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Maillard Reaction Products in Powder Based Food for Infants and Toddlers

Merichel Plaza[1], Elin Östman[2] and Eden Tareke[2*]

[1]Department of Chemistry, Centre for Analysis and Synthesis, Lund University, P.O.Box 124, SE-221 00 Lund, Sweden.
[2]Food for Health Science Centre, Lund University, Sweden.

Authors' contributions

This work was carried out in collaboration between authors MP, EO and ET. Author ET headed the project. Authors EO and ET were involved in the initial design of the study. Author ET conducted CML and CEL analysis and author MP conducted the analysis of the Maillard reaction products, HMF, Furfural, fluorescent MRPs and melanoidins. Authors MP, EO and ET participated in interpretation and drafting of the manuscript. Author ET was in charge of data management. All authors were involved in the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To investigate the formation of Maillard reaction products (MRPs) during 28 days storage of the two most consumed brands (B1, B2) of gruel products on the Swedish market.
Methodology: The MRPs; furfural, 5-hydroxymethyl furfural (HMF), N-(1-Carboxyethyl)-L-Lysine (CEL), N-(1-Carboxymethyl)-L-Lysine (CML), fluorescent advanced glycation end products (AGEs) and melanoidins (brown colour) were selected for analysis. High performance liquid chromatography coupled to UV spectrophotometry, fluorescence spectrophotometry or tandem mass spectrometry was used for analysis.
Results: In general, MRPs were higher in B2 than in B1 at the time of opening the package. The initial content of MRPs in B1 and B2 respectively was as follows: 4.39 and 13.74 µg/g of furfural; 1.11 and 1.47 µg/g of HMF; 73.64 and 134.3 µg/g of total CML; 19.79 and 30.42 µg/g of total CEL; 51.11 and 73.01 AU/g of fluorescent AGEs; 0.52 and 1.45 AU/g of MRPs that absorb light at 420 nm and 1.40 and 3.22 AU/g of MRPs that absorb light at 360 nm. During storage for 28 days, furfural, HMF, MRPs that absorb light at 360 nm and at 420 nm as well as fluorescent MRPs increased significantly by respectively 7, 30, 60, 83 and 21% in B2. In B1, only the fluorescent MRPs (21%) increased during storage.
Conclusion: A higher initial content and more pronounced increase of MRPs during 28 days

**Corresponding author: Email: Eden.tareke@food-health-science.lu.se;*

storage time was observed in B2. Consequently, children consuming gruel from B2 are exposed to 1.3-3.1 times more MRPs compared to B1. Considering that a child often sticks to one gruel brand throughout the first years of life and that some MRPs are inflammatory drivers, more studies are required to understand the role of food-process induced chemicals at an early age for future health of the children.

Keywords: Maillard reaction products; furfural; 5-hydroxymethyl furfural; N-(1-carboxyethyl)-L-lysine; N-(1-carboxymethyl)-L-lysine; advanced glycation end products; HPLC-MS/MS; melanoidins; fluorescence; infant; toddler.

1. INTRODUCTION

The Maillard reaction (MR) is a series of complex sequential and parallel reactions between reducing sugars and carbonyl groups with the nucleophilic groups of different biomolecules such as amino acids, peptides, proteins and lipids, that occur during food-processing and storage [1]. These reactions eventually lead to irreversible modifications of amino acids collectively called advanced glycation end products (AGEs).

Follow-up formula (gruel), containing both proteins and reducing sugars, are nutritionally optimised, powder-based products for infants and toddlers (6-36 months of age). The recommended shelf life for these products is up to 2 months when stored at room temperature in the dark. Recently, when developing an analysis method for markers of AGEs, carboxymethyl lysine (CML) and carboxyethyl lysine (CEL), relatively high and varying AGE-levels were observed in gruel products of 10 different commercial brands available on the Swedish market [2]. This was of concern since in both exposure and restriction studies, AGEs has been associated with systemic inflammation and other elevated risk markers for diabetes in humans [3-7]. Furthermore, in non-obese diabetic (NOD) mice, early life exposure to AGEs was associated with diabetes; both with respect to incidence and onset time [8]. These results suggested that limiting the exposure to AGEs in early life might have positive effects on future health. Considering that infants and toddlers in Sweden consume from 500-1500 mL gruel/day [9] the potential level of exposure to AGEs from this kind of products needs to be investigated.

