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Technical note: Development and validation of a new method for the quantification of soluble and micellar calcium, magnesium, and potassium in milk

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ABSTRACT

Milk mineral content is a key trait for its role in dairy processes such as cheese-making, its use as source of minerals for newborns, and for all traits involving salt-protein interactions. This study investigated a new method for measuring mineral partition between soluble and micellar fractions in bovine milk after rennet coagulation. A new whey dilution step was added to correct the quantification bias due to whey trapped in curd and excluded volume. Moreover, the proposed method allowed the quantification of the diffusible volume after milk coagulation. Milk mineral content and concentration in whey, and diluted whey were quantified by acid digestion and inductively coupled plasma optical emission spectrometry. The repeatability of the method for micellar Ca, Mg, and K was between 2.07 and 8.96%, whereas reproducibility ranged from 4.01 to 9.44%. Recovery of total milk minerals over 3 spiking levels ranged from 92 to 97%. The proposed method provided an accurate estimation of micellar and soluble minerals in milk, and curd diffusible volume.
Key words: milk mineral, cheese making, curd, micellar

Technical Note

Milk has been extensively investigated as source of macro- and micronutrients such as essential minerals. In particular, Ca supply is essential for human health (Caroli et al., 2011; Dror and Allen, 2014; Burckhardt, 2015), growth (Sheikh et al., 1987), and regulation and maintenance of physiological functions (Weaver, 2014). Magnesium intake from dairy food is correlated with insulin sensitivity (Ma et al., 2006), and K is important in physiological functions such as blood pressure control (O'Halloran et al., 2016).

Besides their effects on health, minerals influence milk technological traits (Tsioulpas et al., 2007) and are crucial for cheese manufacturers in countries such as Italy, as well as several other countries where milk is mainly used for cheese making (Cassandro, 2003; Rosa et al., 2016). Calcium and Mg are related to casein structure, which is primarily involved in the coagulation process and curd formation (Malacarne et al., 2014; Toffanin et al., 2015; Visentin et al., 2016). Calcium content plays a fundamental role in the ability of milk to produce cheese, and the addition of Ca salts to milk decreases rennet clotting time and increases curd firmness (Landfeld et al., 2002; Guillaume et al., 2004).

Minerals are present in 2 main forms in milk: soluble (or diffusible) and micellar. Soluble minerals are present in a highly dynamic equilibrium between ionic and associate forms, mainly represented by citrate, phosphate, sulfate, and chloride salts (Holt et al., 1981). Micellar Ca can locate both on the surface or inside the casein micelles. Calcium located on the surface helps micelle aggregation during the formation of the paracasein reticulum (Sandra et al., 2012). At the same time, Ca is present in the internal part of casein micelles as calcium phosphate, also defined as colloidal calcium. Colloidal Ca fraction is essential in the formation and stabilization of casein micelles (Holt et al., 2013; Ingham et al., 2016), and influences milk coagulation ability (Malacarne et al., 2014; Niero et al., 2016).

Currently, different approaches can be used for the separation of aqueous phase milk for the determination of mineral concentration. Dialysis is one of the first proposed methods but has been demonstrated to be strongly affected by osmotic perturbation determined by equilibration with dialysis buffer (White and Davies, 1958; de la Fuente et al., 1996). Ultrafiltration has been effectively used in several studies, despite its tendency to underestimate soluble Ca concentration as a consequence of Ca precipitation during the process (deMan, 1962; Vyas and Tong, 2003). Moreover, UF needs adequate machinery and must be carefully set up. Ultracentrifugation (usually around 80,000 × g)

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gives good estimation of the amount of soluble minerals with an easy sample preparation and can also be used for poor-coagulating milks (Udabage et al., 2000; Jensen et al., 2012), even if high pressure can promote solubilization of minerals from casein micelles (Huppertz and de Kruif, 2006). Finally, rennet coagulation is used to separate micellar from soluble mineral fractions, with good reproducibility, applying the cheesemaking procedure (Malacarne et al., 2014). Nevertheless, it cannot be used for noncoagulating samples. All reported methods need correction factors to take into account the excluded volume effect. Moreover, values obtained from UF require Donnan potential correction factors (Ohshima and Kondo, 1988), which can be taken from the literature or need to be calculated de novo (deMan, 1962; de la Fuente et al., 1996). The present study aimed to develop a new method, based on rennet coagulation, for the determination of soluble and micellar mineral fractions in bovine milk and for overcoming the need of correction factors. In particular, a dilution step was introduced during sample preparation, followed by an hour incubation to allow the complete mineral equilibration between the whey trapped in the paracasein reticulum and the diluted whey. Such a step, with an adequate mathematical transformation, allowed the quantification of the absolute amount of soluble minerals and avoided technical issues due to whey trapped in the curd and excluded volume, which affect the determination of mineral concentration.

