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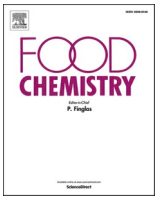

Occurrence of dietary advanced glycation end-products in commercial cow, goat and soy protein based infant formulas

Yajing Xie [a,b], H.J. van der Fels-Klerx [b,*], Stefan P.J. van Leeuwen [b], Vincenzo Fogliano [a]

[a] Food Quality and Design Group, Wageningen University, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[b] Wageningen Food Safety Research, Akkermaalsbos 2, 6708 WB Wageningen, The Netherlands

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ABSTRACT

Thermal treatment is a key step during infant formula (IF) processing which causes protein glycation and formation of dietary advanced glycation end-products (dAGEs). This study aimed to evaluate the glycation degree in IF in relation to the ingredients of the formula. dAGEs concentrations have been determined by UPLC-MS/MS in a range of commercial cow-based, goat-based, and soy-based IF. Results indicated that the protein source, protein composition, and amount and type of carbohydrates determines the level of protein glycation in IFs. The investigated soy-based formula had significant higher concentrations of arginine and arginine-derived dAGEs than cow-based and goat-based formulas. IF containing hydrolyzed proteins had higher dAGEs concentrations than those containing intact proteins. Lactose-containing formula was more prone to glycation than those containing sucrose and maltodextrin. Data showed glycation degree in IF cannot be estimated by a single compound, but the complete picture of the dAGEs should be considered.

1. Introduction

Food industry offers a large variety of infant formula (IF) to meet different nutritional demands for babies and to satisfy market' needs. Cow-based IFs, comprising standard formula, hypoallergenic formula, and low-lactose formula, are the dominantly products on the market. The consumption of goat and soy formulas is increasing in the latest years paralleling the increased interest of consumers towards low allergy or plant-based product.

IFs need to be thermally treated to guarantee aseptic conditions and/or to produce the powdered form which are also convenient for transportation and storage. The heating process, however, will inevitably lead to the formation of thermal processing contaminants, such as Maillard reaction (MR) products (Pischetsrieder & Henle, 2012). The MR occurs between amino groups and carbonyl groups followed by the formation of Amadori products (Yaylayan & Huyghues-Despointes, 1994). Amadori products can be further degraded into Nε-(carboxymethyl)lysine (CML) and dicarbonyls (Hayashi & Namiki, 1986; Nguyen, Van der Fels-Klerx, & Van Boekel, 2014). The modifications of dicarbonyls by lysine residue form CML and Nε-(carboxyethyl)lysine (CEL) (Vistoli et al., 2013); by the modifications from arginine residue, it forms methylglyoxal hydroimidazolones (MG-H), glyoxal hydroimidazolones (G-H), and argpyrimidine. These lysine- and arginine-derived compounds, collectively named as advanced glycation end-products (AGEs), are identified as the key markers of MR (Contreras-Calderón, Guerra-Hernández, & García-Villanova, 2009). Those AGEs specifically present in food items are named dietary AGEs (dAGEs) to distinguish them from endogenous AGEs that are formed in the human body (Delgado-Andrade & Fogliano, 2018).

The MR in IFs is not desirable because of its adverse effect on milk quality. During severe milk processing, about 14–20 % of lysine residues are lactosylated (Henle, Walter, & Klostermeyer, 1991). The glycated lysine is not digestible for infants, hence, the nutritional value of IF and milk protein digestibility are decreased (Mauron, 1990). In addition to the effect on quality, some authors hypothesized that dAGEs might have negative effects on gut microbiota leading to inflammatory bowel diseases (Mastrocola et al., 2020). dAGEs can also be metabolized by gut bacteria leading to the formation of compound such as butyrate having

Abbreviations: 3-DG, 3-deoxyglucosone; IF, infant formula; CEL, Nε-(carboxyethyl)lysine; CML, Nε-(carboxymethyl)lysine; dAGEs, dietary advanced glycation end-products; EFSA, European Food Safety Authority; GO, glyoxal; MR, Maillard reaction; UPLC-MS/MS, ultra performance liquid chromatography with tandem mass spectrometry; G-H, glyoxal hydroimidazolones; MG-H, methylglyoxal hydroimidazolones; MGO, methylglyoxal; MPA, metaphosphoric acid; MRM, multiple reaction monitoring; OPA, orthophosphoric acid; NFPA, nonafluoropentanoic acid; THBQ, tert-butylhydroquinone; SPE, solid phase extraction.

* Corresponding author.
E-mail address: ine.vanderfels@wur.nl (H.J. van der Fels-Klerx).

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positive outcomes on the intestine health (Bui, Troise, Fogliano, & de Vos, 2019; Bui et al., 2020). Despite these controversial hypothesis related to the effects of dAGE on human health, there is a general consensus to mitigate the presence of dAGEs in IF, to improve the nutritional value and the overall product's quality.

Studies performed till to date have provided important information on dAGEs occurrence in IFs, but data gaps related to its formation and presence still remain. dAGEs are a diverse group of compounds including lysine-derived molecules and arginine-derived molecules. This implies that chemical analytical methods should not only include CML as the primary dAGE target but also related dAGEs molecules to accurately understand the glycation degree in IFs. The MR in IFs is recipe- and processing-dependent, which means products with different formulation will have a different development of MR during processing. To the best of our knowledge, previous studies only assessed the glycation degree in cow-based formulas (Contreras-Calderón et al., 2009; Delatour et al., 2009; Fenaille et al., 2006); dAGEs data in goat-based and soy-based formulas have not been reported yet. It is expected that the MR extent in these products might vary greatly due to the differences in amino acid compositions, protein reactivity and overall formula composition.

The objective of this study was to investigate the presence of MR products in different types of commercially available IFs, and to obtain insights into the recipe effects on the presence of MR products. The investigated samples in this study included cow-, goat-, and soy-based formulas. A variety of dAGEs were analyzed using LC-MS/MS to accurately quantify the various dAGEs to fill the above-mentioned data gaps.

