TITLE: ***In vitro* formation of Maillard reaction products (MRPs) during simulated digestion of food models corresponding to average and sugar-containing meals**

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Abbreviations: AGEs, Advanced glycation end products; **BSA,** Bovine serum albumin; **CML**, Carboxymethyl-lysine; **DMF**, 1-deoxy-1-morpholinofructose; **ELISA**, enzyme-linked immunosorbent assay; **HFCS**, High fructose corn syrup; **MGO**, Methyl glyoxal; **MR**, Maillard reaction; **MRPs**, Maillard reaction products; **NBT**, nitroblue tetrazolium; **RFU**, relative fluorescence units.

Abstract

The aim of the present research was to study the formation of Maillard reaction products (MRPs) during digestive process of simplified meal systems. An average meal (protein, starch and oil) and sugar-containing meals (protein and glucose or fructose or high fructose corn syrup (HCFS)) were tested. Intestinal simple amino acid systems were also analyzed to gain insight into their contribution to the Maillard reaction (MR). Decrease of lysine (11.7-34%), arginine (24-35%) and other amino acids occurred after digestion of the meals. Fructosamine (42.6±4.7 and 332.9±10.4 µg/ml) and fluorescent adducts (22270±119.6 and 9283±188.3 RFU) were detected in digests of those meals containing HCFS and starch, respectively. Carboxymethyllysine (CML) (5.03±1.09 µg/ml) and MGO-derivative AGEs (12.2±1.5 µg/ml) were found in the meals composed of fructose and only MGO-

derivative AGEs (12.2±1.6 µg/ml) in presence of glucose. Physiological concentrations (43 mM) of sugars in simplified systems composed by single amino acids caused formation of MRPs under intestinal conditions. Arginine and fructose (314 mM) showed formation of fructosamine and different AGEs. Fructose (43 mM) gave rise to CML by interaction with lysine, which was observed within 1 hour of incubation at intestinal conditions. This may be feasible under the conditions of fructose malabsorption. Results suggested the interest of the use of meal systems for a better understanding of complex chemical events taking place during digestion such as MR. This is the first study proposing the formation of non-fluorescent AGEs associated to the pathogenesis of diabetes during digestion of sugar containing and average meals. Their formation may be possible under those conditions where sugar absorption is delayed such as fructose malabsorption or the intake of a fatty meal. The occurrence of the MR during the digestion process may reduce the bioavailability of essential amino acids and increase the production of MRPs causing health disorders.

1. INTRODUCTION

The Maillard reaction (MR) is a series of non-enzymatic reactions between reducing sugars and proteins firstly described in foods during thermal processing and long-term storage. MR products (MRPs) provide sensory attributes of cooked foods, and are the main determinants of the consumer's quality-oriented food choice. Early products of the MR (Amadori and Heyns compounds) tend to decline after overheating and give rise to advanced glycation end products (AGEs) (Mesías & Delgado-Andrade, 2017).

The modern diet is a large source of MRPs (Goldberg et al., 2004; Uribarri et al., 2010; Vlassara & Uribarri, 2004) significantly contributing to those endogenously produced. Previous studies have showed that dietary AGEs are partially absorbed (Cai et al., 2008; Lin et al., 2003), approximately 10–30% (Uribarri et al., 2007), and they are indistinguishable from endogenous AGEs (Cai et al., 2002). Great interest has been raised due to their association with oxidative stress and inflammation, processes that eventually cause most chronic diseases, including diabetes, and cardiovascular disease (Sayej et al., 2016; Vlassara & Uribarri, 2014). Recently, a relationship has been reported between the intake of high fructose corn syrup (HFCS) sweetened soft drinks, fruit drinks and apple juice and the development of arthritis (DeChristopher, Uribarri, & Tucker, 2016), asthma (DeChristopher, Uribarri, & Tucker, 2015b) and chronic bronchitis (DeChristopher, Uribarri, & Tucker, 2015a). The authors hypothesized that these associations might be mediated through the intestinal in situ formation of fructose-AGEs and their subsequent absorption. This event may be favored by the increased intake of beverages and food containing HFCS (Ventura, Davis, & Goran, 2011) in which the ratio of fructose to glucose is higher than 1:1 therefore creating conditions for potential

fructose malabsorption and intraluminal generation of fructose-AGEs (DeChristopher et al., 2016). In fact, malabsorption of simple carbohydrates affects 20% to 30% of the European population (Raithel et al., 2013). The dietary lipid content can affect the intestinal membrane function (Thomson, 1982) thus attenuating intestinal sugar uptake and therefore potentially creating transient increase in intraluminal concentration of sugars Moreover, the natural conditions of the digestive tract (pH, minerals, temperature) might be a favorable environment for the MR to take place (Nursten, 2005). Based on the above information, the need to study the effect of gastrointestinal digestion process on the MRP formation, a largely unexplored area, becomes very important.

The aim of the present research was to obtain novel information regarding the nature of MRPs formed during *in vitro* oral gastrointestinal digestion (Hollebeeck, Borlon, Schneider, Larondelle, & Rogez, 2013). Simplified meal systems comprising an average meal composed of bovine serum albumin (BSA), digestible starch and oil; as well as, sugar-containing meals prepared with BSA and glucose or fructose or HFCS were tested. We further analyzed simple amino acid systems prepared using lysine or arginine and fructose or glucose under compatible intestinal conditions to evaluate their contribution. Analyses of amino acid content, fructosamine, carboxymethyllysine (CML), methylglyoxal (MGO)-derivative AGEs, and fluorescent adducts were performed. This is the first study reporting the formation of MRPs associated to the pathogenesis of diabetes and its complications during digestion of simplified meals.