Various MRPs are used as markers of how far the reaction cascade has gone during processing and storage. 5-Hydroxymethyl furfural (HMF) and furfural formed early in the MR serve as indicators of food quality/degree of deterioration [10]. Other commonly used markers are CML [11-14] and its analogue CEL [15], which are produced from oxidative degradation of lysine Amadori products, as well as from reaction products of glyoxal and methylglyoxal, respectively, with the ε-amino group in lysine [16]. Both CML and CEL are end products in the MR and have been associated with diabetes [17]. AGEs, may exhibit fluorescence that can be used as indicators of MR progression [14]. At the final reaction stages, MRPs condense and form nitrogen containing polymers and co-polymers, melanoidins, that are responsible for brown colour and can be analysed using UV spectrophotometer [14].

Although the presence of MRPs in milk based infant formulas has been studied previously, no comparative study of MRPs in milk/cereal based gruel is at hand. Neither are there any reports on MRP formation and change during storage of the aforementioned gruel products. Thus, this study aims to investigate content of HMF, furfural, CML, CEL, light-absorbing melanoidins and fluorescent MRPs, in two commonly consumed freshly opened and stored commercial powder based gruel products.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Carboxymethyl Lysine-d4 (d4-CML), CML, Carboxyethyl Lysine-d4 (d4-CEL) and CEL were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Ammonium solution (25%) and formic acid were bought from Merck (Darmstadt, Germany). Methanol (HPLC grade) was provided by Scharlau (Barcelona, Spain). Nonafluoropentanoic acid (NFPA, 97%), HMF and furfural were obtained from Sigma-Aldrich (Steinheim, Germany). SPE columns TELOS neo (PCX 200 mg, 6 mL) were purchased from Sorbent AB (Västra Frölunda, Sweden). All organic solvents were of analytical grade and all aqueous solutions were prepared in water purified from a Milli-Q reagent water system (Millipore SA, Molsheim, France).

2.2 Nutritional Composition and Preparation of Gruel

The two most commonly consumed gruel brands in Sweden (B1 and B2) were selected. According to the product labelling, the nutrient composition of gruels B1 and B2, respectively, was 15 and 16% protein, 17 and 18% fat, and 56 and 52% total carbohydrate. In B1, 25% of carbohydrates were declared as mono- and disaccharides and 25% lactose and in B2, the corresponding numbers were 23.5% mono- and disaccharides, 14% lactose and 6% maltose. The protein was both of animal (milk) and plant origin (B1: wheat and rye and B2: malted wheat and oats).

2.3 Sample Preparation and Analysis

The gruel products were purchased from a local supermarket in Lund, Sweden. When the experiment started (day 0) three gruel packages of B1 and three of B2 were opened and samples were collected for MRP analysis. The respective gruel packages were from the same production batch and thus had similar expiration dates. To investigate the effect of storage on level of MRPs, new samples from the opened packages were collected after two (day 14) and four (day 28) weeks, respectively. It should be noted that the recommended shelf life for gruel is two months but we decided to study a shorter storage time since infants with a regular intake of one portion per day will finish the package (5 L readymade gruel) in about 22 days. To exclude any effects associated with differences between batches of gruel powder, triplicates of three different batches from both brands were analysed.

2.4 HMF and Furfural

Gruel samples (0.3 g) B1 and B2 from three individual gruel packages and from each storage period (days 1, 14 and 28) were dissolved in 45ºC distilled water to make 0.1 g/mL solution (the recommended preparation method is 0.18 g/ml dissolved and heated in microwave for 40 s full effect giving 37ºC). To denature the protein in the gruel solutions, 0.1 N HCL was added to each sample followed by centrifugation at 6000 rpm for 10 min. The supernatants were then used for determination of HMF and furfural.

The analysis of HMF and furfural was performed using an Ultimate-3000 HPLC system from Dionex (Thermo Fisher, Germering, Germany) consisting of an online degasser, a quaternary solvent pump, a thermostated auto-sampler and a column oven. Separation was performed using isocratic mobile phase, with a methanol-water (20:80, v/v) and formic acid (0.5 v %) mobile phase at a flow rate of 0.2 mL/min on a porous-shell fused core Ascentis Express C18 (150 mm x 2.1 mm, 2.7 µm.) from Supelco (Bellefonte, PA, USA) analytical HPLC column with an injection volume of 2 µL. The column temperature was set at 50°C and the vial tray at 4°C. Detection was accomplished using a diode array detector (DAD) at 280 nm, Thermo Fisher software was used and all instruments were controlled by a Chromeleon 6.80.