2. Materials and methods

2.1. Chemicals and reagents

Chloroform, HPLC grade; methanol, HPLC grade; metaphosphoric acid (MPA); boric acid (99.5 %); sodium borohydride (96 %); orthophosphoric acid (OPA); nonafluoropentanoic acid (NFPA, 99 %); formic acid, HPLC grade; acetonitrile, HPLC grade; ammonium formate; D-Methionine, HPLC grade; cellulose; L-Ascorbic Acid; tert-butylhydroquinone (THBQ); and sodium hydroxide (98 %), all reagents and chemicals upon were obtained from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q water purification system (ELGA Veolia, UK). Analytical standards of lysine, arginine, CML, furosine, CEL, G-H1 (TFA salt), MG-H1 (TFA salt), and argpyrimidine (TFA salt), together with the deuterated internal standards lysine-d4, CML-d4, furosine-d4, CEL-d4, G-H1-$^{13}C_2$, and MG-H1-d3 (TFA-salt) were purchased from Iris Biotech (Marktredwitz, Germany). Lysine was prepared to 500 mg/L, 100 mg/L and 10 mg/L in Milli-Q water. Arginine was prepared to 50 mg/L, 10 mg/L and 2 mg/L in Milli-Q water. Furosine was dissolved in Milli-Q water to obtain 5 mg/L, 1 mg/L and 0.1 mg/L solutions. Lysine-d4 and furosine-d4 were mixed in Milli-Q water to reach a concentration of 1 mg/L for each compound. CML, CEL, G-H1, MG-H1, and argpyrimidine were mixed to 1 mg/L and 0.1 mg/L by Milli-Q water. CML-d4, CEL-d4, G-H1-$^{13}C_2$ and MG-H1-d3 were dissolved into Milli-Q water to reach a final concentration of 0.1 mg/L for each compound.

2.2. Samples

Fourteen IF products were purchased online or from local supermarkets. The products included seven different IF types, including standard adapted IFs for babies under six months of age (anonymously named as A1-A3), standard follow-on formulas for babies over one year old (F1-F3), hypoallergenic IFs (HA1-HA2), low-lactose IF (LL), liquid follow-on formulas (L1-L2), goat-based IFs for babies under six months of age (G1-G2), and soy-based formula for babies more than one year old (Soy). For each product, boxes from three different batches were collected, whenever possible, in order to eliminate batch effects and to cover natural variation. All samples had to fully fall within the same three-month production date of March – June 2020 to allow making results comparable among different batches and products. However, given their low production frequency, A2, F2, F3, and HA2 products could only include two samples (batches) in the considered time period; G1, G2, and Soy products only included one sample (batch) with production dates of November 2019, January 2020, and September 2020, respectively. All boxes were left unopened and stored in a 4 °C climate room until further processing and chemical analysis. To mimic the use of the low-lactose IF in practice, we mixed LL formula with A1 formula according to the instructions on the label of the LL product just before the analysis.

The recipe and processing information of each IF product that had been bought was collected from the product labels, official websites of the corresponding IF producing company, and literature which analyzed the compositions of the selected IF in their study. Three IF companies contributed to this study by providing the requested information on their respective products. The collected information primarily focused on whey to casein ratio, carbohydrate compositions and concentrations, protein hydrolysis degree for hypoallergenic IFs, and iron concentration. Unfortunately, processing information of the IF products, such as the heat treatment intensity, could not be obtained due to company confidentiality.

2.3. Methods

2.3.1. Total protein determination

Total protein content was measured by the DUMAS method by using a Flash EA 1112 Protein Analyzer from Thermo Fisher Scientific (USA). The protein analyzer measured nitrogen concentration in samples, the value of which was converted to protein content with a conversion factor. The conversion factor used in this study was 6.38 for cow-based and goat-based formulas, and 5.69 for soy-based formula (World Health, Food, & Agriculture Organization of the United, 2019). For powdered products, 9 mg samples were weighed by microbalance (Mettler-Toledo, XA105DU), followed directly by the DUMAS measurement. For liquid products, 200 μL of samples were weighed and dried overnight until dry material remained in a 60 °C incubator prior to measurement. Methionine was used as standard for quantification, and cellulose was used as blank sample.

2.3.2. Acidic hydrolysis of proteins

The amino acids and MR products were quantified by liquid chromatographic (LC) and mass spectrometry (MS) instrumental methods (e.g. LC-MS/MS) after acid hydrolysis. The detailed procedure was described elsewhere (van der Lugt et al., 2020). Briefly, 0.75 g IF powders were reconstituted into Milli-Q water at room temperature and to make a total volume of 5 mL. 30 μL samples were mixed with 300 μL of boric acid (0.33 M) and 27 μL of sodium borohydride (2 M) solution and reacted for three hours at room temperature to prevent the overestimation of the CML concentration. The above emulsion was defatted by addition of 1 mL of chloroform:methanol (v:v, 2:1) and vortexing for 1 min. The tubes were then centrifuged for 10 min at 3000 rpm. The chloroform layer was discarded. Afterwards, the sample was hydrolyzed by 1 mL concentrated HCl at 110 °C for 18 h. The hydrolysate was evaporated to a dry residue at 50 °C under nitrogen flow and dissolved in 1 mL Milli-Q water for further measurement.

2.3.3. dAGEs determination

Fifty μL of the above extract was pipetted into a HPLC vial and mixed with 100 μL 0.1 mg/L dAGEs internal standard mixture obtaining CML_d4, CEL_d4, MG-H1_d3, and G-H1_13C2, and 50 μL Milli-Q water, after which the sample was ready for dAGEs analysis. The dAGEs were measured by QTRAP 6500 ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, Sciex, MA, USA), equipped with a positive electrospray ionization (ESI+) interface.

Chromatographic separation was achieved on a BEH C18 column (100 × 2.1 mm, 1.7 μm, Waters Corporation, Milford, MA, USA). The UPLC-MS/MS method was applied as previously by van der Lugt et al. (2020) (apart from the column temperature which was 50 °C in our study). The precursor-product ion multiple reaction monitoring (MRM) transitions were presented in Table S1. Calibration of CML, CEL, G-H, and MG-H was done with the respective standard and the deuterated or 13C labelled internal standard. For argpyrimidine, no mass labeled internal standard is available and therefore we used CML-d4 as their internal standard to calculate the final result. Data were acquired and processed with the Multiquant 3.0.2 software (Sciex, MA, USA). For those products having three different batches, singular chemical analysis was conducted for each analyte in each sample. Those products for which two samples (from two different batches) were available, analytical duplicates were conducted for each sample. For the goat-based and the soy-based products, one sample was collected per product, therefore analytical triplicates were given per sample.

The accuracy of the dAGEs data was evaluated by spiking the selected samples with dAGEs standard mixture and by making calibration curves in water and in an IF matrix. Those selected samples were A1, HA1, L2, G2, and Soy, together covering most of the IF types. The recovery of the spiked dAGEs fell in the range of 72–105 %. The linearity of calibration curves ranged from 0.992 to 1.000. Identification of the dAGEs in the samples relied on chromatographic retention times, the MRMs and the ion ratio of the two product ions relative to those observed in the standards. All deviations of ion ratio from standards calibration were acceptable (within 30 % deviation compared to the ratio observed in the standards), whereas deviations of ion ratio were observed for G-H1 which may be explained by the presence of co-eluting isomers of G-H1 (G-H2 and G-H3) (unpublished data).