2. MATERIALS AND METHODS

2.1. Reagents

Alpha-amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3.200–4.500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, BSA, L-lysine, L-arginine, D-(-)-fructose, D-(+)-glucose, soluble starch, cellulose microcrystalline, potassium and sodium chloride, sodium carbonate and bicarbonate, sodium phosphate monobasic and dibasic, 1-deoxy-1-morpholinofructose (DMF), nitroblue tetrazolium (NBT), magnesium chloride, diethanolamine and Tween 20 were from Sigma-Aldrich (St. Louis, MO, USA). Ninhydrin was provided by Biochrom Ldt. (Cambridge, UK). CML standard ((S)-2-amino-6-(carboxymethyl-amino)-hexanoic acid, CAS-No. 5746-04-3) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Competitive ELISA, based on monoclonal antibodies for CML (6C7) and MGO-derivative AGEs (3D11 mAb) (Cai et al., 2002) were used to detect these relevant AGEs. The test sensitivity for CML and MGO-derivatives AGEs was 0.1 U/ml and 0.004 nmol/ml, respectively; the intra-assay variation was ±2.6% (CML) and ±2.8% (MGO-derivatives AGEs) and the inter-assay variation was ±4.1% (CML) and ±5.2% (MGO-

derivatives AGEs). The AGE content of each sample was based on the mean value of at least three measurements per sample and expressed as µg eq./ml. The secondary antibody (goat anti-mouse IgG (H+L)) was obtained from MP Biomedicals (Solon, OH, USA) and the alkaline phosphatase substrate (4-nitrophenyl phosphate disodium salt hexahydrate) from Sigma Aldrich (St. Louis, MO, USA). The blocking buffer (SuperBlock™ (PBS)) was purchased from Thermo Fisher Scientific Inc. (USA). Water was purified using Milli-Q and Elix system. All other chemicals and reagents were of analytical grade.

2.2. Preparation of the simplified meal systems

-An average meal was prepared with BSA (0.18 mM), soluble starch (56 mg/ml) and sunflower oil (8.3 mg/ml).

-Simple sugar-containing meals were prepared as follows: 1) BSA (0.18 mM) and glucose (43 mM); 2) BSA (0.18 mM) and fructose (43 mM); and 3) BSA (0.18 mM) and HFCS (43 mM, 60% fructose and 40% glucose).

These samples were based on the main macronutrients of a meal (carbohydrates, protein and oil). Non-reactive cellulose powder was incorporated to adjust all of them to the same final weight. The concentrations were selected according to the volumes of ingested and secreted fluids, as well as the relative proportions of the dietary macronutrients. For a standard meal, we assumed an intake of 120 g of total carbohydrates, 25 g of protein and 25 g of lipids. In 24 h, a human drinks roughly 2 l, and secretes 1 l of saliva, 2 l of gastric juice, 1 l of bile, 2 l of pancreatic juice, and 1 l of intestinal juice. Considering an average of three meals per day, the physiological concentration of each nutrient was calculated in the gastrointestinal tract (Hollebeeck et al., 2013; Tortora & Grabowski, 1996).

These simplified meals were then digested in duplicate under *in vitro* oral gastrointestinal human digestion conditions (Hollebeeck et al., 2013) with slight modifications, in order to evaluate the formation of MRPs during the digestive process. Briefly, all three stages, salivary (pH 6.9, 10 ml, 5 min, 3.9 U α-amylase/ml, aerobic), gastric (pH 2, 13 ml, 90 min, 71.2 U pepsin/ml, aerobic), and abiotic duodenal step (pH 7, 16 ml, 150 min, 9.2 mg pancreatin, aerobic) were performed in the same flask at 37 ºC. BSA (0.18 mM) was included as the control. A complete digestion of the starch during the digestive process of the average meal could yield up to 314 mM glucose equivalents. The resulting digests were then centrifuged and the soluble fractions were frozen at -20°C, lyophilized (Telstar Lyobeta-15 lyophilizer (Telstar, Spain)) and stored at room temperature until further analysis.

2.3. Preparation of simple amino acid systems

In order to gain insight into the contribution of the single amino acids (lysine and arginine) on the MRP formation during the digestion process of the above described meals, simple systems constituted by basic amino acids (40 mM) and sugars (glucose or fructose) at two different sugar concentrations were prepared as follows: 1) lysine (40 mM); 2) lysine (40 mM) and glucose (43 mM); 3) lysine (40 mM) and glucose (314 mM); 4) lysine (40 mM) and fructose (43 mM); 5) lysine (40 mM) and fructose (314 mM); 6) arginine (40 mM); 7) arginine (40 mM) and glucose (43 mM); 8) arginine (40 mM) and glucose (314 mM); 9) arginine (40 mM) and fructose (43 mM); 10) arginine (40 mM) and fructose (314 mM). These systems were then incubated mimicking lumen intestinal conditions *in vitro* (pH 7, 37 ºC, 2h 30 min).

Simple amino acid systems prepared with lower sugar concentration (43 mM): corresponds to physiological concentrations likely to be present in the lumen of the intestine when ingesting simple sugar-containing meals or ingesting HFCS beverages.

Simple amino acid systems prepared with higher sugar concentration (314 mM): corresponds to a theoretical maximal concentration present as the result of complete digestion of the average meal containing starch. This sugar concentration does not prevail in the lumen under physiological conditions. However, such amount of sugar could be release during the digestion of average meal. As a consequence, it is interesting to study these theoretical sugar digestion conditions to identify the maximal amount of MRPs that can be formed from the precursors without taking into account the rate of sugar absorption. The information to be obtained from such model is very relevant from a food chemistry point of view. The model provides information regarding the reactivity of the different amino acids composing the food proteins and the maximal large number of MRPs that can be generated in the gut during the digestion process. There is a lack of information on this completely new field, which is of great interest because of the impact of MRPs and essential amino acids in human health.

A delay on the absorption of the sugar due to either fructose malabsorption or change in intestinal membrane functionality because of the presence of fat has been assumed either for simplified meal and amino acids systems. Fat present in simplified meal also may be a substrate of the MR causing the formation of non-fluorescence adducts such as CML. Theoretically, intestinal physiological (≤ 50 mM) and non-physiological conditions (50-100 mM) regarding the glucose or fructose concentrations (Ferraris, Yasharpour, Lloyd, Mirzayan, & Diamond, 1990) can be achieved in both cases avoiding clinically evident diarrhea.

In addition, a kinetic study was performed on the system constituted by lysine (40 mM) and fructose (43 mM) in order find out how much time of reaction is necessary for the formation of MRP. This system was incubated under lumen intestinal conditions *in vitro* (pH 7, 37 ºC) for 1h, 2h, and 3h. Samples in duplicate were then lyophilized (Telstar Lyobeta-15 lyophilizer (Telstar, Spain)) and stored at room temperature until further analysis.

