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Research Article

Assessment of bioaccesibility of precursors of advanced glycation end products in puddings under *in vitro* digestive system

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Abstract
Advanced glycation end products (AGEs) are potentially chemical hazardous compounds. Digestive system conditions may effect AGEs formation. Despite puddings have low AGEs content, the effect of *in vitro* digestion on AGEs formation is unknown. This study aims to determine the formation of AGEs precursors as Glyoxal (GO) and Methylglyoxal (MGO) in puddings under *in vitro* digestion. For this purpose, GO and MGO levels of puddings were determined by High-Performance Liquid Chromatography. The amount of GO and MGO in samples ranged between 6.3 to 18.8 µg /100 g and 5.8 to 24.4 µg / 100g, respectively. After *in vitro* digestion, GO and MGO levels were increased to between 18.1 to 47.7 µg / 100g and 8.5 to 31.6 µg/100 g, respectively. Our results indicate that puddings have low AGEs precursors this may be due to bounding capability of milk proteins, water binding capacity of starch, low heat treatment and sugar type.

Keywords: Glyoxal; Methyglyoxal; Pudding; *in vitro*; Bioaccesibility.

Pudinglerde İleri Glikasyon Son Ürünleri Öncüllerinin *in Vitro* Sindirim Sisteminde Biyoerişilebilirliklerinin Belirlenmesi

Öz
İleri glikasyon son ürünleri(AGE) potansiyel kimyasal tehlikeli bileşiklerdir. Sindirim sistemi koşulları da AGE oluşumuna etki edebilir. Pudinglerin düşük AGE içeriğine sahip olduğu bilinmesine rağmen, *in vitro* sindirimin AGE oluşumuna etkisi bilinmemektedir. Bu çalışma *in vitro* sindirim koşullarında pudinglerde AGE öncülü olan Glioksal(GO) ve metilglioksal(MGO) oluşuşumun belirlenmesini amaçlamaktadır. Bu amaçla, pudinglerde GO ve MGO seviyeleri yüksek performanslı sıvı kramatografisi (HPLC) ile belirlenmiştir. Örneklerin GO ve MGO

miktarları sırasıyla 6.3 ila 18.8 µg /100 g ve 5.8 ila 24.4 µg /100 g'dır. *In vitro* sindirimden sonra GO ve MGO seviyeleri sırasıyla 18.1 ila 47.7 µg /100 g'a ve 8.5 ila 31.6 µg /100 g'a kadar artmıştır. Sonuçlarımız pudinglerde süt proteinlerinin bağlama kapasitesi, nişastanın su tutma kapasitesi, düşük ısıl işlem ve şeker türünden dolayı AGE öncüllerinin düşük olabileceğine işaret etmektedir.

Anahtar Kelimeler: Glioksal, Metilglioksal, Puding, İn Vitro, Biyoerişebilirlik.

1. Introduction

Advanced glycation end products (AGEs) are heterogenous group of molecules that can be produced from non-enzymatic reactions between reducing sugars and free amin groups of proteins and lipids [1]. These reactions are also known as Maillard Reaction (MR). At the initial state of MR, the Schiff base is formed by condensation reaction between amine-carbonyl groups. Then Schiff bases undergoes rearrangements to form more stable α-ketoamine, which are Amodori products. Degradation of Amadori products results in the formation of dicarbonyl compounds (α-DCs), which are AGE precursors, such as glyoxal (GO) and methylglyoxal (MGO) [2]. The reactions of AGEs precursors with lysine, N-ξ-carboximethyllysine is formed from GO and N-ξ-carboxiethyllysine from MGO., N-carboxymethyl lysine, Nε-(carboxylethyl)-Llysine, methylglyoxal-lysine dimers, methylglyoxal-lysine dimers, pentosidine and pyrraline are well-known AGEs [3].

AGEs are potentially chemical hazardous compounds that can be formed endogenously or exogenously [4]. AGEs normally occur endogenously during metabolism, however the accumulation of AGEs in organs, tissues and body fluids may cause oxidative stress, inflammation and abnormalities in protein structures and functions [5]. Recent studies found out that excessive AGEs exposure may cause variety of diseases including inflammation, aging, kidney disease, cardiovascular diseases, neurodegenerative diseases, diabetes, and cancer [3,6,7]. In this context, it is highly essential to reduce AGEs exposure by limiting exogen AGE intake [8]

Diet is the main contributor of endogenous AGE exposure [9]. AGEs can be easily formed in foods due to MR, caramelization, sugar autooxidation and lipid peroxidation [10]. Also, food composition, food processing conditions, high temperature, moisture content and storage conditions may contribute AGEs formation in foods [11]. Determination of AGEs formation in foods is great of importance and several studies have focused on this topic with various foods [7,12].

