

Salt equilibria in nutritional formulae for infants and young children

Thom Huppertz^{a, b, *}, Christel Timmer^a

^a FrieslandCampina, Amersfoort, the Netherlands

^b Wageningen University and Research, Wageningen, the Netherlands

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ABSTRACT

The distribution of Ca, Mg, K and Na between the sedimentable (200×g), protein-associated and soluble phase of infant formula (IF; n = 46), follow-on formula (FOF; n = 8), junior growing up milk (jGUM; n = 22) and senior GUM (sGUM; n = 16) were studied. For all product classes, virtually all Na and K was in the soluble phase and small amounts were found as protein-associated, i.e., as counterions. Most Mg was also found in the soluble phase, but a notable proportion (20–30%) was found to be protein-associated. Sedimentable Mg was only found in a few samples. Particularly in IF and FOF products, notable amounts of sedimentable Ca were observed; the proportion of protein-associated Ca increased with increasing casein content of samples, but even after correction for casein content, large differences (>2-fold) remained between products. These differences in casein mineralisation can affect physicochemical properties and colloidal stability.

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1. Introduction

Nutrition in early infancy and childhood is essential in the healthy development of children. Breast milk is considered the best source of nutrition for babies, as it contains the perfect balance of nutrients for the neonate (Andreas, Kampmann, & Le-Doare, 2015). However, in cases where breast milk is not available, infant formula (IF) products have been designed to provide suitable alternatives. As IF products are the only source of nutrition for formula-fed infants, these products need to provide a balance of nutrients for the neonate as close as possible to what breast milk does. IF products are typically intended for infants of 0–6 months of age. For older infants (6–12 mo), so-called follow-on formula (FOF) is available and is typically intended as the liquid part of the weaning diet for the infant. For older children, growing up milk (GUM, also known as young child formula) products are available as a supplementary source of nutrients to the diet of the child. GUM products often distinguish between junior GUM (jGUM; 12–36 mo) and senior GUM (sGUM; >36 mo). For all these nutritional formula products for infants and young children, provision of nutrients to fulfil the requirements is the key purpose.

In addition to macronutrients (protein, fat and carbohydrate), infant and toddler milk products also contain a wide variety of micronutrients. Among the essential micronutrients provided by IF, FOF and GUM products are the salts. Like milk, the products are

balanced and rich sources of essential salts. Even during or after weaning, FOF and GUM are important to supply sufficient levels of e.g., Ca, Mg, Na, K, P and other elements. In addition to the levels, the form in which the salts are provided to infants and children will also be important. For milk of all species studied in sufficient detail to date, it is clear that the concentrations of calcium and phosphate present exceed the aqueous solubility at the specific environmental conditions (Holt, 2016; Holt & Jenness, 1984). Therefore, a partitioning of calcium phosphate occurs whereby parts of calcium and phosphate remain in soluble form in the serum phase and the remainder is associated with the casein fraction. This so-called mineralisation of the casein fraction differs between species, depending on the specific requirements of each species, and is for instance higher in bovine milk (~7.7 mmol micellar Ca per 10 g casein) than in human milk (~3.2 mmol micellar Ca per 10 g casein; Holt & Jenness, 1984). It is also noteworthy that whereas for bovine milk ~30% of total Ca is found in the soluble phase, ~65% of total Ca is found in the soluble phase in human milk (Holt & Jenness, 1984). In addition to calcium and phosphate, magnesium and citrate have also been found to be associated with the casein fraction (Holt, 1982).

The mineralisation of caseins in the mammary gland also forms the key to the formation of casein micelles (Farrell, Malin, Brown, & Qi, 2006; Huppertz et al., 2017). In bovine milk, these micelles are ~150–200 nm in diameter and contain several hundred nanoclusters (4–5 nm diameter) of amorphous calcium phosphate (De Kruif, Huppertz, Urban, & Petukhov, 2012). In contrast, in addition to the aforementioned lower mineralisation, casein micelles in human milk are also notably smaller (50–100 nm diameter) than their bovine

* Corresponding author. Tel.: +31 6 11187512.

E-mail address: thom.huppertz@frieslandcampina.com (T. Huppertz).

counterparts (Ruegg & Blanc, 1982). Casein micelles are susceptible to gastric coagulation, primarily as a result of pepsin-induced hydrolysis of κ -casein (Huppertz & Lambers, 2020; Wang, Ye, Lin, Han, & Singh, 2018). This entails an important role in relation to controlled transit of proteinaceous material (Boirie et al., 1997; Nakai & Li-Chan, 1987).