2.4. Amino acid content analysis

Amino acid analysis was performed directly on digests from the simplified meals, prepared as indicated in section 2.2. The analysis was carried out using an amino acid analyzer (Biochrom 30, Biochrom Ldt. Cambridge, UK). It is based on an acid hydrolysis of the sample followed by cationic exchange chromatography (high pressure PEEK column packed with Ultropac 8 cation exchange resin sodium form) with post column derivatization using ninhydrin. Samples (1 ml) were first hydrolyzed with 3 ml of HCl (10.6 N). The hydrolysates were then filtered through grade filter paper, and filtrate (0.5 ml) was applied to a SepPak® C18 cartridge (Waters Cromatografía, S.A., Barcelona, Spain) previously activated with methanol (5 ml) and water (10 ml). Finally, samples were eluted with 3 ml of HCl (3 N), and 70 µl of this volume were injected in the chromatograph. Analysis was performed in duplicate and results were expressed as mmol/g BSA.

2.5. Early glycation products

The formation of fructosamine was measured on both simplified meals and amino acid systems.

2.5.1. Fructosamine assay

NBT assay was carried out to determine fructosamine following the micromethod of Vlassopoulos, Lean, & Combet (2013) in both the simplified food models and the amino acid systems. Briefly, samples (25 µl) were mixed with 100 µl nitroblue tetrazolium (0.25 mM) previously dissolved in sodium carbonate buffer (100 mM, pH 10.8). Microplates were incubated for 20 min at 37 ºC and measured spectrophotometrically (BioTek powerWaveTM XS microplate spectrometer (BioTek Instruments, U.S.A)) against control at 530 nm. The fructosamine analogue (DMF) was used as standard (0.5-5 mM). All measurements were performed in triplicate and expressed as µg DMF eq./ml.

2.6. Advanced glycation end products (AGEs)

The analysis of CML and MGO-derivative AGEs, and fluorescent adducts was performed in both the average and sugar-containing meals, and in the amino acid systems.

2.6.1. Carboxymethyl lysine (CML) and MGO-derivative AGEs

Competitive ELISA, based on monoclonal antibodies for CML (6C7) and MGO-derivative AGEs (3D11 mAb) (Cai et al., 2002) was used to detect these relevant AGEs. The test sensitivity for CML and MGO-derivatives AGEs was 0.1 U/mL and 0.004 nmol/ml, respectively; the intra-assay variation was ±2.6% (CML) and ±2.8% (MGO-derivatives AGEs) and the inter-assay variation was ±4.1% (CML) and ±5.2% (MGO-derivatives AGEs). The AGE content of each sample was based on the mean value of at least three measurements per sample and expressed as µg eq./ml.

CML content was also quantitated by using an amino acid analyzer (Biochrom 30, Biochrom Ldt. Cambridge, UK). It comprises an ion exchange HPLC column (high pressure PEEK column packed with Ultropac 8 cation exchange resin sodium form) with post column derivatization using ninhydrin Quantitation was carried out according to the external standard method using a commercial standard of pure CML. Results were expressed as µM.

2.6.2. Fluorescent adducts

Fluorescent adducts were measured by fluorescence spectrophotometry (microplate fluorescence reader, FP-6200 (JASCO, Easton, U.S.A)) using 360 ± 40 nm and 460 ± 40 nm as excitation and emission wavelengths, respectively. Samples (150 µl) were added to black microplates in triplicate and results were expressed as relative fluorescence units (RFU).

3. RESULTS

3.1. Progression of the MR during digestion of food simplified meals

3.1.1. Amino acid content as first indicator

Figure 1 illustrates the consumption of the basic amino acids, lysine and arginine, during the digestive process of an average meal and sugar-containing meals. As can be observed, a significant decrease ($p < 0.05$) of both amino acids occurs for all the tested meals. The arginine amino acid presented a decrease 2 fold greater than lysine (24% vs 11.70%). The meal containing HFCS and the average meal with starch and oil demonstrated significantly ($p < 0.05$) higher loss of these amino acids (34.05% lysine and 35.4% arginine; and 23.44% lysine and 32.7% arginine, respectively) than the glucose and fructose-containing meals (12.20% lysine and 23.92% arginine; and 11.70% lysine and 24.51% arginine, respectively). In the case of arginine content, the average meal and the sugar-containing meals with HFCS presented amino acid reduction during digestion of the same order of magnitude ($p > 0.05$). Furthermore, significant ($p < 0.05$) consumption was observed for other amino

acids different from lysine and arginine released from the BSA during the digestive process (table 1). Glycine (42.88-100% blocked), serine (27.78-100% blocked), tyrosine (42.49-54.05% blocked), isoleucine (34.22-45.07% blocked) and phenylalanine (19.93-54.08% blocked) exhibited the greatest reactivity for all the meals tested. Indeed, the average meal showed complete blockage of several amino acids (aspartic, threonine, serine, glutamic, glycine, alanine, cysteine and valine) released from the BSA during the digestion process. Both, the meal composed of BSA and HFCS and the average meal significantly exhibited the largest total amino acid loss ($p < 0.05$) among all the meals.