Puddings are semisolid starch-based pastes which are characterized as a suspension of deformable particles dispersed in a continuous phase containing milk proteins and a gelling agent [13]. Due to their sweet tastes, puddings have strong hedonic appeal, especially for children [14]. The main ingredients of pudding are milk, starch, sugar, and oil. All ingredients have provided desirable texture, aroma, taste and viscosity to puddings [13]. Previous studies indicated that puddings had relatively low AGEs levels due to high moisture levels [7,15]. However, gastrointestinal digestion conditions may affect AGEs formation. Digestive environment factors such as pH, temperature, minerals, and presence of oxygen may increase AGEs formation [16]. To our knowledge, there is no study focused on determining AGEs formation under gastrointestinal system conditions in puddings. There is a need for such study for calculate estimated AGEs exposure with pudding consumption. The present study aims to determining the bioaccessibility of GO and MGO in puddings under *in vitro* digestive system.

2. Materials and Method

2.1. Material

Methanol, GO, MGO, sodium acetate, acetonitrile, 4-nitro-1,2- phenylenediamine, alpha-amylase (1.5 U/mg), pepsin (≥250 units/mg solid), lipase (100-500 units/mg protein), pancreatin (8 × USP), $NaHCO_3$, $CaCl_2 \cdot 2H_2O$, NaCI, KCl serum albumin (bovine), bile salts, uric acid, urea, mucin, fructose, glucose, sucrose, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sampling

In the present study, 15 different pudding samples, 1 whole-fat milk and 1 semi-fat milk were purchased. The content of the pudding and samples are presented in Table 1. Puddings in powder form were prepared with whole-fat milk, or semi-fat milk as indicated instructions in the label.

2.3. Extraction and Derivatization of Glyoxal and Methylglyoxal

Extraction method used for GO and MGO in samples was described by Mahar et al [17]. Each ground sample was weighed in a 50 mL falcon tube to be 2 g, and 25 mL of methanol was added on it. The samples were extracted with an ultra-thorax homogenizer and centrifuged at 8000 rpm for 5 minutes. One mL of liquid sample, 1 mL of CH_3COONa buffer (0.1 M, pH: 3), and 0.5 mL of derivatization solution (1% 4-nitro-1,2-phenylenediamine in methanol) were added in10 mL glass tube. Mixture was incubated at 70 °C for 20 minutes. The samples were filtered using a 0.45 µm cellulose acetate filter and injected into High-Performance Liquid Chromatography (HPLC).

2.4. Determination of Glyoxal and Methylglyoxal

HPLC conditions for the determination of GO described by Cengiz et al. were used [18]. The HPLC system consisted of a Shimadzu LC 20AT pump with a Shimadzu SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan). The mobile phase was consisted of MeOH:water: ACN (42:56:2 v/v/v). The wavelength was set to 254 nm. An Inersil ODS-3 column was used with a flow rate of 1 mL/min and the column oven temperature was set 30 °C.

2.5. *In vitro* Bioaccesibility of GO and MGO

Bioaccessibility of the GO and MGO in pudding samples were determined using an *in vitro* simulated digestive system. This method was a modified version of that described previously by Yaman et al [19]. Gastric, duodenal, bile juices and saliva were prepared as 1000 mL and shown in Figure 1.