Factors affecting salt equilibria and effects thereof on product functionality have been widely studied, particularly for bovine milk (for reviews Holt, 1997; Lucey & Horne, 2009). For human milk, studies on salt equilibria have also been reported (Holt, 1993; Holt & Jenness, 1984; Neville, Keller, Casey, & Allen, 1994). Despite their influence on product functionality, salt distributions have not been reported for IF, FOF and GUM products to date. To provide insights into this area, the distributions of Na, K, Ca and Mg in a series of commercial IF, FOF, jGUM and sGUM products were studied.

2. Materials and methods

2.1. Sample collection and reconstitution

Infant formula (IF; $n = 46$), follow-on formula (FOF; $n = 8$), junior growing up milk (jGUM; $n = 22$) and senior GUM (sGUM; $n = 16$) products were collected from retail outlets in Europe, North America, Asia and Australia. Samples were reconstituted in demineralised water at a level of 12 g powder per 100 g water. Samples were stored overnight at 5 °C to ensure complete hydration.

2.2. Sample fractionation

Samples were equilibrated at 20 °C for 1 h, followed by centrifugation at 200×g for 30 min at 20 °C. Following centrifugation, the

sediment and non-sedimentable phase were separated by decanting. The non-sedimentable phase obtained from this centrifugation step was subsequently centrifuged at 100,000×g for 60 min at 20 °C, after which a cream phase, a sediment and an intermittent layer were obtained. The intermittent layer was carefully removed and subsequently subjected to centrifugal filtration over a 10 kDa membrane and the permeate was collected. The whole sample, the non-sedimentable fraction and the permeate were subsequently used for analysis.

2.3. Analysis

Total nitrogen (TN) and non-protein nitrogen (NPN) content of whole samples were determined using the Kjeldahl method, as described by ISO/IDF (2014) and ISO/IDF (2016), respectively. Protein content was calculated as $TN \times 6.38$. Na, K, Ca and Mg in the whole sample, the non-sedimentable fraction and the permeate were determined by ICP-AES as described by Crujisen, Poitevin, and Brunelle (2019). The ratio of whey protein to casein in the products was calculated from the concentrations of Pro, Phe, Asp + Asn and Ala, according to the method described by Jacobs et al. (2013). For this purpose, amino acid composition of the samples was determined by ion exchange chromatography as described by Windham (1995).

3. Results

3.1. Protein and non-protein nitrogen

Protein content of reconstituted samples is shown in Fig. 1A. As expected, based on the information on the labels, protein content