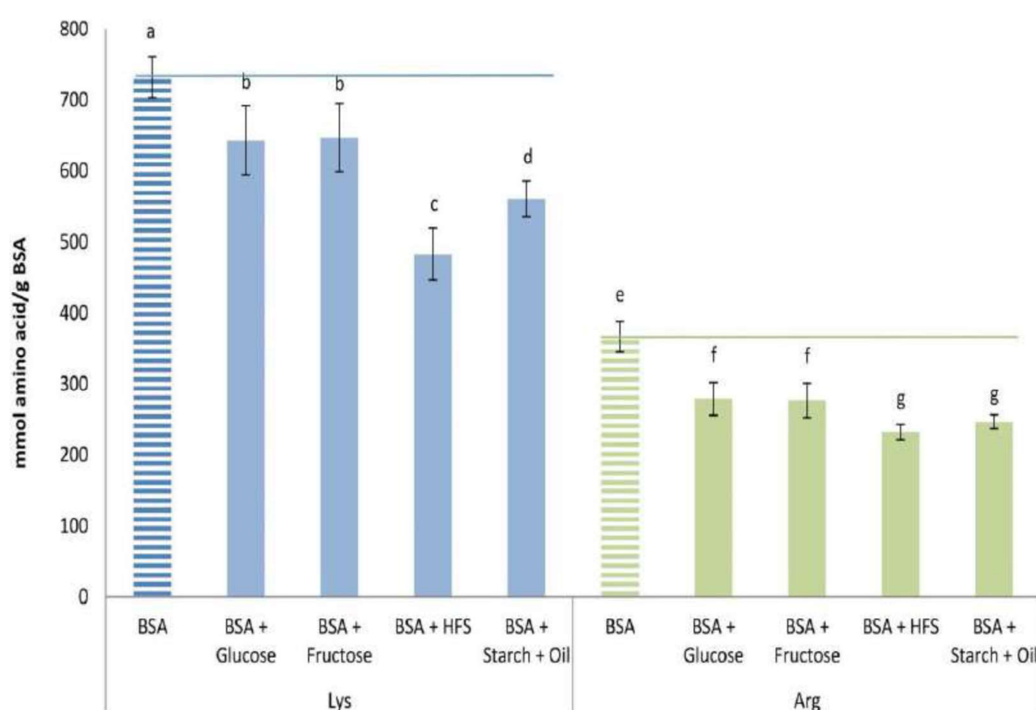

Figure 1. Amino acid content (lysine (lys) and arginine (arg)) of an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and high-sugar meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose] submitted to *in vitro* simulated oral-gastrointestinal digestion. Data are presented as mean (n = 6) ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between samples.

Table 1. Content of other amino acids (AAs) and their percentage of blockage in an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and sugar-containing meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose] submitted to *in vitro* simulated oral-gastrointestinal digestion.

		Sugar-containing meals						Average meal	
	BSA (control)	BSA + Glucose		BSA + Fructose		BSA + HFCS		BSA + Starch + Oil	
Other AAs	µmol/g BSA	µmol/g BSA	% blocked	µmol/g BSA	% blocked	µmol/g BSA	% blocked	µmol/g BSA	% blocked
Aspartic	890.05±35.6^{a}	682.2±54.58^{b}	23.35	690.64±53.1^{b}	22.40	641.7±0.6^{b}	27.90	N.D.	100
Threonine	503.46±23.07^{a}	407.97±32.73^{b}	18.97	415.7±33.71^{b}	17.43	377.17±7.56^{b}	25.08	N.D.	100
Serine	450.02±19.89^{a}	324±23.39^{b}	27.78	334.84±28.39^{b}	25.59	292.37±6.56^{b}	35.03	N.D.	100
Glutamic	1112.25±42.76^{a}	929.92±71.96^{b}	16.39	942.36±70.7^{b}	15.27	858.69±10.74^{b}	22.80	N.D.	100
Glycine	460±16.11^{a}	258.69±21.7^{b}	43.76	262.77±23.49^{b}	42.88	235.27±0.99^{b}	48.85	N.D.	100
Alanine	684.59±29.43^{a}	556.97±46.25^{b}	18.64	565.69±44.83^{b}	17.37	507.68±12.73^{b}	25.84	N.D.	100
Cysteine	171.15±14.12^{a}	153.92±14.33^{a}	10.07	149.63±12.57^{a}	12.57	138.66±4.38^{a}	18.98	N.D.	100
Valine	573.35±17.3^{a}	457.33±34.41^{b}	20.24	466.4±24.65^{b}	18.65	425.41±21.28^{b}	25.80	N.D.	100
Methione	21.24±1.79^{a}	11.74±1.44^{b}	44.70	31.36±1.86^{c}	0.00^{c}	41.06±5.62^{d}	0.00	65.53±4.18^{e}	0.00
Isoleucine	236.54±12.34^{a}	150.26±6.33^{b}	36.47	155.61±6.81^{b}	34.22^{b}	129.94±11.26^{c}	45.07	134.54±6.69^{c}	43.12
Leucine	636.92±30.03^{a}	521.32±15.22^{b,c}	18.15	543.89±22.01^{b}	14.61^{b}	486.72±13.19^{c}	23.58^{c}	551.76±49.56^{b}	13.37
Tyrosine	154.55±13.33^{a}	71.02±7.71^{b}	54.05	81.21±7.81^{b,c}	47.45	88.88±2.3^{c}	42.49	75.57±3.28^{b}	51.11
Phenylalanine	86.07±12.33^{a}	39.52±4.76^{b}	54.08	46.34±5.07^{b}	46.16	68.91±7.39^{c}	19.93	63.47±5.97^{c}	26.25
Histidine	237.95±13.13^{a}	197.45±17.29^{b}	17.02	199.48±17.57^{b}	16.16	167.28±12.56^{c}	29.70	171.08±6.24^{c}	28.10
Total AAs	7320±310^{a}	5460±470^{b}	**25.41**	5690±220^{b}	**22.24**	5280±110^{b}	**27.88**	1900±70^{c}	**74.06**
% Lys+Arg of the total blocked		9.57%		10.87%		17.15%		5.35%	

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and duplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same row.

3.1.2. Early MRPs and AGEs

The formation of MRPs during the *in vitro* simulated oral-gastrointestinal digestion of the average meal and sugar-containing meals is shown in table 2. Regarding the MRPs produced in the early stages of the reaction, fructosamine was detected. This compound was significantly ($p < 0.05$) found, in descending order, in the average meal, HFCS- and glucose-containing meals. Fructosamine was not detected in the meal prepared with BSA and fructose. On the other hand, the analyses of the advanced MRPs showed the presence of CML and MGO-derivatives AGEs determined by ELISA, and fluorescent adducts by fluorescent measurement, differed among the samples (table 2). The meal constituted by BSA and fructose was the only one that presented significant ($p < 0.05$) formation of CML. Of interest, the meal with BSA and HFCS did not showed formation of CML despite the presence of fructose. However, MGO-derivatives AGEs were significantly ($p < 0.05$) detected in both glucose- and fructose-containing meals but no significant ($p > 0.05$) differences were found between these samples. Again, the meal with BSA and HFCS did not show MGO-derivatives AGEs despite the presence of both fructose and glucose. Furthermore, fluorescents MRPs during the digestive process were detected only in the meal composed of BSA, starch and oil

(average meal), and that prepared with BSA and HFCS. The average meal exhibited the highest amount of fluorescents adducts.

Table 2. Formation of Maillard reaction products (MRPs) during *in vitro* simulated oral-gastrointestinal digestion of an average meal [BSA (0.2 mM) + starch (≈314 mM glucose) + oil] and high-sugar meals [BSA (0.2 mM) + glucose (43 mM); BSA (0.2 mM) + fructose (43 mM); BSA (0.2 mM) + high fructose corn syrup (HFCS) (43 mM sugars, 60% fructose : 40% glucose].