2.3.4. Lysine, arginine, and furosine determination

Fifty μL of the hydrolyzed protein extract (paragraph 2.4.3) was mixed with 100 μL 1 mg/L deuterated internal standards lysine-d4 and furosine-d4, and 50 μL Milli-Q water before being purified by solid phase extraction (SPE) method (Delatour et al., 2009). The SPE method was carried out with an equilibrated Oasis HLB 1 cc cartridge (Waters Corporation, Milford, MA, USA). Briefly, the cartridge was washed and activated in sequence of 1 mL methanol, 1 mL of 10 mM NFPA, and 1 mL of 10 mM NFPA:methanol (v:v, 95:5). Two hundreds μL of the extract was then loaded onto the cartridge. The fractions containing lysine, arginine, and furosine were eluted with 2 mL of 10 mM NFPA:methanol (v:v, 50:50), followed by the evaporation step under nitrogen flow using a heating block at 50 °C. The dry residue was dissolved with 200 μL Milli-Q water, centrifuged at 800 × g, and the supernatant was transferred into a HPLC vial.

The SPE efficiency was analyzed by the comparison of peak areas of a standard mixture and internal standard mixture with and without using SPE. Triplicates were performed to calculate the recovery rate for each compound. The SPE cartridge was eluted by 3 sequential volumes of 1 mL 10 mM NFPA:methanol (v:v, 50:50) and the fractions were collected in Greiner tubes for further LC-MS/MS measurement. The peak area of the sample without SPE and the peak area of each eluted fraction were calculated, the ratio of which was used to estimate the recovery rate of each compound. The data showed that the standards eluted entirely in the first fraction. Next, the elution pattern was checked in real food matrix (i.e. infant formula sample).

The matrix effect on SPE efficiency was evaluated by comparing the peak area of sample extracts with and without using SPE (Fig. S1). The recovery rate of each compound were acceptable after two times eluting, ranging from 79 to 85 %. Therefore, 2 mL of 10 mM NFPA:methanol (v:v 50:50) was applied in SPE procedure.

Total lysine, arginine, and furosine concentrations were measured by LC-MS 8050 triple quadrupole mass spectrometer (Shimadzu, Japan), equipped with a ESI+ interface. A Kinetex HILIC column (100 × 2.1 mm, 5 μm, Merck KGaA, USA) was employed for separation of the analytes. The mobile phases, gradient, UPLC, MS/MS conditions, and data quality control were set up as previously reported by Zhang et al. (2021). The selected reaction monitoring mode for these compounds was shown in Table S2. Data were processed with LabSolution software (Shimadzu, Japan).

Data accuracy on lysine, arginine, and furosine was evaluated by spiking each selected sample with lysine and arginine mixture and by making calibration curves in water and in an IF matrix, as mentioned above. The linearity of calibration curve was 1.000, and 0.993 for lysine and arginine. The recovery fell in the range of 80–122 %. All deviations of ion ratio from standards calibration were acceptable (within 30 % deviation compared to the ratio observed in the standards). Data quality for furosine was assessed by making calibration curves in water and in an IF extraction. The linearity of calibration curve in water and in extraction was 1.000 and 0.999, respectively. The slop of the calibration curve in water and in extraction was comparable. The recovery rate that was calculated from extract calibration ranged from 74 to 96 %.

2.3.5. Ascorbic acid determination

The ascorbic acid concentration in each IF sample was analyzed by HPLC after defatting steps. The details on sample preparation, HPLC setting, and results (Fig. S3) are shown in the supplementary materials.

2.4. Statistical analysis

The average concentration of each analyte in each product was calculated from the product replicates. Statistical analysis of the differences in analyte concentration among IF products was performed by one-way ANOVA method and Duncan's test using the SPSS software (version 25). The differences were considered significant when $p < 0.05$. Statistical analysis on arginine, MG-H, and argpyrimidine was performed without the data of soy-based formula because of the high concentrations (see Fig. 5) affecting the statistical analysis of the remaining IFs. Likewise, data for soy-based formula and HA2 formula were removed when analyzed the significance of G-H among formula products.

3. Results and discussion

3.1. Study on different commercially available product formulations

The basic information of each included IF product and the components that may contribute to the formation of dAGEs is summarized in Table 1. From the data in Table 1, it can be seen that the whey to casein ratio was adjusted from 20:80 in raw milk to 60:40 in adapted IFs and to 40:60 in follow-on formulas. The included liquid formulas were not added with whey protein concentrates and, therefore, retained the original cow milk ratio of 20:80. The hypoallergenic IFs of this study had a whey protein-based recipe. Whey protein dominated formulas are beneficial for infants to digest the milk (Martin, Ling, & Blackburn, 2016), but on the other hand, they are more susceptible to MR during manufacturing due to the higher lysine content and lysine molar content of whey proteins as compared to caseins (Cardoso, Wierenga, Gruppen, & Schols, 2018).

The lactose concentration of the powdered formulas A2, F2, and HA2 was higher than that of the other powdered formulas. In addition to lactose, a small portion of glucose was present in A1 and A3 formula, and some maltodextrin was present in F1 and F3 formula. A large difference in sugar contents was observed in different liquid samples: L2 had a very low concentration of sugars respect to L1. The total sugar concentrations in goat-based IFs were similar to that in cow-based IFs, and no additional sugars beyond lactose were added. The composition and concentration of sugar in soy-based formula was different from the animal milk-based formulas. Sugar in soy-based formula mainly covered sucrose, in a low amount of 2.5 g/100 mL. The sugar formulation had a major influence on the MR development: disaccharides, especially

Table 1
Infant formulas key ingredients and overall composition. The data for LL formula was calculated from a mixture of A1 and LL formula according to the instruction of LL formula. NA: not available or not applicable.