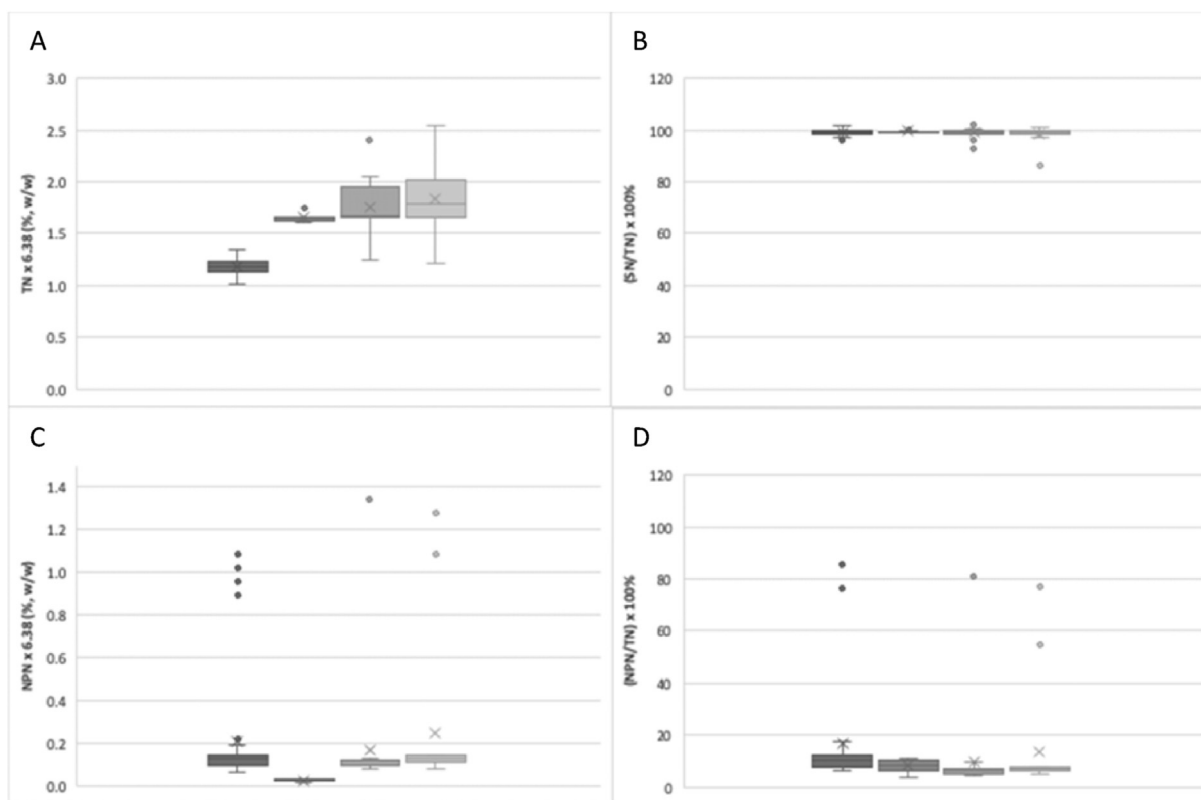


Fig. 1. Total nitrogen (TN) × 6.38 (A), soluble nitrogen (SN) as a percentage of TN (B), non-protein nitrogen (NPN) × 6.38 (C), and NPN as a % of TN (D) for (left to right in each panel) infant formula (■; $n = 46$), follow-on formula (▒; $n = 8$), junior growing up milk (▓; $n = 22$) or senior growing up milk (□; $n = 16$) products prepared by reconstitution of 12 g powder in 100 g water. Data are presented in Box-Whisker plots, where x represented the mean, the middle line in the box represents the median and the bottom and top line of the box represent the lower and upper quartile, respectively. The whiskers extend from the lowest to highest value, excluding outliers, with outliers shown as individual points and consider as such if their distance from the upper or lower quartile value exceeds $1.5 \times$ the interquartile distance.