		Early MRPs	Advanced MRPs		
		Fructosamine (µg DMF eq./ml)	CML (µg eq./ml)	MGO-derivative AGEs (µg eq./ml)	Fluorescent adducts (RFU)
	BSA	N.D.	3.91 ± 0.90^{a}	10.22 ± 1.57^{d}	1316.17 ± 54.71^{a}
Sugar-containing meals	BSA + Glucose	29.64 ± 1.05^{a}	4.12 ± 0.56^{a}	12.20 ± 1.63^{a}	1387.17 ± 75.96^{a}
	BSA + Fructose	N.D.	5.03 ± 1.09^{b}	12.23 ± 1.50^{a}	1039.78 ± 52.68^{b}
	BSA + HFS	42.57 ± 4.70^{b}	1.63 ± 0.37^{c}	$8.96 \pm 1.27^{b,d}$	2270.17 ± 119.55^{c}
Average meal	BSA + Starch + Oil	332.89 ± 10.37^{c}	3.17 ± 1.02^{c}	7.16 ± 1.11^{b}	9282.50 ± 188.33^{d}

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and triplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same column.

3.2. Progression of the MR in simple amino acid systems

3.2.1. Early MRPs and non-fluorescent AGEs

In order to better understand the formation of MRPs during the human digestion, simple systems constituted by lysine or arginine and glucose or fructose were studied. Table 3 presents the contribution of these amino acids and sugars to the intestinal MRPs' formation. Fructosamine was detected only in those systems containing high sugar concentrations (314 mM). The presence of fructose significantly ($p < 0.05$) caused greater formation of fructosamine (3 and 6 times more, for lysine and arginine systems, respectively) than glucose suggesting that our assay can also detect Heyns products. Lysine system presented higher amount of fructosamine than arginine ($p < 0.05$) when incubated with glucose (314 mM). Fructosamine was of the same order of magnitude ($p > 0.05$) for both amino acids in presence of fructose (314 mM). Fructosamine was not detected in any of the systems with physiologiccal concentration of either glucose or fructose (43 mM).

In the analysis of advanced MRPs measured by ELISA (table 3), the presence of fructose led to a significantly ($p < 0.05$) greater amount of these advanced compounds than glucose. Lysine incubated with 43 mM of fructose showed 14.69±2.19 µg CML eq./ml while 20.21±3.55 µg CML eq./ml ($p < 0.05$) was detected in presence of 314 mM, and both values were significantly higher (p <

0.05) than the control (lysine, 11.42±1.84 µg eq./ml). This result was confirmed by the identification of CML by using an amino acid analyzer as described in section 2.6.2. An amount of 1.86 µM for CML was detected in the intestinal lysine and fructose (314 mM) system, and also several unidentified peaks of compounds formed were revealed (chromatograms provided as supplementary material, figure 1S). In addition, it was already observed significant ($p < 0.05$) formation of CML (13.16±1.75 µg/ml) within 1 hour of incubation of lysine and fructose (43 mM) at intestinal conditions (pH 7, 37ºC) compared to the control (lysine, 9.77±1.20 µg/ml). In contrast, MGO-derivatives AGEs were detected only in the arginine systems containing high fructose concentrations (314 mM).

Table 3. Contribution of the basic amino acids, arginine (Arg, 40 mM) and lysine (Lys, 40 mM) to the formation of Maillard reaction products (MRPs) mimicking lumen intestinal conditions *in vitro* (2h 30 min, pH 7, 37 ºC) when incubated with glucose or fructose at 43 or 314 mM under the conditions described in section 2.3.

	Early MRPs	Advanced MRPs
	Fructosamine (µg DMF eq./ml)	MGO-derivative AGEs (µg eq./ml)
Lys	N.D.	$5.63 \pm 0.17^{a,d}$
Lys + Glucose (43 mM)	N.D.	$5.75 \pm 0.76^{a,d}$
Lys + Glucose (314 mM)	51.21 ± 2.53^{a}	$4.53 \pm 0.47^{b,c,d,e}$
Lys + Fructose (43 mM)	N.D.	5.79 ± 0.71^{a}
Lys + Fructose (314 mM)	165.43 ± 5.78^{b}	$5.29 \pm 0.57^{a,b,d}$
Arg	N.D.	$4.54 \pm 0.24^{b,c,e}$
Arg + Glucose (43 mM)	N.D.	3.71 ± 0.53^{c}
Arg + Glucose (314 mM)	30.77 ± 1.24^{c}	$5.07 \pm 0.56^{d,e}$
Arg + Fructose (43 mM)	N.D.	$4.44 \pm 0.76^{c,e}$
Arg + Fructose (314 mM)	178.99 ± 15.00^{b}	18.86 ± 0.97^{f}

N.D. Not Detected. Data are presented as mean ± standard deviation; duplicate of sample preparation and triplicate of analysis (n = 6). Different letters indicate significant differences ($p < 0.05$) between the samples of the same column.

3.2.2. Fluorescent AGEs

The formation of fluorescent adducts in the amino acid systems incubated under intestinal conditions is shown in Figure 2. All systems composed of lysine and glucose or fructose presented significant ($p < 0.05$) formation of fluorescent adducts (≈ 20% increase) but differences were not detected ($p > 0.05$) among them. In the case of the amino acid systems constituted by arginine, a very significantly lower fluorescence ($p < 0.05$) was found for the arginine control compared to lysine control. The formation of fluorescent adducts experimented a 3-fold increase for those arginine systems prepared

with glucose and a 5-fold increase for those prepared with fructose. A dose-response relationship was not detected for any of the systems