Brand (anonymized)	Infant formula (anonymized)	Protein source	Infant formula type	Whey to casein ratio (%)	Sugars (g/100 mL milk)	Lactose (g/100 mL milk)	Other sugars (in per 100 mL milk)	Iron (mg/100 mL milk)
1	A1	cow protein	standard adapted infant formula [a]	50: 50	7.3	7.0	0.2 g glucose	0.53
2	A2	cow protein	standard adapted infant formula [a]	60: 40	8.2	8.2	0	0.31
3	A3	cow protein	standard adapted infant formula [a]	63: 37	7.0	6.8	0.2 g glucose	0.40
1	F1	cow protein	standard follow-on formula [b]	NA	6.6	6.1	0.5 g maltodextrin	1.2
2	F2	cow protein	standard follow-on formula [b]	40: 60	9.0	9.0	0	1.2
3	F3	cow protein	standard follow-on formula [b]	35: 65	5.8	5.6	0.2 g maltodextrin	1.2
1	HA1	cow protein	hypoallergenic infant formula	whey proteins-dominated formula. All proteins are hydrolyzed into larger-than-dipeptide size [c]	7.1	6.9	0.2 g glucose	0.53
2	HA2	cow protein	hypoallergenic infant formula	whey proteins-dominated formula [c]	7.8	7.8	0	0.69
1	LL	cow protein	low-lactose infant formula	50 %: 50 %	5.4	5.1	0.17 g glucose, 0.11 g maltose, around 1.45 g maltodextrin	0.6
1	L1	cow protein	liquid formula	20 %: 80 %	8.4	8.1	sugars from milk flavor	1.2
2	L2	cow protein	liquid formula	20 %: 80 %	2.7	NA	maltodextrin, sugars from milk flavor	1.3
4	G1	goat protein	goat-based infant formula	63 %: 37 %	6.9	6.9	0	0.50
5	G2	goat protein	goat-based infant formula	NA	7.3	7.0	0	0.70
6	Soy	soy protein	soy-based formula	NA	2.5	0	0.1 g glucose, 0.7 g fructose, 1.7 g sucrose, maltodextrin	2.1

[a] Adapted infant formula are intended for babies under six months of old.
[b] Follow-on formula are intended for babies over one year old.
[c] The whey to casein ratio is not available.

lactose, are less active than monosaccharides; sucrose is a non-reducing sugar and cannot react with proteins unless is hydrolyzed; maltodextrin has the lowest reactivity amongst these sugars (Ames, 2003; Contreras-Calderón et al., 2009).

The presence of iron in IFs promotes the occurrence of MR, especially when ascorbic acid is simultaneously present. Iron acts as a catalyst during MR. It can accelerate the oxidation of lactose into dicarbonyls and can accelerate the oxidation of ascorbic acid into L-threose participating in glycation (Leclère, Birlouez-Aragon, & Meli, 2002). The latter reaction is assumed to be more predominant. In our study, soy-based formula had the highest iron concentration. Follow-on formulas had an over-two-times higher iron concentration than adapted IFs. Iron is crucial for the growth of infants. EFSA (2014) recommended no less than 0.4 mg/100 mL of iron for cow/goat-based adapted IFs, 0.9 mg/100 mL for cow-based follow-on formulas, and 1.4 mg/100 mL for soy-based follow-on formulas. Iron concentration in all sampled products complied to this minimal requirement.

3.2. Total protein concentrations

Fig. 1 presents the concentration of proteins in all samples. The total protein content was compared with the concentration reported on the nutrition fact label of each product. Only small differences were found between the measured data and those indicated on the product label, noticeably the values on the label were slightly higher than the measured ones for most products. Soy-based formula had a significantly higher protein content than cow-based and goat-based formulas. This is in accordance with the advice of the European Food Safety Authority (EFSA) to increase the content of total proteins for plant-based formula as the plant proteins are less digestible than the mammalian milk proteins (EFSA, 2014). F2 formula showed the lowest protein concentration among all products ($p < 0.05$). Liquid formulas contained higher protein concentration than powdered IFs regardless of formula type or protein source.

3.3. Protein profiles in IFs

The amounts of lysine and arginine calculated on milk basis and protein basis are reported in Fig. 2. As expected, different milk protein sources resulted into different amino acid compositions; the concentrations of lysine and arginine in different types of IFs were mostly in line with the levels of the respective protein contents when calculated on milk basis. The lysine and arginine concentration in IFs was 97.5–204 mg/100 mL milk (69.6–136 g/kg protein) and 36.7–307 mg/100 mL milk (27.0–151 g/kg protein), respectively. Based on our data and from the nutritional perspective, infants can ingest remarkably higher lysine and arginine from soy-based formula as compared to mammalian milk-based formula (given the same volumes consumed). The higher amino acids in soy formula can be explained by its higher protein content in the recipe. Cow-based and goat-based formulas contained two-times more lysine residues than arginine, while soy formula contained higher arginine residues than lysine. Notably, the arginine concentration in soy

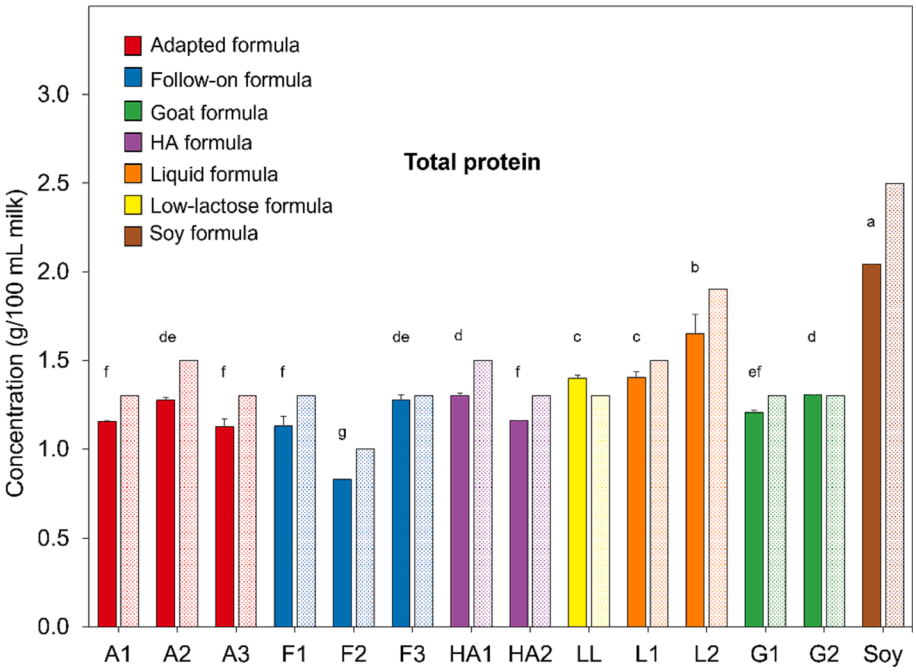

Fig. 1. Total protein concentration in different types of infant formulas. The full colored bar on the left is the measured protein concentration; the shaded bar on the right is the protein concentration reported on the nutrition fact label of each infant formula. Different letters indicate significant differences ($p < 0.05$).

