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**Type I caramel products of fructose with water and their bioactivities**

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**Abstract**

To investigate the fructose-water caramel reactions, the type I fructose caramel products (FCPs) were prepared and collected at different reaction stages. The reaction rate, caramel flavor compounds, UV absorptions, antioxidant, and antibacterial activities were determined. The results showed that the reaction rate was increased quickly to 90.1% at the end of middle stage. The exponentially growth of UV305nm suggested the caramel pigments and flavor compounds were rapidly formed along with the reaction. The FCP obtained at arriving of 180 °C possessed the highest contents of total flavor compounds and 5-hydroxymethylfurfural, and the fructose caramel reaction was fast than those of glucose. Total eight characteristic caramel flavor compounds were revealed from FCPs and their formation pathway were also speculated. An optimized condition was suggested at arriving of 180 °C and the FCP under this condition also exhibited potent antioxidant and antibacterial activities. All these results suggested that such kind of type I fructose caramel products could be used as food additive in food industry for the further development.

**Keywords**: caramel reactions; fructose caramel products; flavor compounds; antibacterial activities

**Practical Application**: caramel manufacture, quality control, and caramel flavors.

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**1 Introduction**

Caramel colors are a kind of popular food additive that used world-wide, which are a complicated ingredient including of flavor compounds and polymerized sugars (Golon & Kuhnert, 2012). During the manufacture of caramel colors, accessory chemicals will be used or not, thus give four different types of caramel colors. In the production of type III and type IV caramels, accessory chemicals like ammonia and ammonium salts are used, and both Maillard and caramel reactions could be occurred (Golon & Kuhnert, 2012; Moon & Shibamoto, 2011). Under some circumstances, several cancerogenic and harmful compounds such as 2-acetyl-4-methylimidazole (4-MEI) and 4(5)-tetrahydroxybutylimidazole (THI) will be generated, which lead to the restricted application of type III and type IV caramels (Moon & Shibamoto, 2011; Vollmuth, 2018). The type II caramels (caustic sulfite caramels) can only be applied to a few special foods and medicines. Thus, according to the safety and rich flavor, the type I caramels (plain caramels) are more promising.

The plain caramels are manufactured through typical caramel reactions using multiple kinds of sugars alone or sugars with water. With the participation of water, the caramel reactions can be more accurately and easily to control. Previous studies have disclosed the characteristic flavor compounds of waterless caramel reactions (Golon & Kuhnert, 2013; Pons et al., 1991). It has been revealed that sugar-water reactions could give richer flavors, but the representative flavor compounds and their formation pathway in this system are not known clearly enough (Li et al., 2020; Wei et al., 2019). Moreover, type I caramel color is known to exhibit antioxidant and antibacterial activities, which could extend the storage period of foods and enhance its application as a kind of food additive (Golon & Kuhnert, 2012; Li et al., 2020). Thus, the flavor and bioactive studies of caramels are of great importance for their production and application. In this study, to investigate the dynamic changes and characteristics of fructose-water caramel reactions, the type I fructose caramel products (FCPs) were yielded and collected at the early, middle, and late reaction stages. Their reaction rate, flavor compounds, and UV absorptions were determined, as well as the antibacterial and antioxidant activities. The preferred processing condition and the flavor compound formation pathway were also studied.

**2 Materials and methods**

***2.1 General experimental procedures***

The heating procedure of caramel reaction was performed with a DF-101S reaction system with oil bath and magnetic stirrer (Yuhua Equipment Co., Ltd, Zhengzhou, China). OD (optical density) and UV data were measured on an F-50 microplate reader (Tecan, Mannedorf, Switzerland) and Ultra 3400 equipment (Rigol Technologies Co., Ltd, Suzhou, China). An Agilent 1290 HPLC was chosen for HPLC-ELSD measurement, which was possessed with a 385 ELSD detector (Agilent, Santa Clara, USA). GC-MS was performed by an Agilent 6890GC/5973MS GC-MS.

D-fructose (> 99.0%) was purchased from Merck Co., Ltd (Merck, Darmstadt, Germany). Furfural, DPPH (2,2-diphenyl-1-picrylhydrazyl), and 5-hydroxymethylfurfural (5-HMF) were obtained from Macklin Reagent (Macklin Chemical Co., Ltd, Shanghai, China). 2,5-Furandicarboxaldehyde, ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), 2-furanmethanol, streptomycin, and emodin were obtained from Aladdin Reagent (Aladdin Chemical Co., Ltd, Shanghai, China).