Quantification of HMF and furfural was carried out by an external standard method using a mixture of furfural and HMF in concentrations from 0.1 to 5 µg/mL each.

2.5 CML and CEL

For determination of free CML and CEL, gruel powder were dissolved in water (0.1 g/mL) at 45ºC [2]. Methanol was then added to make a 1:3 solution of water:methanol (v/v). For quantification of free CML and CEL, 0.1 µg/mL of CML-d4 and CEL-d4, respectively, was added to each sample as internal standards (IS). After lowering pH to 2.0 using NFPA, the samples were centrifuged and CML and CEL were extracted from a 4 mL solution with, in total, 0.3 g gruel/sample using solid phase extraction (SPE) according to Tareke et al. [2]. For the determination of total CML, 10 µg/mL IS was added to 0.3 g of each sample and incubated with 2 mL 6N HCl for 18 h at 110°C under nitrogen atmosphere, in order to prevent formation of artefacts. The samples were then filtered and evaporated to dryness under nitrogen stream and reconstituted in 1 mL 5 mM NFPA prior to SPE. The extracts for determination of free and total CML and CEL levels were evaporated, reconstituted in 200 µL 5 mM NFPA H_2O and kept at -20°C until analysis using HPLC coupled to tandem mass spectrometry (HPLC-MS/MS).

The HPLC instrument used for the quantification of CML and CEL was an Accela (Thermo Scientific) UHPLC pump with an auto-injector. Detection was performed by a LTQ VelosPro Orbitrap mass spectrometer run in positive electrospray ionization ion trap MS/MS mode, detecting two SRM transitions for each analyte, and two for the internal standard. The Xcalibur software (Thermo Scientific) was used both for data acquisition and evaluation. The

chromatographic separation was performed using Genesis column (Lightn AQ 4.6 mm x 250 mm, 4 µm particles; Grace Vydac, Hesperia, CA, USA). The mobile phases were: eluent A, aqueous 0.1% formic acid and eluent B, acetonitrile (ACN). Eluent A (7%) was maintained for 2 min followed by a linear gradient from 7 to 93% of eluent A over 5 min and finally remaining at 93% for 3 min. The flow rate was 0.7 mL/min and the injection volume was 20 µL.

The ion source parameters, based on the Xcalibur software settings, were source voltage 4 kV, capillary and source temperatures 375°C, sheath gas flow 60 L h^{-1}, auxillary gas flow 320 L h^{-1}, sweep gas flow 5 L h^{-1}, and an S-lens setting at 60%. Collision-induced fragmentation was achieved by a collision energy of 35 eV and the following transitions were monitored: m/z 205.08 – 130 (±0.5) and 205.08 – 84 (±0.5) for compound CML, 219.08 – 130 (±0.5) and 219.08 – 84 (±0.5) for compound CEL, and similarly for the deuterated standards 209.08 – 134 (±0.5) and 209.08 – 88 (±0.5) for d4-CML and 223.08 – 134 (±0.5) and 223.8 – 88 (±0.5) for d4-CEL. Mass MS/MS detection was performed between 5 – 10 minutes; otherwise, the flow was directed to waste. Transitions m/z 205.08 – 130 (±0.5) and m/z 219.08 – 130 (±0.5) for the analytes CML and CEL, respectively, and transitions m/z 209.08 – 134 (±0.5) and 223.08 – 134 (±0.5) for d4-CML and d4-CEL, respectively, were used as quantifiers and the other transitions as qualifiers.

2.6 UV Absorbing Melanoidins and Fluorescent MRPs

Sample preparation for UV absorbing melanoidins and fluorescent MRPs was as described for HMF and furfural above. Hydrolysis in 1 N HCL by incubation glucose:glycine (1:1) solution for 1 h at room temperature was used to solubilize melanoidins [18]. In this study, a final concentration and incubation time of 0.1 N for 10 min was used to denature the proteins prior analysis of furfural, HMF, fluorescent MRPs, and melanoidins. The possible effect of 0.1 N HCL, for 10 min, compared to the effect observed when using 1 N for 1 h [18], in the composition of analytes was expected to be minimal. Moreover, since all the samples were treated in the same way, the possible change inferred due to treatment with HCL is expected to have affected all samples equally.