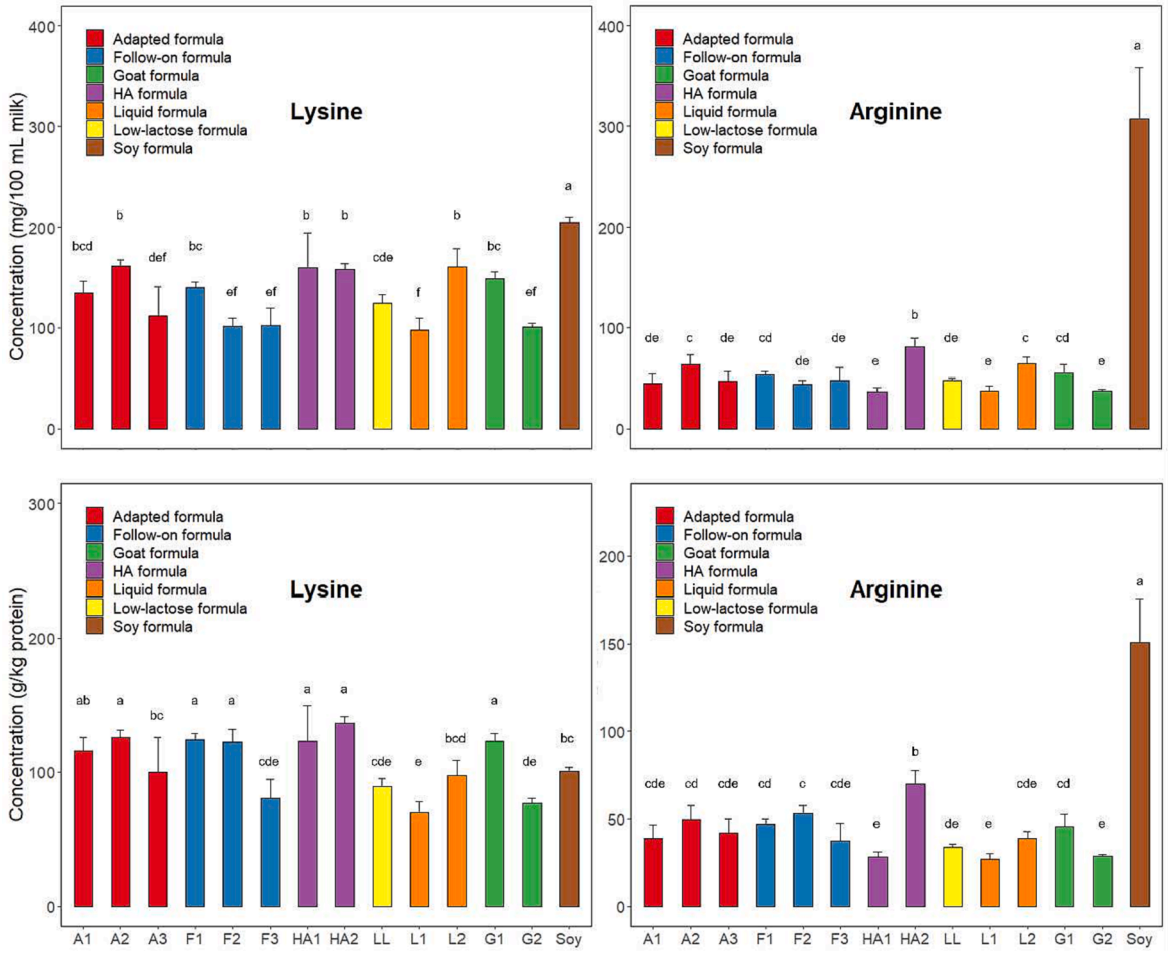

Fig. 2. Lysine and arginine concentrations in per 100 mL milk and in per kg proteins in infant formulas. Different letters indicate significant differences ($p < 0.05$).

formula was 307 mg/100 mL milk which was 2.8–7.4-fold higher than the concentration in cow/goat-based formulas.

Lysine and arginine expressed on a protein basis showed a slightly different pattern than when expressed on a milk basis. Soy protein had relatively lower lysine and a significant higher arginine content as compared to cow/goat milk protein. Lysine concentrations in mammalian milk proteins are influenced by the ratio of whey proteins to caseins in their protein profile, as explained in section 3.1. F3 formula had comparable total proteins as A2 and HA1 formula, but the lysine concentration in F3 was statistically lower; Liquid formulas had higher total protein contents but lower lysine contents than powdered formulas. Arginine concentration was the highest in the soy formula, followed by HA2 formula. It did not show much differences among the remaining products.

Processing effects on protein glycation could be visible looking at goat IFs: G1 had a lower protein but a higher lysine concentration as compared to G2. We speculate that heating procedures during the production of G2 could be more intensive and harsher than that of G1 production, which blocks more lysine and forms more furosine, as indicated in Fig. 3.

Our data regarding the presence of lysine and arginine residues that were measured after acid hydrolysis are in accordance with the earlier reported data in IFs (Xie, van der Fels-Klerx, van Leeuwen, & Fogliano, 2021). EFSA (2014) indicated that lysine should be higher than 63 g per kg protein for adapted IFs and follow-on formulas regardless of the protein source used. All measured lysine contents in the studied products were above this threshold. Amino acid lateral chains of milk proteins participate through the entire MR in the presence of reactive carbonyl groups. Münch et al. (1999) proposed that lysine residues are more reactive in the formation of protein-bound dAGEs than arginine residues and other N-terminal amino acids. The authors explained this as the side chain of amino acid is easier to react with sugars when its α-amino group is easier to approach. MR forms a large number of lysine derivatives and arginine derivatives during milk processing. Therefore, the compositions of lysine and arginine in the recipe directly influence the amounts and varieties of dAGEs molecules formed during processing. Lysine has been commonly targeted to study MR in IFs by either evaluating the lysine blockage extent or the available lysine contents. However, the effects of arginine contents on the occurrence of MR were not taken into account: only one study quantified dAGEs in IFs together with arginine concentrations (Akıllıoğlu & Lund, 2022).

3.4. Furosine concentration

As shown in Fig. 3, furosine concentrations varied largely among the IFs containing different protein sources, ranging from 152 to 2750 mg/kg protein. Soy-based formula had an equivalent amount of furosine as liquid formula and it is 90 % lower than the furosine in powdered formulas ($p < 0.05$). Goat-based formulas (2252–2750 mg/kg protein) had the highest furosine concentrations among all products. Among the cow-based formulas, the highest furosine concentration was observed in HA2 formula, while the lowest furosine concentration was seen in liquid formulas.

Results on furosine concentrations from the current study are in line with the data from previous studies, as recently reviewed by Xie et al. (2021). To give a more precise comparison of the MR extent across studies, scientists need to further convert furosine concentration into the concentration of individual Amadori products according to the acid molar concentration used in their study (Krause, Knoll, & Henle, 2003). Data variation among the studies may be caused by the reduction step and/or the efficiency of acidic hydrolysis and/or different analytical techniques (Aktag, Hamzalioglu, & Gokmen, 2019; Troise, Fiore, Wiltafsky, & Fogliano, 2015).

