.

Peak identification was accomplished using pure standards and quantification was performed using calibration curves. The purity of the standards used was monitored by UV–vis spectra. Using the described method, hydrolysis efficacy was >97% and, based on stoichiometric calculations, the recoveries of the added analytes were 71% and 81% for retinol and the different tocopherol forms.

2.4. Near infrared spectroscopy

The milk samples were kept frozen at −30 °C in vessels with a capacity of 250 mL. The samples were thawed at room temperature on the day before the NIR recordings. Once thawed, the samples were heated in a water bath until the milk had reached a temperature of 40 °C and were then homogenized by shaking. Between 5 and 10 mL of sample was collected and cooled rapidly to room temperature (23–25 °C). When working with liquid milk samples, 250 μL of sample was placed on a circular aluminium capsule with an optical pathway of 0.1 mm. When working with samples on paper, another 250 μL of sample was placed on a circle of filter paper (Albet paper, without ashes, ref 1150) with the same size as the aluminium support and was spread across the whole of the paper circle. Each circle of paper was placed in a small Petri dish. Once the sample had been placed on the paper, it was placed in an oven at 50 °C for 2 days to remove all the water present. After this time, the circle of paper was placed on the cam-lock and spectrum was recorded with a Foss NIRSystems 5000. Measurement was performed in reflectance mode between 1100 and 2498 nm. In both cases, the spectra were recorded at intervals of 2 nm, performing 32 scans both for the reference and for the sample. To minimise sample error, all samples were analyzed in triplicate. The average spectrum was used for NIR analysis. The software used was Win ISI 1.05, installed on a Hewlett Packard Pentium III computer.

2.5. Statistical analyses

Milk fatty acids and fat-soluble vitamins were quantified with the modified partial least squares (MPLS) regression method to