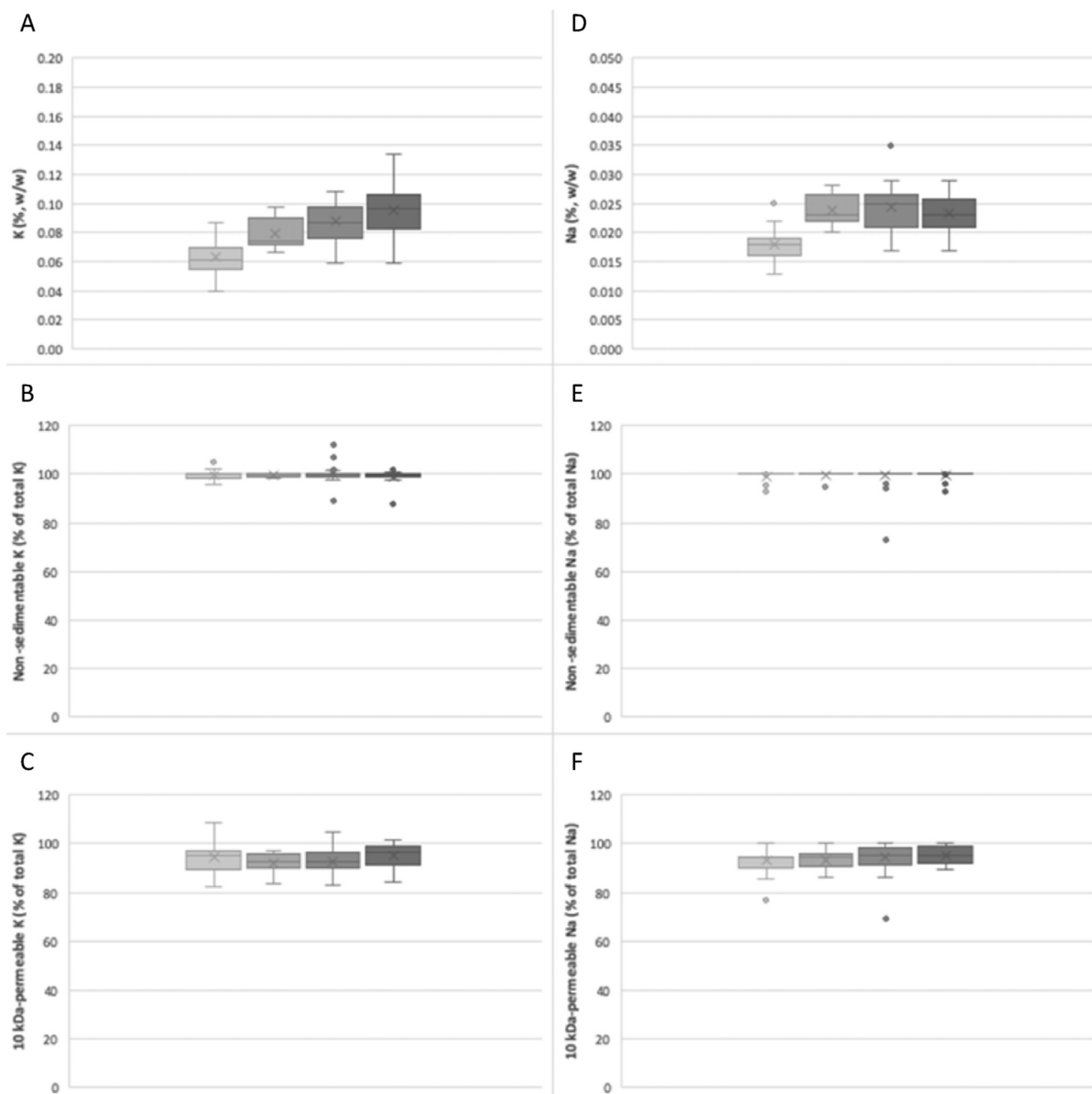


Fig. 2. Total K (A), non-sedimentable (200×g) K as a percentage of total K (B), 10 kDa-permeable K as a percentage of total K (C), total Na (D), non-sedimentable (200×g) Na as a percentage of total Na (E), and 10 kDa-permeable Na as a percentage of total Na (F) for (left to right in each panel) infant formula (■; n = 46), follow-on formula (■; n = 8), junior growing up milk (■; n = 22) or senior growing up milk (■; n = 16) products prepared by reconstitution of 12 g powder in 100 g water. Data are presented in Box–Whisker plots. For explanation see Fig. 1.

increased with the stage of product, particularly from IF to FOF. Variation in total protein for each product category was found to be larger for jGUM and sGUM products than for IF and FOF products. With the exception of one sGUM product, >95% of total protein in all samples remained in solution after centrifugation at 200×g for 30 min (Fig. 1B). NPN was also determined for all samples (Fig. 1C and D). NPN levels were low for most products (i.e., <10% of total N), but in several products, NPN represented >50% of total N (Fig. 1D); these were products containing protein hydrolysate as ingredients. Typically, free amino acid and peptides with a chain length up to ~6

amino acids remain soluble in the presence of 12% trichloroacetic acid (Yvon, Chabanet, & Pelissier, 1989), as used in the determination of NPN. In addition, some strongly glycosylated CMP is also soluble under these conditions (El-Salam, El-Shibiny, & Buchheim, 1996). NPN/TN decreased from IF to FOF to jGUM and sGUM (Fig. 1D). This can probably be attributed to decreasing proportions of whey ingredients being used in GUM products. Whey protein content, as a percentage of total protein, was found to range from 30 to 45% for IF, 30–60% for FOF, 40–80% for jGUM and 60–80% for sGUM (data not shown).