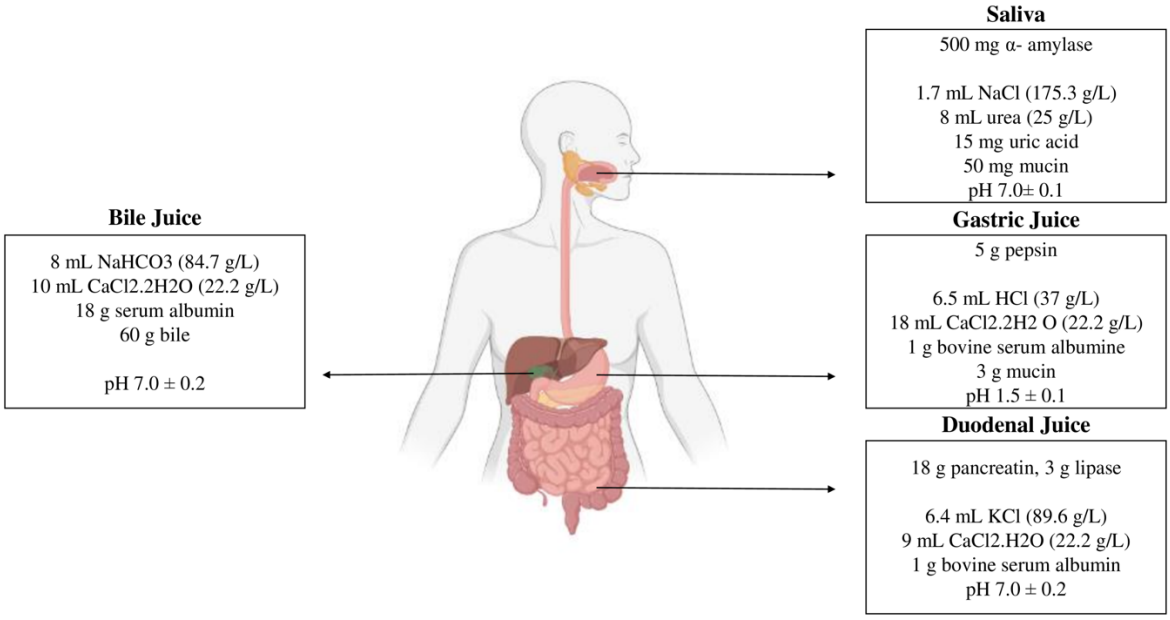

Figure 1. Saliva, gastric, duodenal, and bile juices used in the in vitro human digestion model

Five grams of sample was mixed with 5 mL saliva juice in a 50 mL falcon tube and incubated for 5 min at 37 °C in a shaking water bath. 10 mL gastric juice was added and incubated for 30 min at 37 °C in a shaking water bath. 5 mL bile juice was added and then, 10 mL duodenal juice solution was added. Intestinal phase was incubated for 2 hours at 37 °C in a shaking water bath. The final volume was adjusted with deionized water to 50 mL and the solution was centrifuged for 5 min at 8000 rpm.

2.6. Determination of Sugars Using HPLC

The extraction method of sugars were used described by Richmond et al. (1981). Five g of sample was weighed into a 50 mL plastic falcon tube. Then, extracted with 25 mL deionized water and volume was completed to 50 mL with deionized water. After that, the mixture was centrifuged at 8000 rpm for 10 min. The samples were filtered with a 0.45 µm cellulose acetate filter and injected into the HPLC.

HPLC system coupled with Shimadzu RI-20A (Shimadzu Corporation, Kyoto, Japan) refractive detector was used for the determination of sugar in cookies. The mobile phase composed of acetonitrile: water (80:20 v/v). The Separation was achieved using Agilent NH_2, 250x4.6 mm, 5 µm column (Santa Clara, CA, USA) with 1 ml/min flow rate. Oven temperature was 40 °C [20].

2.7. Statistical Analysis

Multivariate statistical analyses were performed to associate the sugar type and content and the formation of GO and MGO. The results were discussed based on the correlation between sugars and GO and MGO values before and after digestion. All statistical analyses were conducted using JMP PRO 16.

3. Results and Discussion

The chromatograms of GO and MGO standards and pudding sample are shown in Figure 2 and Figure 3.