Chen et al. (2019) and Penndorf et al. (2007) reported higher furosine concentration in HA formulas as compared to the IFs containing intact proteins. The same finding was also observed in our study. The only difference between the composition of HA formulas and adapted IFs in our study is the degree of protein hydrolysis. Adapted IFs had intact proteins, whereas HA formulas consisted of oligopeptides. Peptides are more reactive to MR than intact proteins and this contributed to the higher furosine concentration in HA formulas. Fenaille et al. (2006) observed lower furosine concentrations in HA formulas, in contrast to our findings. However, the authors also agreed that MR is more advanced in HA formulas than in standard IFs. They explained that

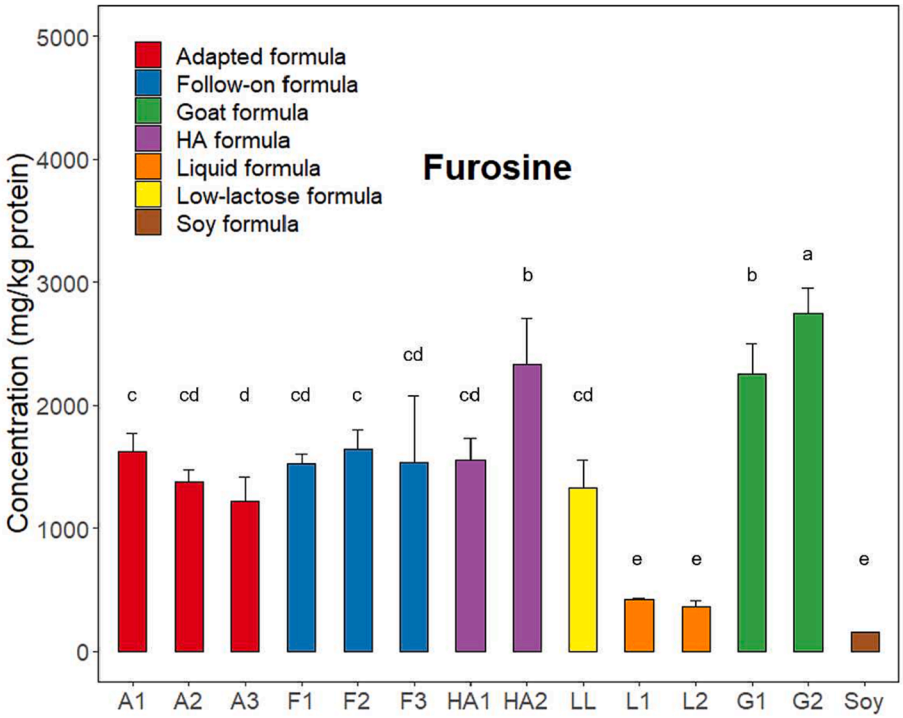

Fig. 3. Furosine concentrations in different types of infant formulas. Different letters indicate significant differences ($p < 0.05$).

furosine in their HA formula was further converted into advanced products, resulting in a lower furosine present at the end. Indeed, our study confirmed this hypothesis when CML data are considered together with those of furosine (see later). From our data, furosine concentration in HA1 formula did not show a significant difference compared to standard IFs possibly because furosine from HA1 formula was further degraded into CML and other dAGEs and this led to less furosine remaining in HA1 formula. This revealed that, to draw a full picture of glycation progress in food items, it is essential to perform a comprehensive analysis involving the quantitation of as many dAGEs molecules as possible.

The liquid formulas in our study showed a significantly lower furosine concentration as compared to powdered IFs, which is in line with the studies by Akıllıoğlu et al. (2022) and Fenaille et al. (2006). Powdered IF is reported to have a stronger early glycation degree than liquid formula because of its low water activity (Van Boekel, 1998; Xie et al, 2021). Additionally, it could be explained by the significantly lower lysine in the protein of liquid formulas, as suggested by Fig. 2. Therefore, less formation of lactosyllysine is expected in liquid samples. The lower furosine in L2 formula can also be partly due to the much lower sugar concentration in its recipe.

To date, the presence of MR products has rarely been investigated for either goat-based milk or goat-based IF. Schwarzenbolz et al. (2016) measured free fructosyllysine in milk from different animals. Results indicated a lower glycation degree in goat's milk as compared to that in cow's milk. In contrast, data reported in Fig. 3 showed that goat-based IFs had significantly higher furosine concentrations than cow-based formulas that having a similar formulation. The difference in findings might be related to a different storage time of our goat-based products and those previously investigated.

Only a few studies have investigated the MR products in soy milk and no one looked at those designed for infants (Amigo-Benavent, Villamiel, & del Castillo, 2007; Rada-Mendoza, Villamiel, Ramírez, Usuriaga, & Montilla, 2021). Our furosine data on soy-based formula agree well with the data on soy drink from Amigo-Benavent et al. (2007) and Rada-Mendoza et al. (2021), who observed 256 mg furosine per kg proteins in soy milk, and 127–251 mg furosine per kg proteins in soy beverages, respectively. Rada-Mendoza et al. (2021) also measured furosine in cow's milk and also observed that soy milk contained a much lower furosine concentration than cow's milk. This is most likely due to the lower sugar content in soy-based formula in our study.

3.5. Lysine-derived glycation products

CML and CEL are lysine-derived dAGEs and their concentrations in IFs are largely related to lysine amounts. The results are shown in Fig. 4. HA formulas had the highest amounts of CML (223–281 mg/kg protein), whereas LL formula showed the lowest CML concentrations ($p < 0.05$). No significant difference was found for CML concentrations between liquid and follow-on formulas, suggesting the different physical forms of IF may not influence the advanced stage of glycation. Comparing CML concentrations among the liquid formula consisting of different protein sources, we found that goat-based IFs obtained a significant higher CML than cow-based and soy-based formulas, which is in line with the observed furosine data in Fig. 3.

Data regarding the concentration of CEL in our sampling are summarized in Fig. 4 and they showed that CEL data have a different pattern than CML among different IF products. The CEL concentration in soy-based formula was 2.8–13.9 times higher than that in the other samples. This is unexpected as the soy-based formula had a comparable lysine concentration as the other formulas. The reason for this different behavior is unknown and we suspect that dicarbonyls, particularly the presence in the product of methylglyoxal (MGO), played a vital role in this case. A3 and HA2 formulas had a relatively low CEL concentration, while L2 had a higher CEL concentration among cow-based formulas ($p < 0.05$). In general, the CEL concentrations in the studied samples were lower than CML concentrations regardless of the IF type, physical form, and protein source, except for the soy-based formula in which the concentrations were in the same range.

CML and CEL concentrations in cow-based formulas of our study are consistent with those observed in most of the previous studies (Xie et al., 2021) with few exceptions likely due to a different sample preparation protocol (e.g. microwave hydrolysis, GC–MS) (Akıllıoğlu & Lund, 2022; Charissou et al., 2007). It should be noted that the samples used in different studies are produced in different conditions, such as different storage time and environment, ingredient compositions, shelf life and production dates of the samples, etc. This hampers an exact comparison across different studies.