Ultrapure water was produced with Arium 611 UV (Sartorius, Monza Brianza, Italy), and all chemicals, if the supplier is not mentioned, were bought from Sigma-Aldrich (St. Louis, MO) at the highest available purity. Bulk whole raw milk was collected directly by a fluid milk farm distributor (Legnaro, Italy) in March 2017. Milk was analyzed using Milkoscan FT2 (Foss Electronic A/S, Hillerød, Denmark) for major components, resulting in 3.92%, 3.60%, 2.75%, 4.73%, and 6.60 for fat, protein, casein, lactose, and pH, respectively. The milk sample was split in five 100-mL aliquots and processed in less than 3 h without preservative. Ten milliliters of milk was preheated to 38°C for 25 min, added to calf rennet 1:3,000 wt/wt (80% chimosin, 20% pepsin, and strength 1:10,000; Clerici-Sacco Group, Codorago, Italy), and incubated for 30 min at 36°C in a water bath. The curd was cut in the tube in 4 pieces using a round-shaped knife and further incubated for 30 min at 36°C. Samples were centrifuged for 15 min at 20°C and 10,000 × *g*, and 5 mL of whey was carefully collected. Five milliliters of ultrapure water was added to the remaining curd, vortexed for 20 s, and left 60 min at room temperature for equilibration. The mixture was centrifuged as previously described and 5 mL of diluted whey was collected. All fractions were stored frozen until inductively coupled plasma optical emission spectrometer (**ICP-OES**) analysis. A schematic view of sample preparation is depicted in Figure 1. Calcium, Mg, and K concentrations in samples were determined after digestion using nitric acid in a microwave closed vessel (Ethos 1600 Milestone S.r.l., Sorisole, Italy) as reported in Visentin et al. (2016) and Manuelian et al. (2017). Briefly, ICP-OES Ciros Vision EOP (Spectro Analytical Instruments GmbH, Kleve, Germany) was used for determination of Ca at 317.933 nm, Mg at 285.213 nm, and K at 766.941 nm after proper dilution. Calibrations for single minerals were prepared in a range between 0 and 100 mg/L using single-element solutions (Inorganic Ventures, Christiansburg, VA). Mineral concentrations in whey ($\mathbf{C_w}$) and diluted whey ($\mathbf{C_d}$) were calculated as

$$C_w = M_w/V_w, \quad [1]$$

$$C_d = M_d/V_d, \quad [2]$$

where M_w is the absolute amount of mineral in whey, V_w is the volume of the whey, M_d is the absolute amount of mineral in diluted whey, and V_d is the volume of diluted whey. According to the experimental design of the present study, V_d was equal to V_w. The M_d can be expressed in function of M_w as

$$M_d = M_w - D \times C_w, \quad [3]$$

with D being the volume of water added for dilution (i.e., 5 mL for the present study). Combining [1], [2], and [3]:

$$M_w/C_w = (M_w - DC_w)/C_d,$$

$$M_w = (DC_w{}^2)/(C_w - C_d), \quad [4]$$

with C_w and C_d obtained from ICP-OES. The V_w was then calculated from [1]. The amount of micellar mineral was expressed as

$$M_c = M_t - M_w, \quad [5]$$

where M_c is the amount of micellar mineral expressed in milligrams and M_t is the total minerals in starting milk from ICP-OES analysis. Final mineral concentrations were expressed as mg/100 mL of initial milk volume. Repeatability was expressed as relative standard deviation ($\mathbf{RSD_r}$) of 5 aliquots from the same bulk milk, processed separately. Reproducibility was calculated as relative standard deviation ($\mathbf{RSD_R}$) of 15 samples measured across 3 d (Niero et al., 2017).