Melanoidins were estimated as browning intensity of the extracted samples at wavelengths of 360 nm and 420 nm, respectively, using a microplate spectrophotometer reader Multiskan GO (Thermo Fisher). The fluorescent MRPs were measured according to Wang et al. [19] by the fluorescence intensity of the sample at an excitation wavelength of 337 nm and emission wavelengths ranging from 350 nm to 550 nm using a microplate reader spectrofluorometer (Cary Eclipse, Varian, Les Ulis, France). Supernatant (550 µL) from gruel samples (0.1 g/mL) were collected and placed in a 96-well microplate. Fluorescence analysis was performed at λEx = 337 nm and λEm of 425 nm. Data is expressed as the mean values (n=3) in arbitrary units (AU/g sample).

2.7 Statistical Evaluation

Significant differences between gruel samples and storage times were determined using One-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism ver 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) A probability value of $P \leq 0.05$ was considered significant. Data in the histograms is expressed as the average ± SD of the values for measurements performed in at least triplicate. Significances are presented with * for $p < 0.05$, ** for $p < 0.001$ and *** for $p < 0.0001$.

3. RESULTS

Noteworthy amounts of furfural, HMF, CML, CEL and melanoidins, expressed as MRPs that absorb light at 360 and 420 nm, were observed in gruel powder of brands B1 and B2. The RSDs for the analysis of furfural, HMF, light absorption and fluorescent MRPs (n=9 of each) was less than 5%. For CML and CEL levels, less than 10% RSDs was recorded for 83% of the analysed samples (n=61) and 11-16% for the rest (n=12). Analysis of three different batches of B1 and B2, respectively, with three independent packages from each batch, showed that there was no batch-to-batch difference in CML and CEL levels (RSDs < 10%).

The study revealed significant increases in furfural (13.74 day 1 to 14.8 µg/g day 28, $p< 0.05$), HMF (1.14 µg/g day 1 to 1.48 µg/g day 28, $p< 0.05$), MRPs that absorb light at 360 nm (0.30 AU/g day 1 to 0.48 AU/g day 28, $p< 0.05$) and MRPs that absorb light at 420 nm (0.12 AU/g day 1 to 0.22 AU/g day 28, $p< 0.05$) as well as fluorescent MRPs (73.11 ug/g day 1 to 88.94 ug/g day 28, $p<0.05$) in B2 during storage for 28 days. The increase ranged from 7% for furfural to 83% for MRPs that absorb light at 420 nm.

Fluorescent MRPs (10.97 day 1 to 13.34 AU/g day 28) and free CML and CEL were the only analytes that showed significant ($p < 0.05$) increases during storage of B1.

The study revealed that MRPs were significantly higher in B2 than in B1 in all time points except for HMF that was significantly higher only after 28 days of storage. In gruel B2, the furfural level (13.74±0.17 µg/g sample) was three times higher than in B1 (4.38±0.16 µg/g sample, Fig. 1). Unlike in B1, HMF levels in B2 were affected by storage and after 28 days, the HMF levels were 1.3 times higher in B2 compared to day 1.

On day 1, the total CML levels were 72-82 µg/g and 124-135 µg/g in B1 and B2, respectively (Fig. 2a). Total CML and CEL levels in B2 were 1.7 and 1.5 times higher compared to corresponding levels in gruel B1. Free CML and CEL levels were the only MRPs that were lower in B2 compared to B1. On day 1 the average ratio between B1 and B2 of free CML and free CEL, respectively, was 1:0.9 and 1:0.7. On day 14, free CML and CEL levels increased to respectively 0.85±0.06 and 0.04±0.02 µg/g for B1; and 0.51±0.07 and 0.02±0.03 µg/g for B2. The levels of free CML (0.68±0.10 and 0.34±0.04 µg/g in B1 and B2, respectively) and CEL (0.01±0.002 and 0.01±0.002 µg/g in B1 and B2, respectively) were lower after storage for 28 days compared to day 14. Free CEL levels in day 28 were also lower than levels in day 1 (Fig. 2b).