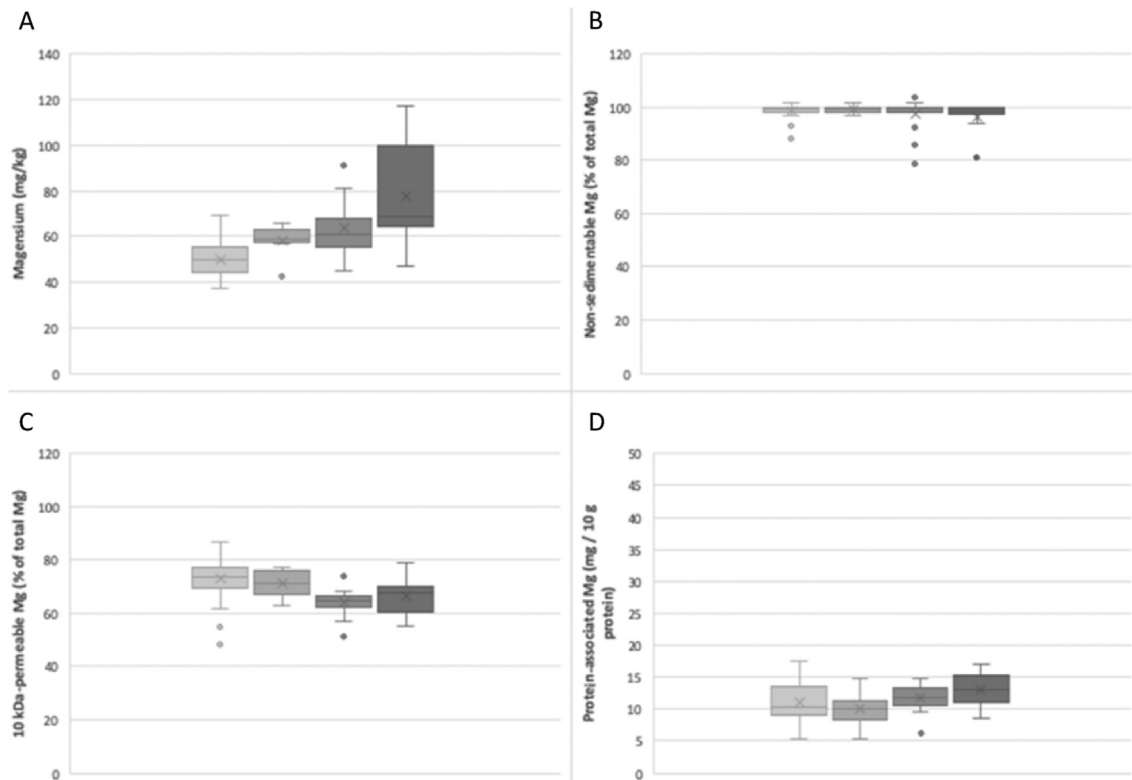


Fig. 3. Total Mg (A), non-sedimentable ($200\times g$) Mg as a percentage of total Mg (B), 10 kDa-permeable Mg as a percentage of total Mg (C), protein associated Mg ($\text{mg } 10 \text{ g}^{-1}$ protein) (D) for (left to right in each panel) infant formula (■; $n = 46$), follow-on formula (■; $n = 8$), junior growing up milk (■; $n = 22$) or senior growing up milk (■; $n = 16$) products prepared by reconstitution of 12 g powder in 100 g water. Data are presented in Box–Whisker plots. For explanation see Fig. 1.

3.2. Sodium, potassium and magnesium distribution

Potassium content of products increased progressively from IF to sGUM (Fig. 2A). All potassium was found to be non-sedimentable (Fig. 2B), whereas ~90% of potassium in all product classes was found to be permeable through 10 kDa filters as well (Fig. 2C). The remainder of the potassium, which was not 10 kDa-permeable, is probably associated with the proteins as counterions for negatively-charged amino acid residues. A similar trend was also observed for sodium (Fig. 2D–F) for which virtually all material was non-sedimentable and only a small fraction was not 10 kDa-permeable, again as it is present as a counterion for negatively-charged amino acid residues.

Total magnesium in products increased progressively from IF to FOF, jGUM and sGUM (Fig. 3A). In most samples, all magnesium was in the soluble phase, but for some samples, up to 20% of magnesium was found to be sedimentable at $200\times g$. This could be the result of adding magnesium to formulations in the form of poorly-soluble salts. Compared with potassium and sodium, notable lower percentages of total magnesium were found to be 10 kDa-permeable and the proportion of magnesium that was 10 kDa-permeable decreased from IF to FOF to jGUM to sGUM (Fig. 3C). This may be linked to non-10 kDa-permeable magnesium being primarily associated with the protein fraction (Fig. 3D).

3.3. Calcium distribution

In contrast to sodium, potassium and magnesium, a large proportion of total Ca (Fig. 4A) was found to be sedimentable at $200\times g$ in all formulations (Fig. 4B), particularly in IF and FOF products. For IF products, samples where $>50\%$ of Ca was

sedimentable at $200\times g$ were observed. Ca sedimentable at these conditions may be in the form of poorly dissolved powder particles or as sparingly-soluble calcium salts added to the products. Considering that little or no protein was found to be sedimentable at $200\times g$ (see Fig. 1B) in these samples, sedimentable Ca is considered to be Ca in the form of sparingly-soluble Ca salts, e.g., calcium phosphates or calcium carbonate, all of which are permitted for use in these products in many jurisdictions. As in the case for Mg, but unlike for Na and K, not all Ca in samples was found to be permeable though a 10 kDa membrane (Fig. 4C) and protein-bound Ca was found to represent in a major proportion of Ca in the samples (Fig. 4D). The proportion of protein-bound Ca increased from ~40% of total Ca for IF to ~60% of total Ca for jGUM and sGUM. Within all sample categories, but particularly for IF, notable variation between products was observed, which, as discussed later, may be related to formulation options for these products. When expressing protein-associated Ca as a function of protein, rather than total Ca, similar trends were observed with increases from IF to FOF to jGUM and sGUM. However, when expressing protein-associated Ca as a function of casein, much more comparable values were observed for all sample categories. However, variation within sample categories remained.