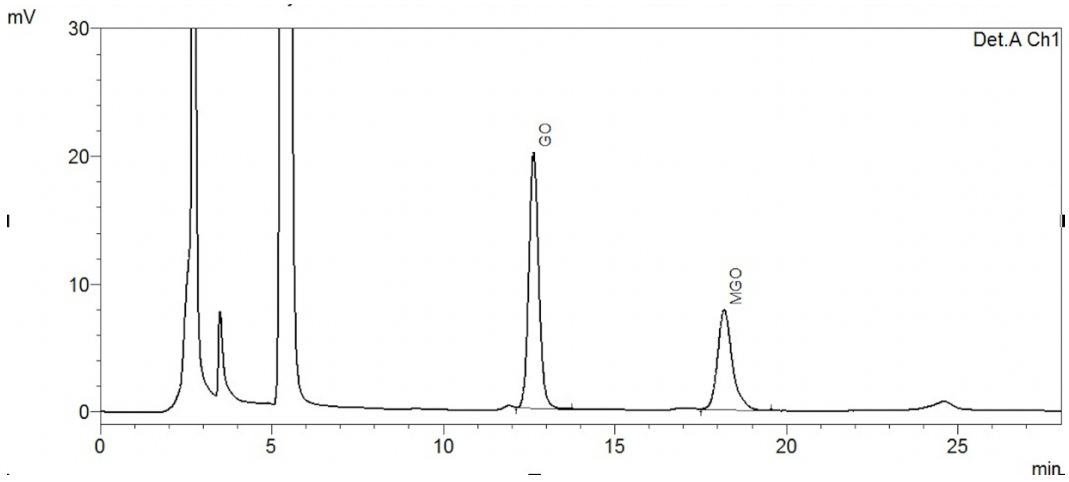

Figure 2. HPLC chromatogram of GO and MGO standards.

Table 1. The declared amounts of macronutrients, sugar and energy value on the label of pudding samples and milks.

Sample Number	Sample type and main content	Energy (kcal/100g)	Carbohydrate (g/100g)	Protein (g/100g)	Fat (g/100g)	Saturated Fat (g/100g)	Sugar (g/100g)
1	Pudding, chocolate	117 kcal	20	2.6	2.9	1.7	15.2
2	Pudding, chocolate, cup	125 kcal	19.3	3.3	3.3	1.86	12.76
3	Pudding, chocolate, mint	131 kcal	22.7	3.2	3	2	17.5
4	Pudding, chocolate, orange	129 kcal	22.4	3.2	3	2	17.1
5	Pudding, strawberry	106 kcal	18.6	2.4	2.4	1.7	14.2
6	Pudding, strawberry	121 kcal	19.6	3	3.4	1.9	14.2
7	Pudding, forest fruit	122 kcal	19	3.2	3.5	2.3	15
8	Pudding, banana, cup	117 kcal	19	2.8	3 g	2	13
9	Pudding, banana	113 kcal	18.8	2.9	2.9	1.9	14.3
10	Pudding, banana	104 kcal	17.8	2.4	2.6	1.7	13.8
11	Pudding, caramel	108 kcal	18.3	2.7	2.7	1.8	13.9
12	Pudding, vanilla, light	57 kcal	10.7	3.1	0.2	0	5.05
13	Pudding, vanilla	114 kcal	19.4	2.8	2.8	0	0
14	Pudding, vanillin, chocolate chips, cup	128 kcal	20.8	2.7	3.6	2.08	13.8
15	Pudding, coffee, cup	120 kcal	19.9	2.7	3	1.7	12.66

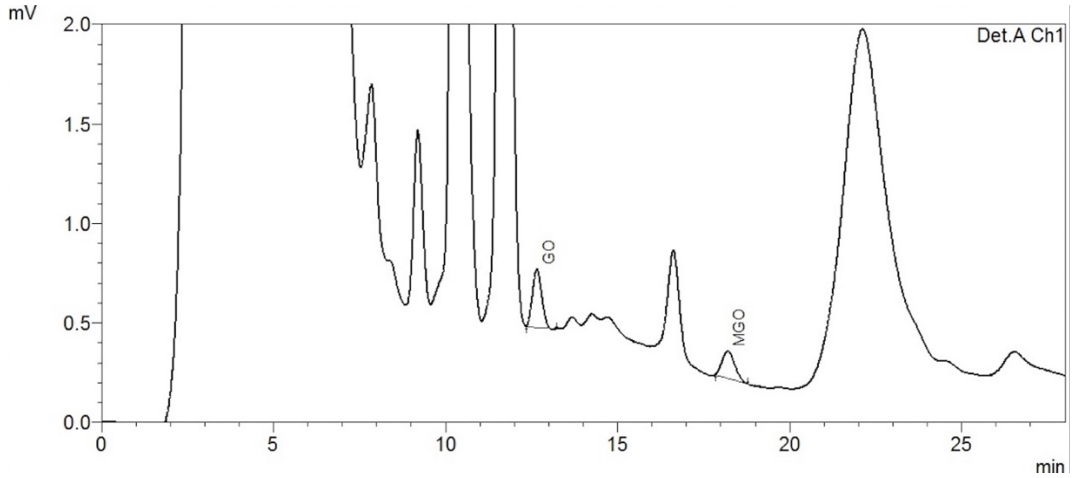

Figure 3. HPLC chromatogram of GO and MGO in sample 1.