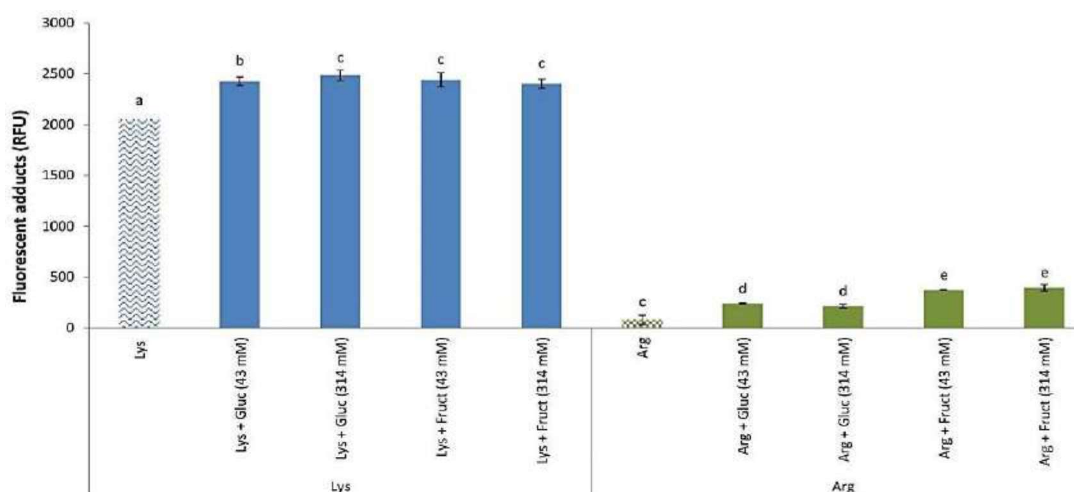

Figure 2. Formation of fluorescent adducts during incubation of basic amino acids (lysine (lys) and arginine (arg), 40 mM) with glucose (gluc) or fructose (fruct) at 43 and 314 mM under lumen intestinal conditions (2h 30 min, pH 7). Data are presented as mean (n = 6) ± standard deviation. Different letters indicate significant differences ($p < 0.05$) between samples.

4. DISCUSSION

The data generated confirm the occurrence of the MR during the digestive process of an average meal (BSA, starch and oil) and sugar-containing meals (BSA and glucose or fructose or HFCS); as well as, during incubation of systems with only a reactive amino acid and fructose within the time period expected for a normal digestion and assuming delay of the sugar absorption due to its malabsorption or fat presence. As expected, the formation of the MRPs differed depending on the nature of the meal.

The loss of free amino acids is the first indicator of the occurrence of the MR during the digestion process of the meals (table 1 and figure 1). During gastrointestinal digestion of dietary proteins (BSA), amino acids and peptides can be released during the gastric and intestinal steps by chemical and enzymatic hydrolysis. The amino groups, from the released amino acids and peptides, are susceptible to react with reactive carbonyl groups present in the intestinal lumen. Therefore, the reaction comprises not only the formation of MRPs from the amino groups of the arginine and lysine side chains, but also the alpha amino groups of all the released amino acids (figure 3). Basic amino acids (arginine, lysine) and others with different polarity (glycine, serine, tyrosine, isoleucine and

phenyl alanine) were highly blocked. These results are in line with data previously reported for amino acid reactivity in the MR at different studied conditions (Golon, Kropf, Vockenroth, & Kuhnert, 2014; Kwak & Lim, 2004; Mennella, Visciano, Napolitano, del Castillo, & Fogliano, 2006; P. Yu, Xu, & Yu, 2016).

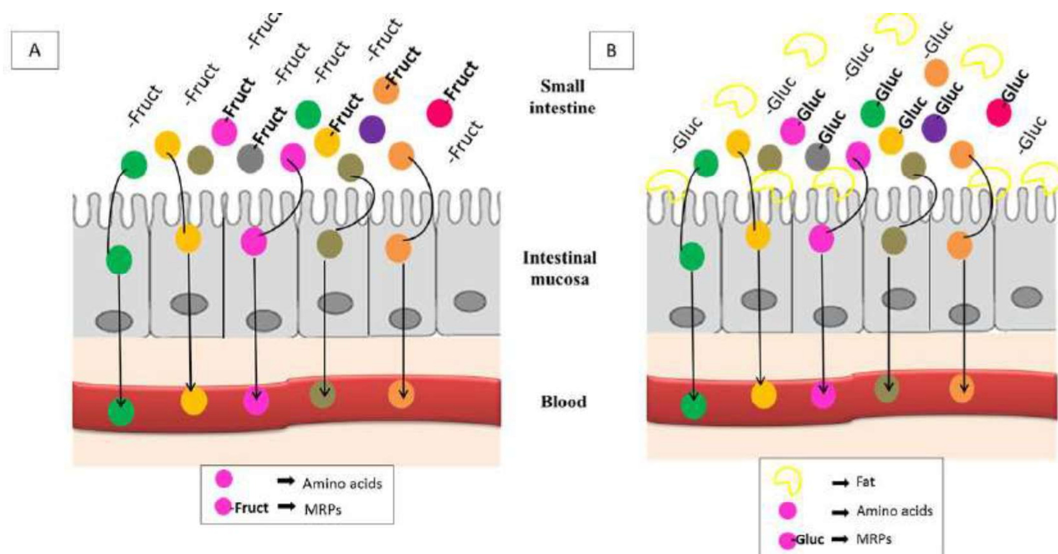

Figure 3. Formation of Maillard reaction products (MRPs) in the intestinal lumen through the reaction between different amino acids released from the BSA protein during the digestive process and (A) fructose at physiological concentrations (43 mM) under malabsorption state and (B) glucose at a maximum theoretical concentration released from the complete digestion of starch of an average meal (314 mM) containing fat which modulates the intestinal epithelial membrane.

Of interest, it has been described that in malabsorption state, the fructose not absorbed in the intestine may react with tryptophan from food through the MR and block its absorption, which causes a potential reduction in the synthesis of serotonin and thereby symptoms of depression (Huether, Kochen, Simat, & Steinhart, 1999; Ledochowski, Widner, Bair, Probst, & Fuchs, 2000). It is described that fructose possess higher proportion of open-chain form compared to glucose and therefore greater active form to react in the MR (Laroque et al., 2008). This may be the reason why HFCS, which presents both sugars combined in a 60:40 ratio (fructose : glucose), achieved higher loss of amino acids than glucose and fructose individually This result might support recent epidemiological investigations describing a relationship between intake of HFCS-containing beverages and development of asthma, bronchitis and arthritis as a consequence of glycation reactions within the lumen of the intestine (DeChristopher et al., 2015a, 2015b, 2016).

Furthermore, the remarkable loss of amino acids in the average meal may be due to its content in starch and oil. The high glucose amount potentially released from the starch (≈314 mM) during the digestion suggests greater formation of MRPs. Likewise, many researchers reported a positive role of dietary lipids promoting the MR by the formation of lipid-derived reactive carbonyls from the lipid oxidation (Hidalgo, León, & Zamora, 2016; L. Yu et al., 2016; Zamora & Hidalgo, 2011). *In vivo* digestion, however, is rapidly followed by absorption leaving little time for the MR unless there is a significant simultaneous sugar malabsorption. The latter is likely during ingestion of large loads of HFCS-rich beverages or foods containing fat.