To exemplify variation, the level of casein mineralisation, expressed in millimoles of protein-associated Ca 10 g^{-1} casein within IF, FOF, jGUM and sGUM products studied, is shown in Fig. 5. From this, the variation in particularly IF products is very notable. With an average of $7.7 \text{ mmol Ca } 10 \text{ g}^{-1}$ casein for the samples in this product class, minimum and maximum values of 4.9 and $11.9 \text{ mmol Ca } 10 \text{ g}^{-1}$ casein were observed. For FOF, jGUM and sGUM products, averages and maximum values were comparable with IF products, but minimum values were notably higher, with no samples

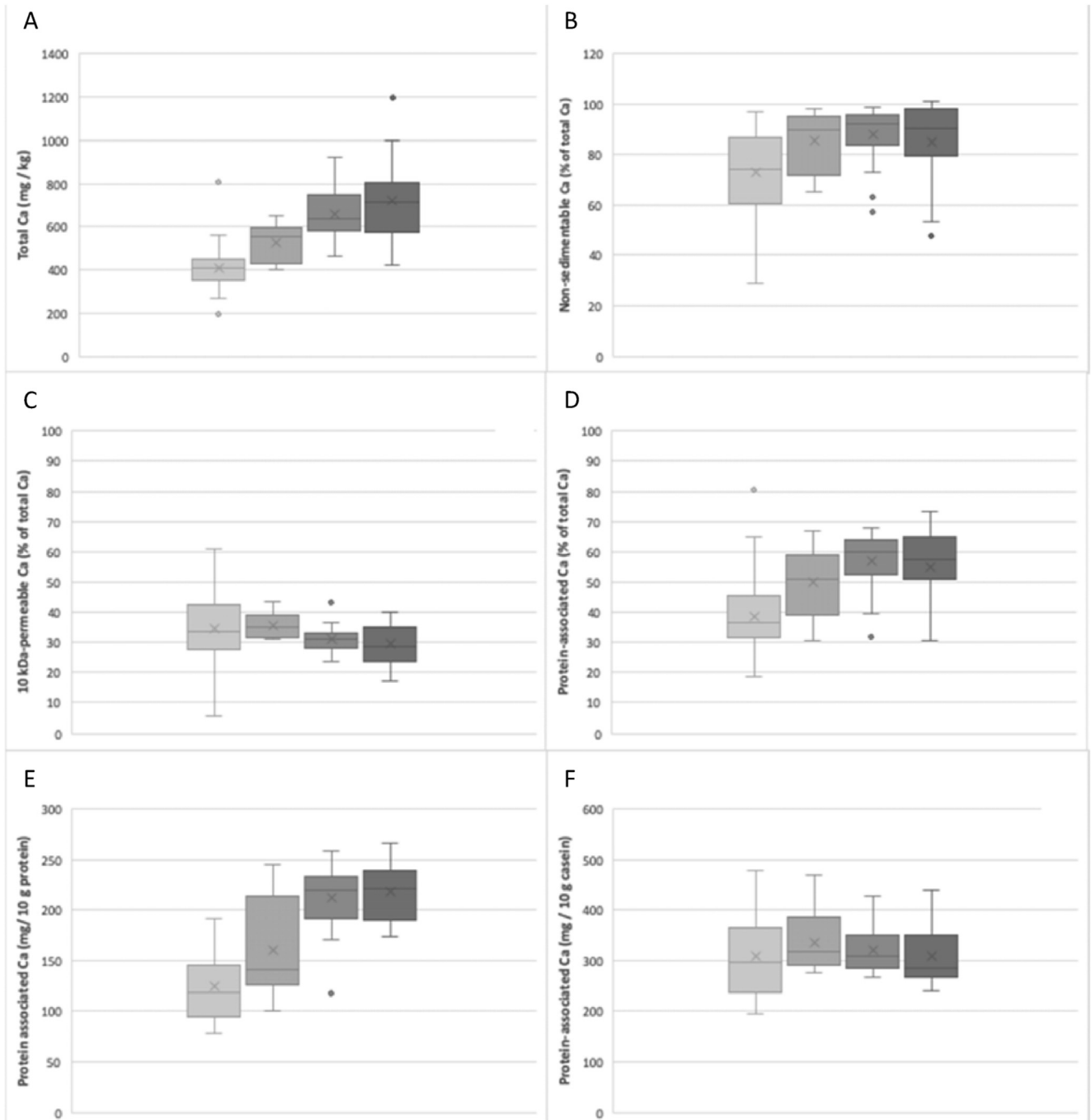


Fig. 4. Total Ca (A), non-sedimentable (200×g) Ca as a percentage of total Ca (B), 10 kDa-permeable Ca as a percentage of total Ca (C), protein associated Ca as a percentage of total Ca (D), protein-associated Ca (mg 10 g⁻¹ protein) (E), and protein associated Ca (mg 10 g⁻¹ casein) (F) for (left to right in each panel) infant formula (■; n = 46), follow-on formula (■; n = 8), junior growing up milk (■; n = 22) or senior growing up milk (■; n = 16) products prepared by reconstitution of 12 g powder in 100 g water. Data are presented in Box–Whisker plots. For explanation see Fig. 1.