Declared amount of macronutrients, sugar and energy values on the label of puddings are given in Table I.

As seen in from Table I puddings have high carbohydrate levels ranged between 18.3 to 22.7 g/100 g whereas relatively have low levels of fat and protein ranged between 0.20 to 3.6 and 2.4 to 3.3 g/100 g respectively. These carbohydrate levels may contribute AGEs formation in puddings via sugar autooxidation [21].

The measured sugar content of puddings and milks including fructose, glycose, sucrose and lactose are given in Table 2. As given in Table 2 pudding samples had high sucrose content up to 11.9 g/100g and had moderate amount of lactose up to 4.9 g/100 g whereas had low levels of fructose and glycose levels up to 0.9 and 2.1 g/100 g, respectively.

The measured amount of GO and MGO of pudding samples and milks at initial and after *in vitro* digestion are given in Table 3. Initial amount of GO and MGO of puddings were found to range between 6.3 to 18.8 µg / 100g and 5.8 to 24.4 µg/100 g, respectively. Also the amount of GO and MGO in were found 7.4 µg / 100 g and 5.8 µg/ 100 g for semi-fat milk and; 10.3 µg / 100g and 7.5 µg / 100g for whole-fat milk, respectively. Similar to our results, Urribari et al found GO levels between 110-160 nmol/100 mL and CML levels 1316 kU/100 mL in puddings [7]. Kureatani et al found CML levels in puddings 892 ± 32 nmol/g. In line with these studies, we found low levels of AGE precursors in puddings [15]. Since these studies indicated low AGEs or AGEs precursors in puddings, our results were similar in terms of having low levels of AGEs precursors.

There are some reasons that pudding samples had low GO and MGO values. Firstly, puddings are milk based pastes, milk proteins consist binding proteins. The binding proteins of milk may bound to GO and MGO, which results in decrease in the formation of AGEs [5]. Another factor that may affect the formation of GO and MGO in puddings would be related with its' moisture content. Starch, one of the main ingredients of pudding, plays critical role in the desired texture. Starch has functional properties including water binding capacity, swelling power, paste viscosity, solubility and retrogradation[6]. The water binding capacity of starch increases the moisture content, which slow down the formation of AGEs in puddings. Heat temperature is also an important determinant in the formation of α-DCs. α-DCs could be derived from reaction of MR, caramelization, or lipid peroxidation over 120 °C [7]. Puddings are prepared by heating at 85-95 °C for about 6 minutes[8]. The low cooking temperature may be another reason the low α-DCs content in puddings.

Additionally, sugar types may also affect the AGEs formation in puddings. The measured amount of sugar type content of pudding is given in Table 2. As seen from the table pudding samples had low fructose and glycose content up to 0.9 and 2.1 g/100 g respectively whereas had high sucrose and lactose amount up to 11.9 and 4.9 g/100g respectively. Several studies point out higher correlation between fructose and AGEs [9]–[11]. Also, correlations graphs of GO content versus sugars were given in the Figure 4.

Table 2. The measured amount of sugar type content of pudding and milks samples.

Sample Number	Sample type and main content	Fructose (g/100g)	Glycose (g/100g)	Sucrose (g/100g)	Lactose (g/100g)
1	Pudding, chocolate	nd	nd	11.7±0.5	4.6±0.2
2	Pudding, chocolate, cup	0.8±0.4	2.1±0.9	3.2±0.1	2.9±0.1
3	Pudding, chocolate, mint	nd	nd	11.9±0.5	0.01±0.0
4	Pudding, chocolate, orange	nd	nd	11.5±0.5	3.9±0.1
5	Pudding, strawberry	nd	nd	10.8±0.4	4.5±0.2
6	Pudding, strawberry	nd	nd	11.4±0.5	3.9±0.1
7	Pudding, forest fruit	nd	nd	9.5±0.4	4.4±0.1
8	Pudding, banana, cup	nd	nd	8.1±0.3	4.3±0.1
9	Pudding, banana	nd	nd	9.4±0.4	4.9±0.2
10	Pudding, banana	nd	nd	nd	nd
11	Pudding, caramel	nd	nd	8.4±0.3	4.1±0.1
12	Pudding, vanilla, light	nd	nd	nd	4.4±0.2
13	Pudding, vanilla	nd	nd	9.7±0.4	4.1±0.1
14	Pudding, vanillin, chocolate chips, cup	0.9±0.4	2.1±0.9	4.4±0.2	3.2±0.1
15	Puddind, coffee, cup	0.9±0.4	2.1±1.0	2.9±0.1	3.4±0.1
	Milk 1, whole fat	nd	nd	nd	4.2±0.1
	Milk 2, semi-fat	nd	nd	nd	4.4±0.2