The simplified pathways for the formation of early and advanced MRPs during the digestive process of the studied meals and amino acid systems are schematically presented in the figure 4. Briefly, MR includes an initial formation of Schiff's base, followed by intermolecular rearrangement and conversion into Amadori/Heyns products. They undergo further processing to form a heterogeneous group of amino acid-bound moieties, such as non-fluorescent adducts (e.g., CML) and cross-linking fluorescent products (e.g., pentosidine), called AGEs. Pathways of AGE formation involve glucose autoxidation through the generation of α-oxoaldehydes, such as MGO, which is a major precursor of AGEs. Of note, during the digestive process different length peptides with different glycation reactivities are also released along with the release of free amino groups.

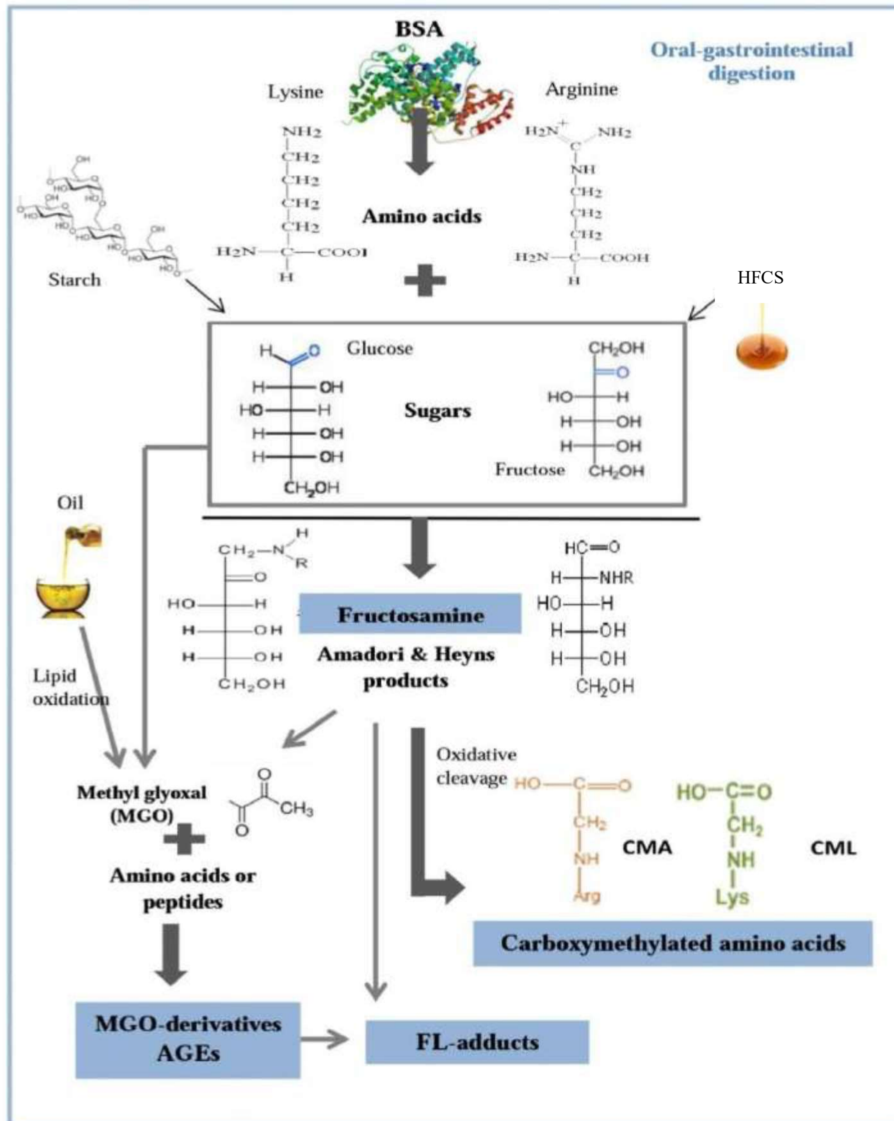

Figure 4. Simplified pathways for the formation of early and advanced Maillard reaction products (MRPs) during the digestive process of an average meal (BSA, starch and oil), high-sugar meals (BSA and glucose or fructose or high fructose corn syrup (HFCS) and simple amino acids systems (lysine or arginine and glucose or fructose), as described in section 2.2 and 2.3

Fructosamine, an early MRP, was found in most meal systems, except in those containing only BSA and fructose. Formation of fluorescent adducts occurred mostly in the meal systems with the highest formation of fructosamine, which suggests pathways going mostly into the formation of cross-linking fluorescent products such as pentosidine rather than CML. The non-fluorescent AGEs (CML and MGO-derivative AGEs) were found exclusively in the meal prepared with BSA and fructose (43 mM)

(table 2) which is supported by the previously commented results. The current results confirm the formation of non-fluorescent products during the human digestive process. To date, there are not studies in this area. New evidence is presented in this study. As regards the fluorescent adducts with characteristic fluorescence of AGEs, 7-fold and 2-fold increases were detected for the meals containing starch and HFCS, respectively, during the digestive process (table 2). These results are supported by the analysis of amino acid systems incubated at conditions similar to those in the intestinal lumen (figure 2). In line with these findings, a very recent study showed formation of fluorescent adducts, when incubating free amino acids (arginine, lysine and glycine, 50mM) with fructose or glucose (50 mM) under conditions compatible with those of the digestive system (Bains & Gugliucci, 2017). Unlike what these authors described, we observed high fluorescence of the lysine solution itself (control). This may be due to the capacity of lysine in high concentrations to form self-assembling aggregates (Homchaudhuri & Swaminathan, 2001). Moreover, we could not demonstrate a dose dependent formation of fluorescent AGEs between fructose or glucose and amino acids. The analysis of fluorescent AGEs by measurement of fluorescence intensity is somehow limited and nonspecific. Pentosidine, crossline and pyrropyridine may be some of the fluorescent cross-linked AGEs formed during digestive process (Nursten, 2005). Pentosidine can be formed by the reaction of lysine and arginine, forming a fluorescent crosslink with any of several carbohydrate precursors including glucose (Hatfield, 2005). The identification of several other unidentified peaks in the lysine and fructose (314 mM) system (supplementary material, figure 1S) suggests the formation of compounds that require further studies.