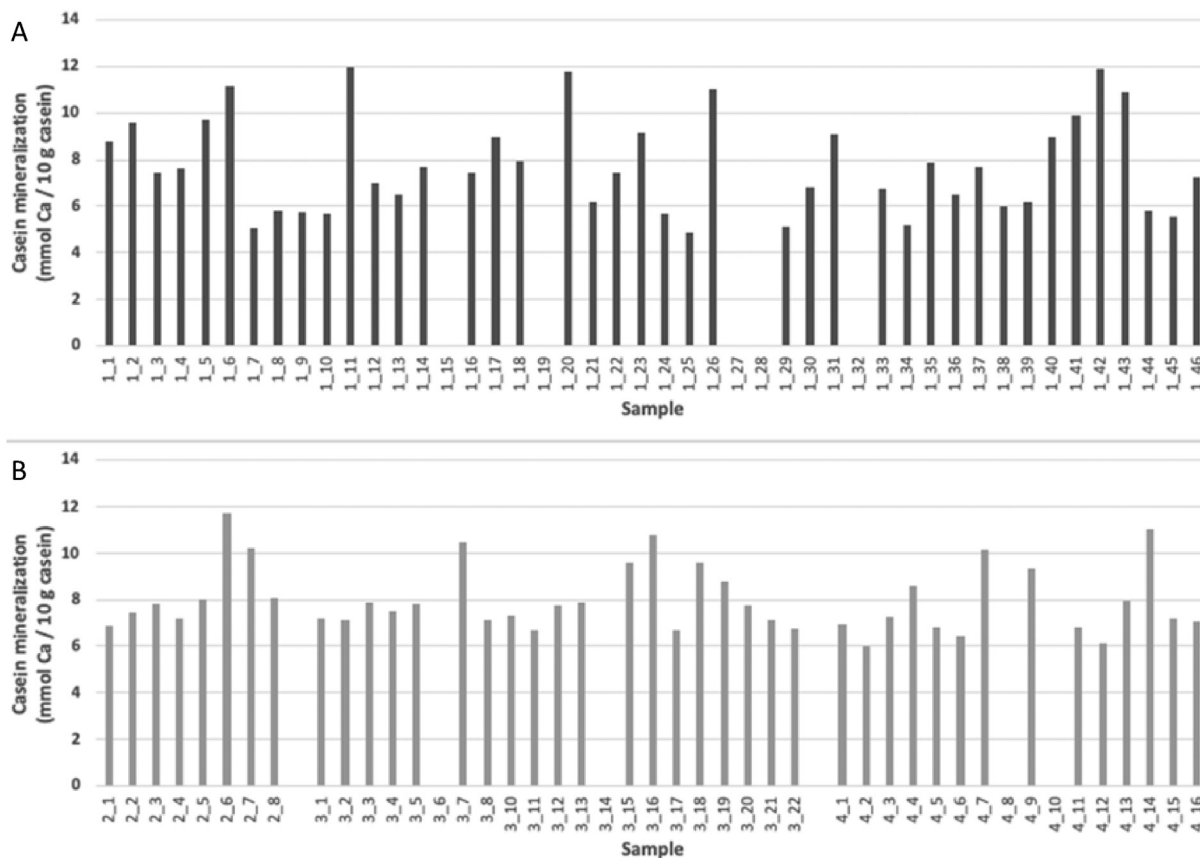


Fig. 5. Casein mineralisation, expressed as mmol protein associated Ca 10 g^{-1} casein, for (A) infant formula (samples 1_1 to 1_46) and (B) follow-on formula (samples 2_1 to 2_8), junior growing up milk (samples 3_1 to 3_22) or senior growing up milk (samples 4_1 to 4_16) products prepared by reconstitution of 12 g powder in 100 g water. No values are shown for samples based on protein hydrolysate rather than intact protein (NPN/TN > 0.5), i.e., samples 1_15, 1_19, 1_27, 1_28, 1_32, 3_6, 3_14, 4_8 and 4_10.

<6.0 mmol Ca 10 g^{-1} casein. In contrast, 10 of the 41 IF products tested had mineralisation levels <6.0 mmol Ca 10 g^{-1} casein. This highlights a strong variation between products in the product classes, particularly for IF.