Multivariate statistical analyses were performed to associate the sugar type and content and the formation of GO and MGO. Values are means ± range, n = 3.

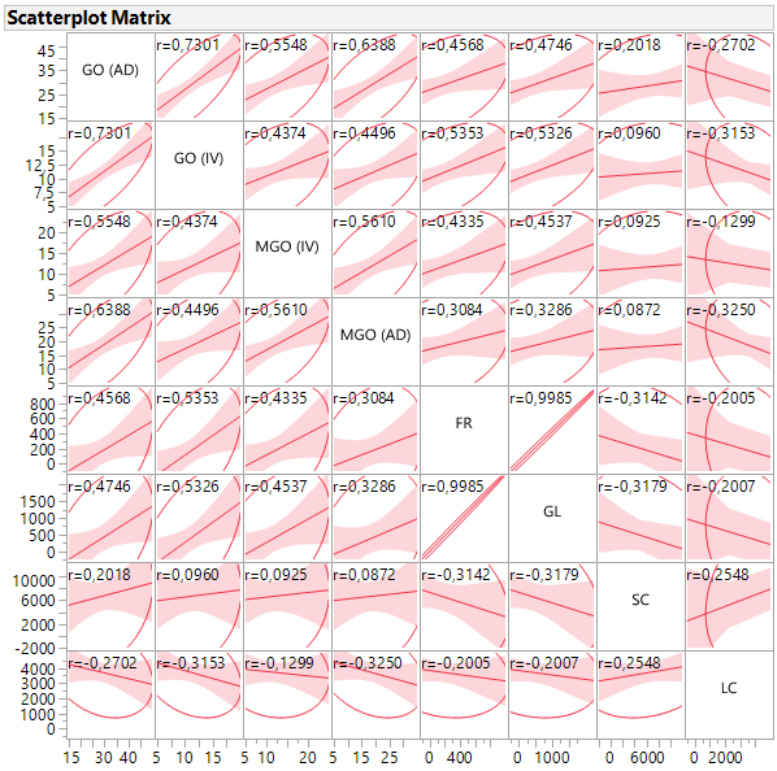

Figure 4. Correlations graphs and correlation coefficients of GO content versus sugars

As presented, while there was a positive correlation between fructose, glucose and sucrose content of pudding and initial GO content, negative correlation was observed between lactose and initial GO content. Effect of fructose and glucose content of puddings was found to be higher than that of sucrose. The *r* values of fructose and glucose were found to be as 0,4568 and 0, 4746, respectively. Since several studies point out higher correlation between fructose and AGEs [9-11] we thought that the high amount of lactose and sucrose and the low amount of fructose may be another factor affect the low GO and MGO values in puddings.

Table 3. GO and MGO contents of pudding samples and milks at initial and after digestion, and in vitro Formation.