Our results indicated a higher glycation reactivity of peptides vs intact proteins, which was particularly clear from the CML concentrations. This finding has widely been observed and is supported by previous studies (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Chen et al., 2019; Delatour et al., 2009; Fenaille et al., 2006). LL formula in our study had the lowest CML concentration, suggesting a low extent of MR progress. A similar result was also observed in the study of Delatour et al. (2009) in which the lowest CML level was found in hydrolyzed

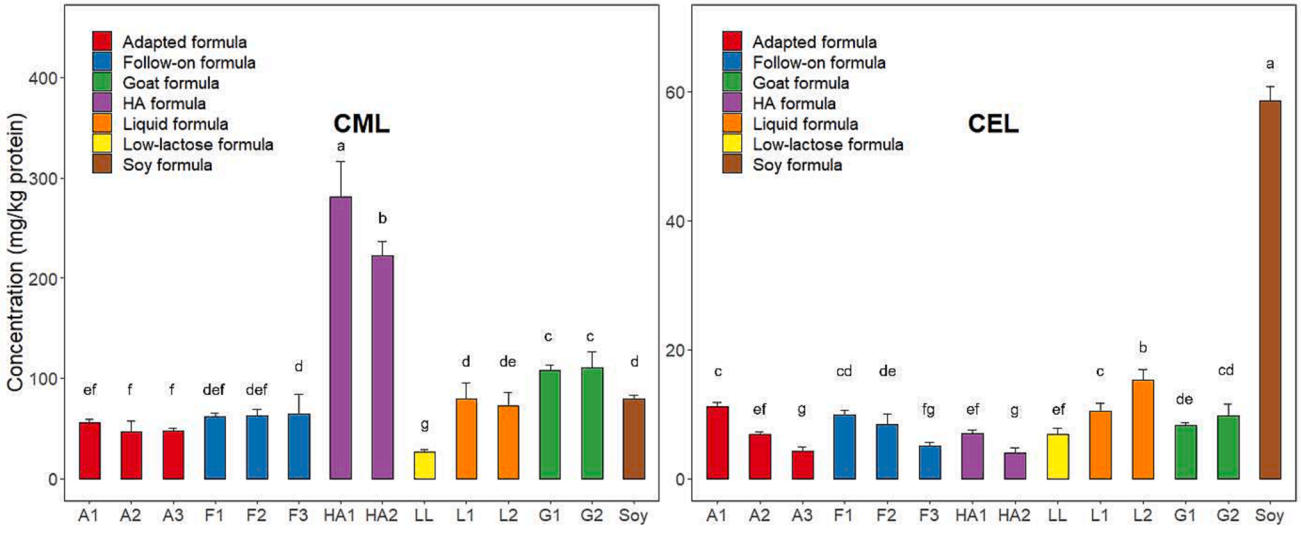

Fig. 4. Lysine-derived dAGEs concentrations in different types of infant formulas. Different letters indicate significant differences ($p < 0.05$).

lactose-free IFs regardless of the physical form. The main factor to explain these results is the carbohydrate source: the LL formula analyzed in our study was produced primarily with maltodextrin and little lactose; maltodextrin is less prone to MR than lactose. As reported by Xie et al. (2021), powdered IFs tend to have a higher amount of furosine and an equivalent amount of CML as compared to liquid formulas because of the spray drying processing. However, no solid conclusion can be drawn due to the limited data from liquid formulas. In the current study further confirmed that powdered IFs have higher furosine and equivalent CML levels compared to liquid formulas. The heat load due to the processing needed to obtain powdered IFs may influence more the early stage of protein glycation than the advanced stage. Powdered IFs could be more susceptible to the early glycation reaction and mitigation of the protein glycation for powdered IFs should focus more on the early stage (i.e. the formation of Amadori products). This hypothesis requires confirmation by other studies.

Glycation in goat IF/milk has been studied by Prosser et al. (2019) and Schwarzenbolz et al. (2016). Prosser et al. (2019) observed that goat-based IFs had significantly lower CML concentration than cow-based IFs. Schwarzenbolz et al. (2016) investigated the glycated proteins in goat milk and cow's milk with free CML as the indicator. They also found goat milk contained a slightly lower level of free CML than cow's milk. An opposite result was seen in the current study since the goat-based IFs showed statistically significant higher CML than standard cow-based formulas. This could be attributed to a longer storage time (shelf life) as G1 and G2 was produced 2–6 months earlier than the other products. However, because of the limited set of samples in the present study, more research is needed to explore the potential difference of glycation patterns in cow-based IF and goat-based IF. To the best of our knowledge, no assays have been conducted on the presence of dAGEs in soy-based formulas/milk.

Our study firstly reported CEL concentration in a large variety of IFs involving cow-based, goat-based, and soy-based products. The formation of CEL is associated with the occurrence of dicarbonyl compounds, particularly of MGO (Vistoli et al., 2013). CEL concentrations in IFs were lower than CML, which is consistent with previous studies (Aktag et al., 2019; Li, Zhang, Gao, Lai, & Dong, 2021; Li et al., 2021; Troise et al., 2015). This is explained by the fact that CML can be formed from either the oxidation of Amadori products, or the modification of glyoxal (GO) or 3-deoxyglucosone (3-DG), whereas the formation of CEL relies only on MGO reaction on N-amino terminal group of lysine. A recent study detected CML and CEL in powdered IF with 14 mg/kg protein and 74 mg/kg protein, respectively (Akıllıoğlu & Lund, 2022). However, the authors did not apply a reduction step for the CEL measurement, which