4. Discussion

The results of this study highlight notable variation in salts distribution between product classes (IF, FOF, jGUM, sGUM) but also between products within a class. For the salt distribution, distinction was made between sedimentable (at $200\times\text{g}$), protein-associated and soluble (10 kDa permeable) fractions. The majority of Na and K present in the samples was found to be soluble, which is in line with observations in milk (Holt, 1997). A small proportion of Na and K was found to be protein-associated, probably due to their role as counter-ions for negatively charged amino acid residues. For Ca and Mg, however, a more intricate distribution over the different phases was observed. Particularly for IF products, notable levels of sedimentable Ca were observed, which, as outlined earlier, is probably the result of adding permitted sparingly-soluble Ca salts (i.e., calcium phosphates or calcium carbonate) to formulations. Furthermore, a large proportion of non-sedimentable Ca and Mg in products were found to be protein associated. This is also observed in milk, where $\sim 2/3$ of Ca and $\sim 1/3$ of Mg appears to be associated with the casein micelles since the solubility products for its respective phosphate salts are exceeded at the concentrations present in milk (Holt, 1997). The use of milk as an ingredient thus introduces the protein-associated Ca in the formulations. Although some Ca and Mg may also be associated with the whey proteins,

>95% of all protein-associated Ca and Mg may be expected to be associated with the casein fraction, even in a whey protein-enriched IFT product. Hence, the levels of protein-associated Ca and Mg essentially reflect the mineralisation of the casein fraction of the product.

For all product categories, casein mineralisation was found to vary notably between products. Various factors can be identified contributing to this variation. As mineralisation of casein micelles is governed to a large extent by solubility products of calcium phosphates, any factors affecting calcium phosphate solubility will also affect casein mineralisation, in milk and IFT products. pH is in this respect an important factor, with lower pH resulting in increased solubility of calcium phosphate (Holt, 1997; Lucey & Horne, 2009). However, the variation in pH observed between samples (6.6–7.2) cannot account for the large differences in mineralisation observed. Added salts in the formulation can also affect casein mineralisation. For milk, it is well-known that addition of soluble calcium salts (e.g., CaCl_2) or soluble phosphate salts (e.g., Na_2HPO_4) increases casein mineralisation due to further supersaturation of the product with respect to calcium phosphate (Holt, 1997; Lucey & Horne, 2009). Addition of soluble citrate salts, on the other hand, can reduce casein mineralisation due to the Ca-chelating ability of citrate (Udabege, McKinnon, & Augustin, 2000). In addition to added salts, some salts are also added though the whey protein ingredients. Depending on the choice of ingredient, e.g., demineralised whey versus WPC35 versus WPC80, the levels and types of salt added via this route may vary. Finally, the source of milk may also affect casein mineralisation in products. Malacarne et al. (2014) showed differences in casein mineralisation between 118 herds in

Italy, whereas Auld, Johnston, White, Fitzsimons, and Boland (2004), showed a notable difference in Ca/casein ratio between milk from Friesian cows and Jersey cows. Considering that the level of soluble Ca tends to be rather constant, this highlights a difference in mineralisation between breeds. From the data of White and Davies (1958), it is also clear that variation in mineralisation between different milk samples from Ayrshire cows is apparent.

Variations in casein mineralisation can also notably affect product properties. In their study, Malacarne et al. (2014) showed that milk with lower levels of casein mineralisation yielded poorer rennet coagulation properties. A similar finding was reported by Shalabi and Fox (1982), who observed that reducing casein mineralisation of milk impaired rennet-induced coagulation. A recent study by Huppertz and Lambers (2020) showed that not only rennet-induced coagulation, but also pepsin-induced coagulation of casein during in-vitro gastric digestion of model infant formula products was strongly affected by casein mineralisation. The authors hypothesised that the higher levels of non-micellar casein in samples with lower levels of casein mineralisation could impair coagulation of para-casein after enzymatic hydrolysis (Huppertz & Lambers, 2020), similar to effects previously shown for the addition of Na-caseinate on the rennet-induced coagulation of milk (Gaygadhiev, Massel, Alexander, & Corredig, 2012; Lin, Kelly, O'Mahony, & Guinee, 2016). Overall, these findings highlight the importance of casein mineralisation and salt equilibria in nutritional products, such as IF, FOF, jGUM and sGUM. With small difference in salt equilibria having the potential to impact significantly on functional properties, the distribution of the salts in these product classes should clearly be considered beyond purely nutritional needs for salts.

5. Conclusions

This study clearly highlights the differences in salt distribution both between and within classes of nutritional formulae for infants and young children. Protein-associated Ca and Mg are particularly important, because they are directly related to the mineralisation of casein micelles and hence their stability. As the role of Ca and Mg clearly goes beyond direct nutrient provision, not only concentrations, but also distribution of salts in nutritional products should be considered.

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