Sample Number	Sample type and main content	Initial value GO (μg/100 g)	Initial MGO (μg/100 g)	After Digestion GO (μg/100 g)	After Digestion MGO (μg/100 g)	Formation %	
						GO	MGO
1	Pudding, chocolate	10.8±0.5	11.8±0.5	25.7±1.2	20.9±0.9	239.4±19.2	178.3±13.7
2	Pudding, chocolate, cup	17.4±0.8	16.7±0.8	47.7±2.2	31.6±1.4	274.3±22.0	189.1±14.5
3	Pudding, chocolate, mint	14.9±0.7	13.9±0.6	39.8±1.8	19.3±0.9	268.3±21.5	139.9±10.7
4	Pudding, chocolate, orange	18.8±0.9	16.9±0.8	43.5±2.0	30.3±1.4	231.2±18.6	179.2±13.8
5	Pudding, strawberry	9.4±0.4	9.9±0.4	34.4±1.6	15.2±0.7	367.8±29.5	154.9±11.9
6	Pudding, strawberry	8.5±0.4	9.2±0.4	32.1±1.5	15.5±0.7	379.6±30.5	169.9±13.1
7	Pudding, forest fruit	7.3±0.3	7.8±0.4	21.5±1.0	11.7±0.5	296.5±23.8	150.3±11.5
8	Pudding, banana, cup	8.3±0.4	24.4±1.1	23.4±1.1	18.9±0.9	283.7±22.8	77.7±6.0
9	Pudding, banana	6.3±0.3	7.8±0.4	24.9±1.1	15.5±0.7	397.7±31.9	200.4±15.4
10	Pudding, banana	9.1±0.4	9.1±0.4	23.5±1.1	23.5±1.1	259.9±20.9	259.9±20.0
11	Pudding, caramel	7.8±0.4	7.7±0.3	21.5±1.0	26.5±1.2	277.5±22.3	346.2±26.6
12	Pudding, vanilla, light	9.0±0.4	9.0±0.4	23.8±1.1	22.1±1.0	266.1±21.4	247.2±19.0
13	Pudding, vanilla	12.9±0.6	7.4±0.3	18.1±0.8	8.5±0.4	141.4±11.3	115.1±8.8
14	Pudding, vanillin, chocolate chips, cup	15.4±0.7	11.0±0.5	27.1±1.2	13.5±0.6	175.9±14.1	123.0±9.4
15	Pudding, coffee, cup	12.8±0.6	22.9±1.0	38.0±1.7	26.1±1.2	298.3±23.9	114.1±8.8
	Milk 1, whole fat	10.3±0.5	7.5±0.3	23.5±1.1	5.6±0.3	229.6±18.4	74.8±5.7
	Milk 2, semi-fat	7.4±0.3	5.8±0.3	19.7±0.9	5.9±0.3	268.1±21.5	101.9±7.8

Multivariate statistical analyses were performed to associate the sugar type and content and the formation of GO and MGO.Values are means ± range, n = 3.

Although our results showed that puddings have low AGE precursor levels, it is important to evaluate the AGE precursors under digestive system conditions. Gastrointestinal digestive conditions may increase or decrease α-DCs in foods [16, 28]. However, best of our knowledge, no study had examined the effect of *in vitro* digestive system conditions in puddings. As seen from the Table 3 GO and MGO contents of pudding samples increased after *in vitro* digestion, except banana cup pudding. While the GO values, after digestion, were ranged from 18.1 to 47.7 μg/100 g, MGO values, after digestion were 8.5 and 31.6 μg/100 g. The increase rate of GO and MGO levels of puddings were ranged between 141.4 to 397.7% and 77.7 to 346.2%, respectively. These results showed that *in vitro* digestive system conditions slightly increased the formation of GO and MGO, except for banana cup pudding. The increase in the banana cup pudding may be related its food matrix. On the other hand, the general increase in our samples may be related their lipid content. Because prooxidant condition in gastrointestinal system is a convenient environment for lipid oxidation [29]. Also, AGE precursors can be derived from lipid oxidation. Besides, after digestion, *r* values of fructose and glucose was decreased and effect of glucose on GO formation was found to be higher than that of fructose. Effect of sucrose content on GO formation increased after digestion comparing to the initial one. Regarding MGO content, similar results was found. While the glucose, fructose and sucrose had a positive r value, which indicates positive correlation; lactose content showed a negative correlation. After digestion effect of fructose and glucose on MGO formation was decreased but effect of sucrose was increased.

4. Conclusions

Briefly, this study examined the GO and MGO levels in puddings, also the effect of digestion system on bioaccessibility of GO and MGO. Our results revealed that puddings have low α-DCs content. The bounding capability of milk proteins, water binding capacity of starch, high moisture, low heat treatment, low fructose content may be contributed to inhibit AGE precursors formation in puddings. Also, gastrointestinal digestive conditions may slightly increase GO and MGO may be related to lipid peroxidation. It can be concluded that puddings are relatively low in terms of AGEs precursors exposure. Despite puddings have low AGE precursors levels, they have rich sugar values, for this reason they have some potential risk in terms of healthy nutrition especially for diabetic patients. Since this study had revealed that puddings are safe for AGE precursors exposure, further studies could focus developing new pudding models.

5. Acknowledge

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