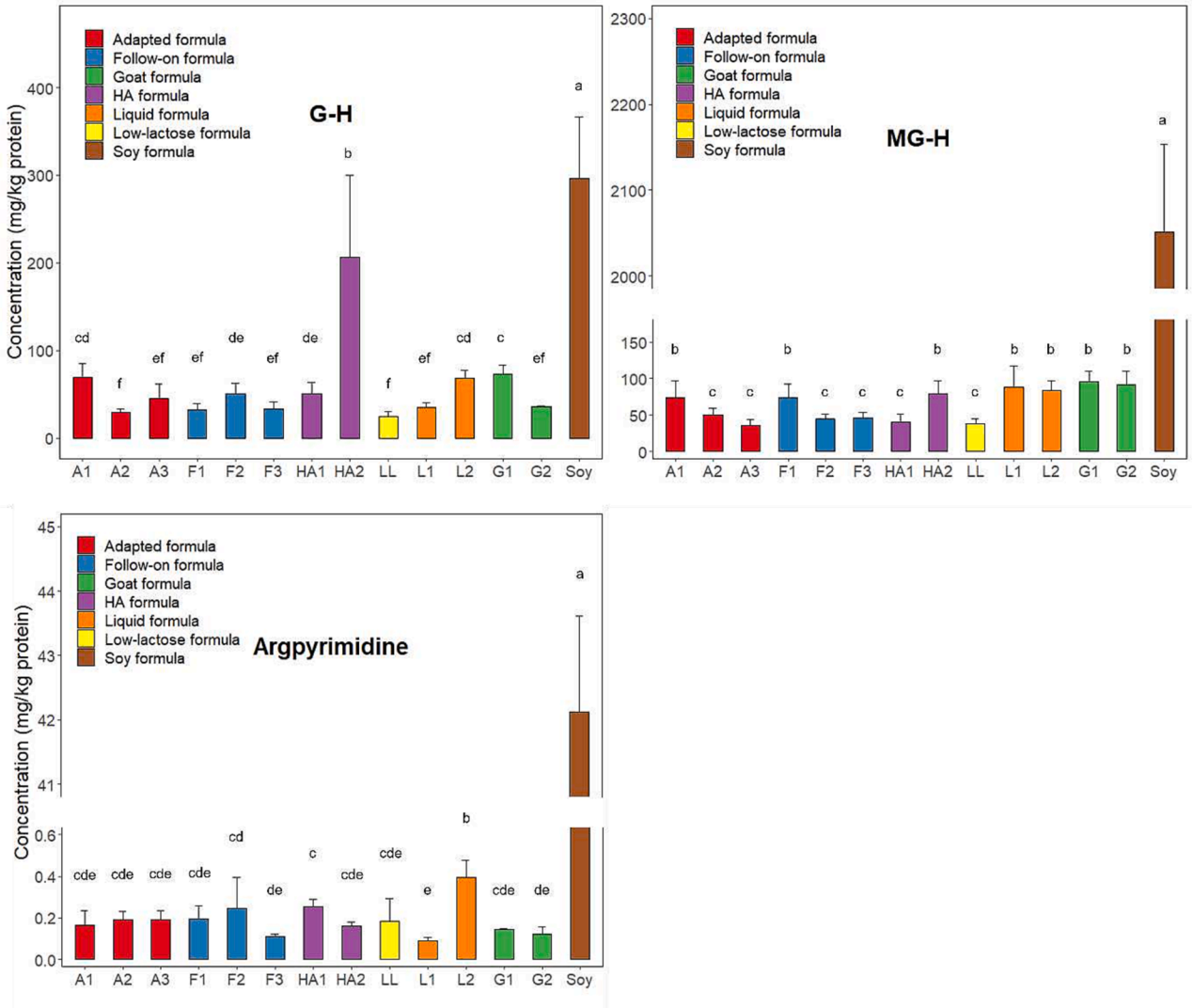

Fig. 5. Arginine-derived dAGEs concentrations in different types of infant formulas. Different letters indicate significant differences ($p < 0.05$). The statistical analysis of G-H concentration was calculated by SPSS without the consideration of Soy and HA2 formulas. The statistical analysis of MG-H and argpyrimidine concentration was calculated by SPSS without the consideration of Soy formulas.

may have caused the higher CEL than CML concentration in their study.

3.6. Arginine-derived glycation products

The concentrations of arginine-derived dAGEs comprising G-H, MG-H, and argpyrimidine are shown in Fig. 5. G-H concentrations in cow-based, goat-based, and soy-based formulas were 25–207, 35–73, and 296 mg/kg protein, respectively. Soy-based formula showed the highest G-H concentration, followed by HA2 formula. The remaining IFs showed less variation in G-H concentrations. MG-H in cow-based formulas ranged from 36 to 88 mg/kg protein, a lower level as the goat-based formulas (91–96 mg/kg protein). A two magnitude orders higher MG-H concentration was observed in soy-based formula (2051 mg/kg protein). Argpyrimidine presented a low-level amount in IFs in general, being 0.11–0.40 mg/kg protein in cow-based formulas, 0.12–0.15 mg/kg protein in goat-based formulas, and 42 mg/kg protein in soy-based formula.

Akıllıoğlu and Lund (2022) reported a trace amount of G-H and 33–34 mg/kg protein of MG-H in IFs, which are in line with the data from our study. The soy-based formula included in this assay was richer in arginine than the other IFs, which explains the significant higher G-H, MG-H, and argpyrimidine concentrations in the soy-based formula.

Up to date, some studies used CML as general indicator for dAGEs formation, whereas the role of arginine-derived dAGEs in processed foods was largely underestimated. Our results revealed that CML is one of the many dAGEs in food items and rarely the surely not the most abundant in IF. The pattern of relevant dAGEs in each products depends on the compositions of amino acids in the food matrix and, even in the same category, such as in the IF sampling investigated in this paper, different AGEs can become dominant. This was also stated by Akıllıoğlu and Lund (2022) who concluded that one indicator cannot represent the extent of glycation and protein damage in foods. In the current study, furosine is the most dominant dAGE in mammalian-based milk formulas, followed by CML, G-H and MG-H. In soy-based formula furosine is almost absent whereas MG-H is the most abundant dAGE, followed by G-H.

4. Conclusions

This study investigated a wide range of dAGEs compounds in different types of IFs, along with nutritional factors such as protein, arginine, lysine, and ascorbic acid contents. Results substantially add to the existing knowledge about dAGEs in IFs and effects of the starting ingredients on dAGEs formation, especially for plant-based IFs. Collected data provide a good overview to evaluating the quality and safety of IFs regarding the dAGEs occurrence.

The formation of dAGEs in IFs is greatly influenced by proteins and carbohydrates in the recipe. Peptide-based IF is more prone to glycation than IF containing intact proteins. Soy-based formula showed an unexpectedly high amount of CEL and more mechanistic studies should be carried out to be able to explain this. After thermal processing, soy proteins are dominated by arginine-derived dAGEs, whereas cow's proteins and goat proteins are dominated by lysine-derived dAGEs. As expected, sucrose and maltodextrin in IF recipe are less active MR ingredients than lactose. Powdered IFs have a higher concentration of the Amadori products (i.e. furosine) but lower concentration of the other dAGEs compared to liquid formulas. This again highlights the possibility to fine tuning the MR progress in the various food product categories. In addition, our data provide further evidence CML is not fully representative for all dAGEs and thus cannot be used as the sole marker to indicate the glycation degree and protein damage degree in IF.

CRediT authorship contribution statement

Yajing Xie: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **H.J. van der Fels-Klerx:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Stefan P.J. van Leeuwen:** Conceptualization, Supervision, Writing - review & editing. **Vincenzo Fogliano:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135424.

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