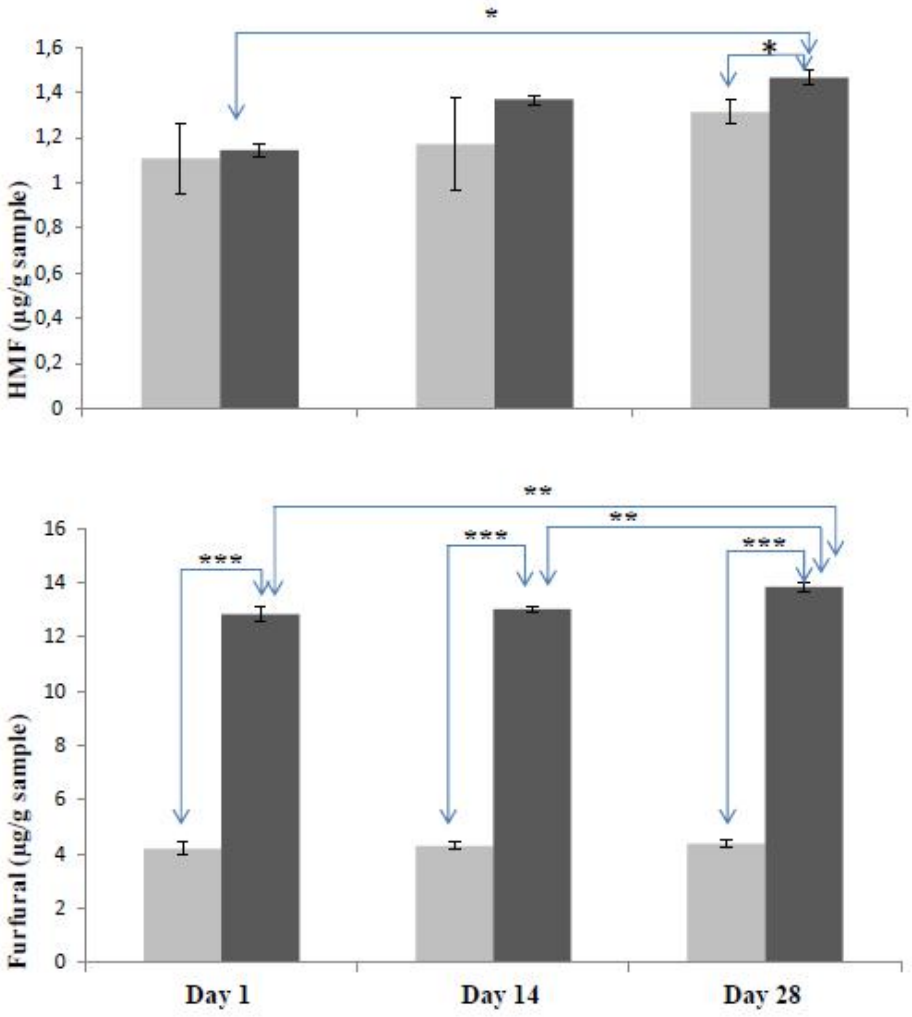

Fig. 1. HMF and furfural levels (n= 3, average ± SD) determined in gruel samples from two different brands, B1 and B2. B1 is denoted by grey bars and B2 by black bars. Day 1 was analysed immediately after opening the packages, with repeated analysis on days 14 and 28