Our finding on formation of MRPs, both early and advanced, during conditions mimicking the digestive process, including concentration of reactants and time of reaction, supports the concept of intraluminal generation of AGEs as another source of exogenous AGEs (DeChristopher et al., 2016). Currently, most workers in the field have suggested that exogenous AGEs come already preformed in certain AGE-rich food we ingest (Uribarri et al., 2010). The fate of exogenous and intestinally generated AGEs is an area that requires intensive further study. We know that a small percent of the ingested AGEs are absorbed into the circulation becoming incorporated into the body pool of AGEs and therefore contributing to an overall state of chronic oxidative stress and inflammation (Vlassara & Uribarri, 2014) and probably the same happens with the compounds generated intraluminally. The largest amount of unabsorbed AGEs will cotinue its transit into the colon where it can interact with the microbiota, react with AGE receptors (RAGE) within the colonic wall initiating a localized inflammation that may eventually propagate systemically or simply be excreted in the stool.

Several studies have reported human colonic microbiota degrading selected glycated amino acids. Higher degradability of early MRPs than advanced products have been shown due to the their lower chemical stability (Hellwig et al., 2015). Some of these MRPs can play a role in colon toxicology, through increased colonic protein fermentation, and may also act as systemic toxicants and inducers of inflammation (Tuohy et al., 2006). Low-grade intestinal inflammation for example plays a key role in the pathophysiology of irritable bowel syndrome (Sinagra et al., 2016). Moreover, these MRPs can modulate gut microbiota composition (Seiquer, Rubio, Peinado, Delgado-Andrade, & Navarro, 2014; Tuohy et al., 2006) and they may act as a growth substrate for detrimental bacteria such as some Clostridium and Bacteroides species (Mills et al., 2008). A relationship between unabsorbed AGEs and the greater prevalence of allergies/inflammation has recently been postulated (Smith, Masilamani, Li, & Sampson, 2015). Moreover, a common colonic bacteria, E. Coli, produces, accumulates and releases AGEs into the medium (Cohen-Or, Katz, Ron, Shanley, & Wheeler, 2011). Therefore they may adversely alter their colonic microbial composition, potentially enhancing their risk for the development of metabolic diseases such as obesity and type 2 diabetes (Cani, Osto, Geurts, & Everard, 2012). As mentioned above, fructose reaction with tryptophan can prevents its absorption decreasing levels of serotonin and perhaps inducing depression (Huether et al., 1999; Ledochowski et al., 2000). The marked loss of amino acids released from proteins though the MR during digestion demonstrated in our data an interesting but usually forgotten issue, which is the loss of the nutritional value of foods resulting from the MR.

5. CONCLUSIONS

Novel information regarding the nature of MRPs formed during the digestive process of simplified average meal and sugar-containing meals was obtained. Content of different amino acids decreased after digestion. Early MRPs and fluorescent adducts were detected in digests of those meals containing HFCS and starch. CML and MGO-derivatives AGEs were found in the meals composed of fructose. Moreover, fluorescent adducts were detected in the control intestinal systems (amino acids alone). According with the results on simplified amino acids systems nature of both components, amino acids and sugars, has an impact on the formation of specific MRPs. Results on the identity of the MR found in simplified amino acids systems and meal agreed. This is the first study proposing the formation of a non-fluorescent AGE associated to the pathogenesis of diabetes and other intestinal inflammatory diseases during digestion of simplified meals containing fructose. On the other hand, our results support that the bioavailability of other amino acids beside arginine and lysine may be greatly reduced by MR during the digestion process which may affect human health

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Author's contributions: "del Castillo, M.D. and Uribarri, J. designed the experiments, supervised the investigation and revised the manuscript. Martinez-Saez, N., Fernandez-Gomez, B., and Cain, W. performed the experiments and analyzed the data; Martinez-Saez, N. is the principal author of the investigation since it is part of her PhD thesis supervised by del Castillo, PhD."

Conflicts of Interest: Declare conflicts of interest or state "The authors declare no conflict of interest."

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Supplementary material

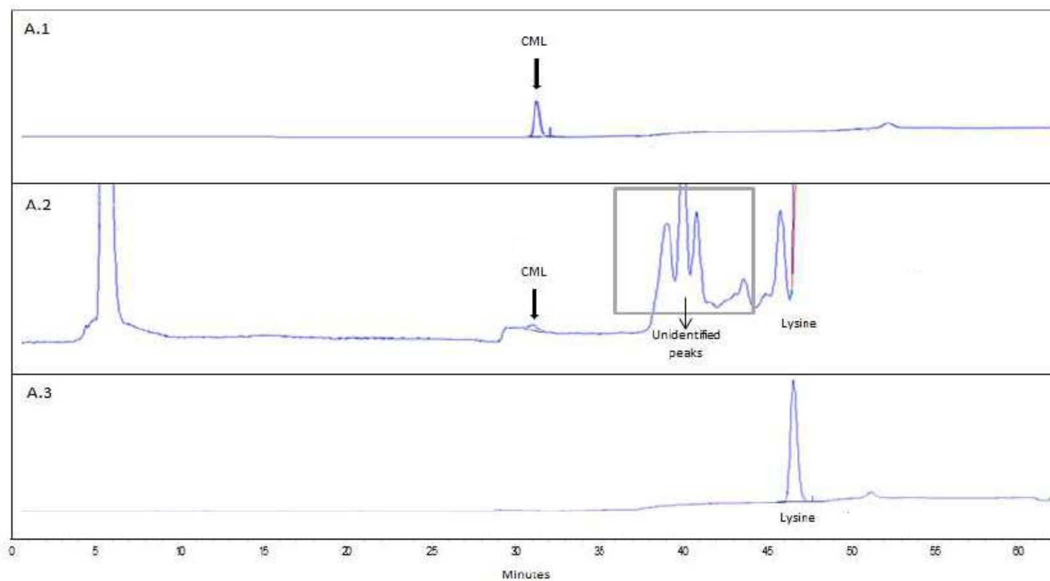

Figure 1S. Identification of carboxymethyl lysine (CML) by amino acid analysis as described in Materials and Methods, in (A.1) a solution of standard CML (5 µg/ml = 26.7µM) and in (A.2) an intestinal system prepared with lysine (40 mM) and fructose (314 mM). CML appeared with a retention time of 31.5 min and unidentified peaks were also present in A.2. Intestinal control of lysine (40 mM) was also included (A.3) which did not show no significant peak other than that of the native amino acid.