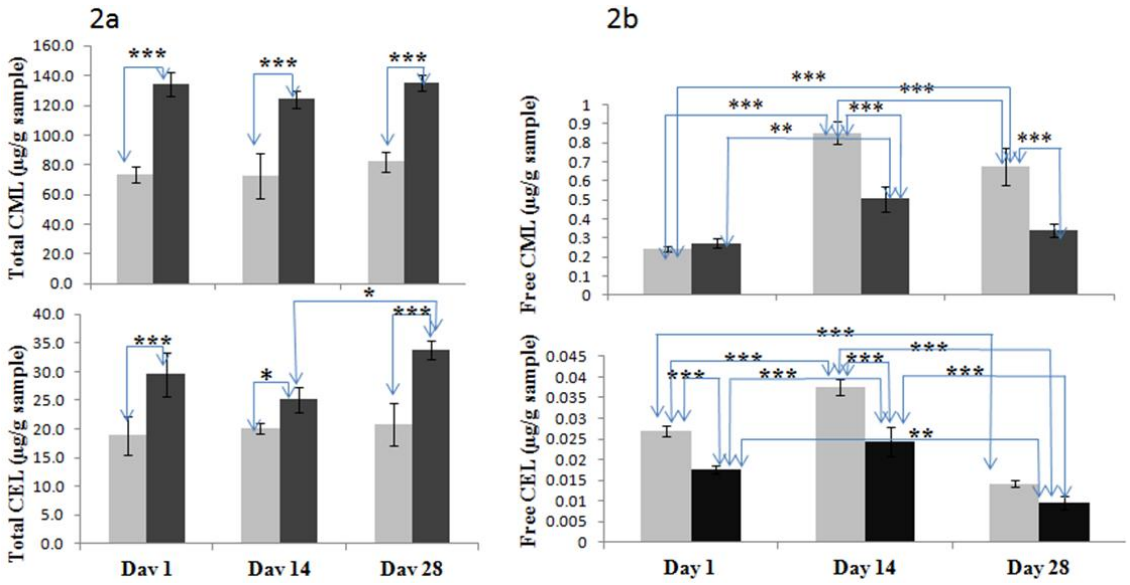

Fig. 2. Total (2a) and free (2b) CML and CEL levels (n= 10, average ± SD) determined in gruel samples from two different brands, B1 and B2. B1 denoted by grey bars and B2 by black bars. Day 1 was analysed immediately after opening the packages, with repeated analysis on days 14 and 28

As shown in Fig. 3, MRPs that absorb light at 420 nm and 360 nm were respectively 1.5 and 1.4 times higher in gruel B2 than in B1 on days 1 and 14. On day 28 the ratio of MRPs between B1 and B2 was increased to 1:2.8 for those that absorb light at 420 nm and 1:2.3 for MRPs that absorb light at 360 nm. There was no significant increase in MRPs (that absorb light at both 420 and 360 nm) in B2 after storage for 14 days. The formation of MRPs that absorb light at 420 nm and 360 nm in gruel B2 was however, augmented after 14 days of storage. Similarly, MRPs measured using fluorescence spectrometry was on average 1.4 times higher in gruel B2 than B1 on days 1, 14 and 28 and there was significant increase in fluorescence in gruel B2. Fluorescent AGEs were also increased in B1 during storage for 28 days (Fig. 4).

4. DISCUSSION

Measurable increases in MRPs were observed in powdered gruel products during shorter storage time than the shelf-life of 2 months communicated by the manufacturer. Results in this study show that gruel B2 is more prone than B1 to storage induced formation of MRs. In addition, B2 exhibited higher levels of MRPs and AGEs than B1 at start. Considering the nutritional similarities in composition, a possible explanation for the higher levels and enhanced formation of MRPs during storage in B2 could be the use of malted grains, which may contain a higher proportion of simple sugars that are readily available for Maillard reactions. In line with this, a saccharide-lysine model system has shown that lactose has higher potency for formation of CML compared to glucose and sucrose [20]. However, despite higher lactose levels in B1 than in B2 (25% and 14% of total carbohydrates, respectively), the latter exhibited higher CML levels. The presence of 6% maltose and 1.5% fructose in B2 (according to manufacturer) may also be a possible reason for the higher MRPs observed in B2. In order to pin point the exact reasons for higher MPRs and storage induced formation in B2, more detailed information about processing conditions and ingredients is required.

The estimated intakes of furfural and HMF, respectively, from 200 ml gruel of B1 and B2, respectively is 1.60±0.03 and 4.90±0.06 mg and 0.40±0.02 and 0.52±0.01 mg (Table 1). The assumed intake of 200 mL of gruel/day is probably an underestimation considering that daily consumption of gruel in Swedish children at six months of age is one to two bottles (prepared by adding 45 g gruel in a bottle of 250 mL water) for about 60% and up to six bottles (1.5 L) a day for 14% of the children [9].

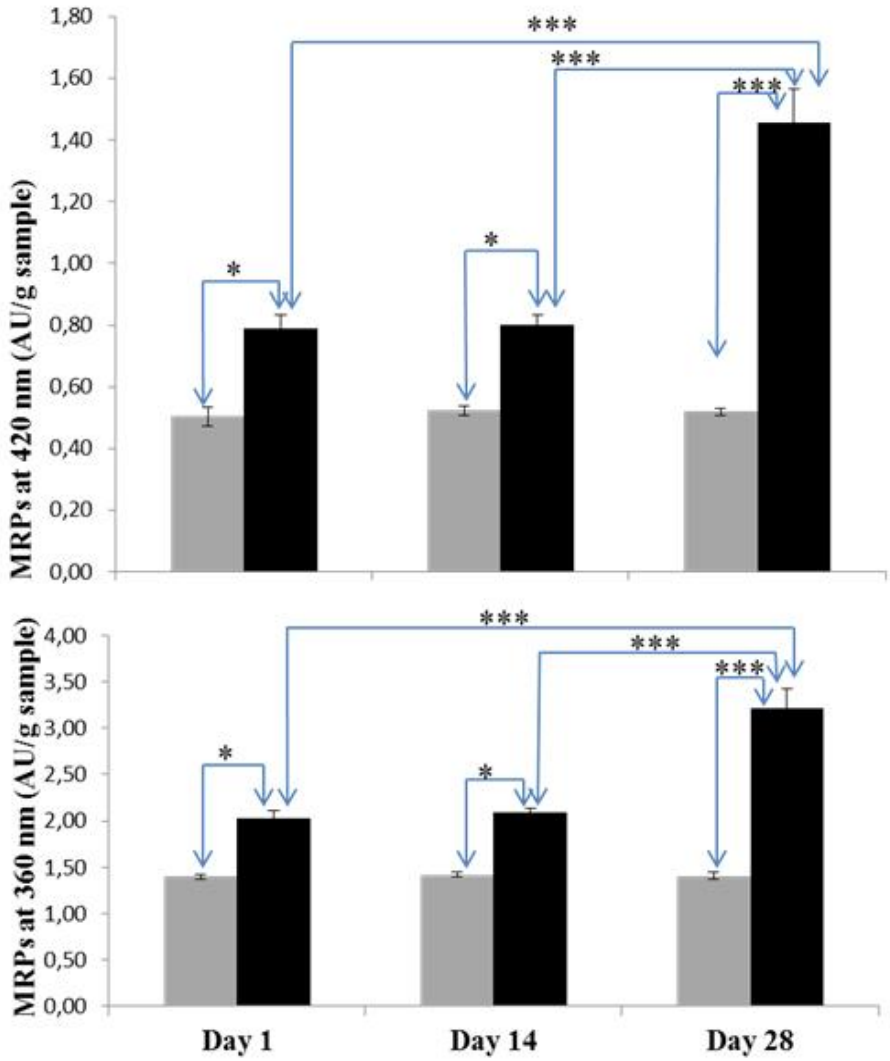

Fig. 3. Light determination (absorption 420 and 360) of melanoidins (n= 3, average ± SD) determined in gruel samples from two different brands, B1 and B2. B1 denoted by grey bars and B2 by black bars. Day 1 was analysed immediately after opening the packages, with repeated analysis on days 14 and 28

Table 1. Daily intake of Maillard reaction products (MRPs) from gruels B1 and B2 on day 1. The levels were calculated per gruel portion (200 ml)

	Gruel B1 (mg/day) X±SD	Gruel B2 (mg/day) X±SD	Gruel B1 (µg/day) X±SD	Gruel B2 (µg /day) X±SD
Furfural	1.57±0.03	4.98±0.06		
HMF	0.40±0.02	0.53±0.01		
CML	2.65±0.20[a]	4.84±0.28[a]	9.82±1.0[b]	8.76±0.60[b]
CEL	0.70±0.1[a]	1.08±0.11[a]	0.96±0.03[b]	0.63±0.03[b]

[a] Total; [b] Free

Based on the same intake estimations, exposure to CML and CEL, respectively, from B2 would be 1.7±0.1 and 1.5±0.2 times higher than that from B1. This difference in CML-levels is close to the factor two difference in CML-levels reported between a high heat treated and a steamed diet

used in a human intervention (64 men and women; mean BMI: 21.8 kg/m^2) by Birlouez-Aragon et al. [3]. The latter results showed that one month exposure to the higher CML-levels promoted risk factors for diabetes, such as lower insulin senstivity, lower omega-3 fatty acid-levels and higher concentration of plasma cholesterol and triglycerides [3]. Furthermore, in NOD mice, a five-fold restriction from dietary AGEs/CML in early life lowered the prevalence and delayed the onset of diabetes type 1 [8]. In addition, there are more studies showing an association between intake of AGEs and elevated risk markers for diabetes in humans [4-7].

Free CML and CEL were the only parameters that were lower in B1 compared to B2. Nevertheless, since the free CML and CEL levels are about 100-1000 times lower than the total CML and CEL levels, the observed difference may be considered negligible.

The largest difference between B1 and B2 was observed in furfural levels. The evidence for carcinogenicity of furfural is contradictory and limited, however considered strong enough to classify furfural as an oral genotoxic carcinogen of low potency [21]. On the other hand, HMF is known to be metabolised to 5-sulphoxymethyl furfural (SMF), which can be converted to a highly reactive intermediate allyl carbocation that may react with vital macromolecules such as DNA [22] and thereby cause damage. Though *in vitro* studies have revealed the potential toxicity of HMF, sufficient animal data is lacking to form convincing proof [23].

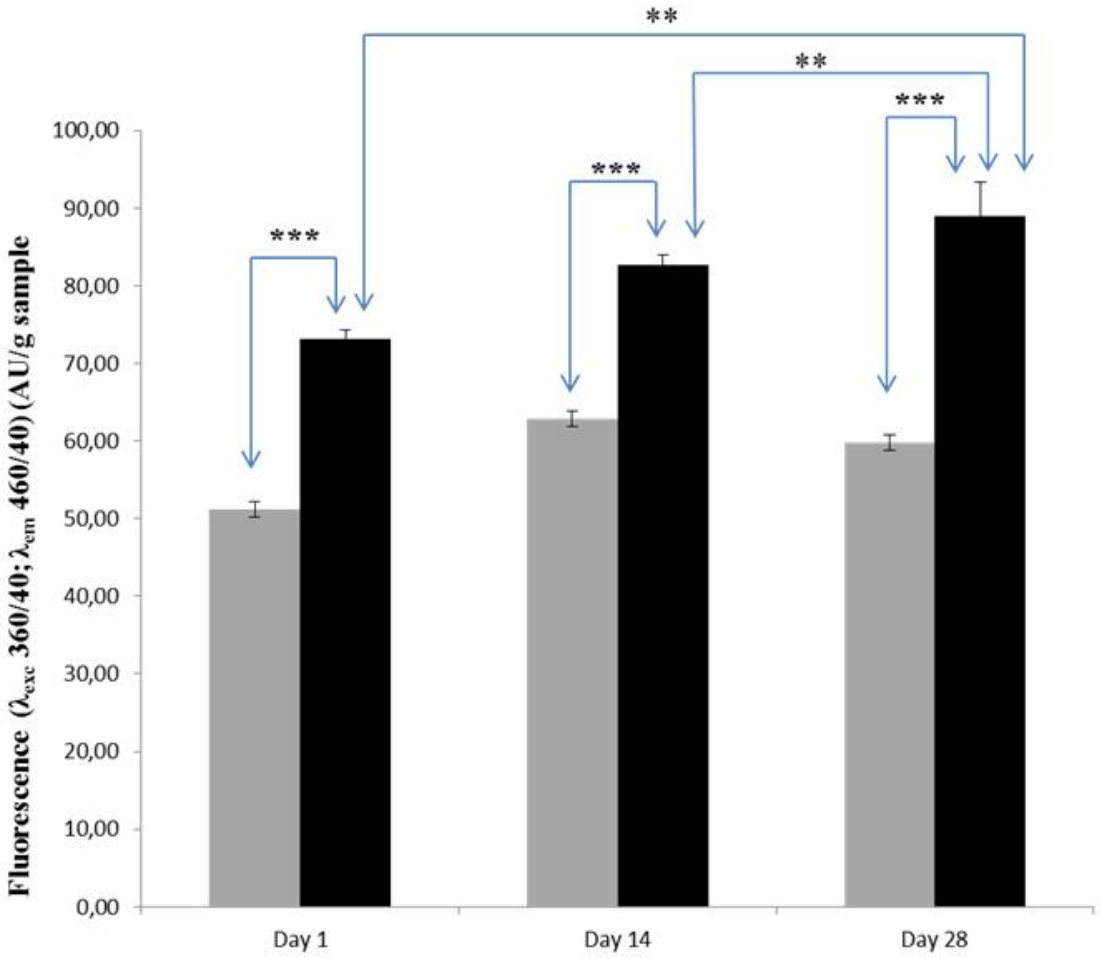

Fig. 4. Fluorescent Maillard reaction products (MRPs) (n= 3, average ± SD) determined in gruel samples from two different brands, B1 and B2. B1 denoted by grey bars and B2 by black bars. Day 1 was analysed immediately after opening the packages, with repeated analysis on days 14 and 28

5. CONCLUSION

In general, MRPs were higher in B2 than in B1 at the time of opening and a more pronounced increase was observed in B2 during storage. Considering that children are often conservative in their choice of food, those consuming B2 are exposed to 1.3-3.1 times more MRPs compared to those that consume B1.

While the role of exposure to AGEs in chronic inflammation and associated risk factors in human adults and animal studies is getting increased attention, corresponding associations for infants and toddlers have been neglected. Therefore, bearing in mind that gruel intake constitutes a relatively large and consistent proportion of food intake in toddlers, the relatively high dose/kg body weight of process and storage-induced MPRs/AGEs on inflammatory markers in children must be further evaluated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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