***2.2 Heating procedure***

Purified water (100 g) and D-fructose (200 g) were added in round-bottomed flasks (1000 mL), which were reacted in the oil bath with stirring speed of 1000 rpm and oil temperature of 187 °C (Li et al., 2020). A temperature probe was put into the caramel mixtures to control the reaction temperatures in the range of 140-180 °C, and a thermometer was fixed in the oil bath to avoid the excess temperature of oil. When the caramel mixture temperature reached at 140 (**a**), 170 (**b**), and 180 °C (**c**), each of caramel products (30.0 g) was taken out quickly. FCPs **d** and **e** were obtained using the subsequent reaction of residual mixture **c** at 180 °C for 2 min (**d**) and 4 min (**e**) respectively. With the supplementation with 50% weight of water, FCP **f** was obtained by the heating **e** at 180 °C for another 2 min. At last, FCPs **a**-**f** were weighted, which were further dissolved in 10% alcohol (1.0 g/mL) as the stock solutions for the following measurements.

***2.3 Reaction rate determination***

D-fructose was dissolved in 10% alcohol and prepared to the concentrations of 10.0, 2.0, 1.0, 0.5, and 0.1 mg/mL. After the HPLC-ELSD measurement, a standard curve was established using D-fructose concentration and ELSD peak area. FCPs were diluted to 10.0 mg/mL using the stock solutions with 10% alcohol and further analyzed by HPLC-ELSD. Based on the peak areas and the standard curve, the concentrations of D-fructose and the reaction rates of FCPs were calculated. A reaction rate curve was established using reaction rates of FCPs.

HPLC-ELSD was performed on an Agilent 1290 HPLC system, which was equipped with a pentafluorophenyl (PFP) column (5.0 *μ*m, 250 mm × 4.6 mm, Agilent Pursuit 5) and a 385 ELSD detector. The 5% methanol in addition with 0.1% HCOOH was chosen as the optimized mobile phase (flow rate, 1.0 mL/min; injection volume, 5.0 *µ*L). The ELSD evaporator temperature was set at 45 °C and the gas carrier was high-purity N2 (flow rate, 1.6 L/min).

***2.4 UV analysis of FCPs***

FCPs were prepared to the concentration of 2.0 mg/mL with water using the stock solutions, which were used for UV determination. The UV wavelength coverage was 200 to 550 nm. UV absorption curves were displayed by Origin 8.0.

***2.5 GC-MS analysis***

FCP stock solutions (1.0 g/mL) were deal with CH2Cl2 according to the following method twice. FCP **a**-**f** solutions (15.0 mL) were extracted with 12.0 mL of CH2Cl2 under ultrasonic condition for 3 min, which were further heated under reflux for 20.0 min (60 °C). The two collected CH2Cl2 layers were combined and volatilized to the volume of 0.9 mL. Finally, 0.1 mL of 2-phenylethyl acetate (0.821 mg/mL, internal standard) was added.

GC-MS was performed by an Agilent 6890-5973 GC-MS (injection volume, 1.0 *µ*L). The GC-MS column was chosen as HP-5 (60 m × 0.25 mm i.d. × 0.25 μm d.f.). Helium was used as the gas carrier with a flow rate of 1.0 mL/min. The column temperature was held at 50 °C for 2 min, raised from 50 °C to 120 °C at a rate of 6 °C/min, and increased from 120 °C to 240 °C at a rate of 12 °C/min. Then the column temperature was held at 240 °C for 5 min and raised from 240 °C to 280 °C at a rate of 15 °C/min. The identification of flavor compounds was carried out through comparing their measured mass data with those in the mass spectral library 2017 of National Institute of Standards and Technology (NIST), and the components with more than 85% matching scores were chosen. The identification of key flavor compounds was further verified by their retention times with those of the standard compounds available in the authors’ laboratory such as furfural, 5-HMF, 2,5-furandicarboxaldehyde, and 2-furanmethanol.

***2.6 Antioxidant assays***

The antioxidant effects of FCPs were determined by DPPH and ABTS free radical scavenging assays according to the previous method (Li et al., 2018). In brief, FCP was diluted to the concentration range of 50.0–0.5 mg/mL using the stock solutions with 50% alcohol. ABTS+ stock solution was obtained by potassium persulfate together with equal volume of ABTS+ solution, which was stored avoid light at room temperature for 20 h. The ABTS working solution was yielded by mixing the blue mixture with 50% alcohol to the UV absorption of 0.70 ± 0.05 (A734nm). In the 96-well plates, 180 *μ*L of ABTS working solutions and 20 *μ*L of FCP diluted solutions were added, and the ODs of A734nm were determined after scavenging for 6.0 min in the darkness. For DPPH assays, 100 *μ*L of DPPH (0.30 mM) working solutions together with 100 *μ*L of FCP diluted solutions were added, and the ODs of A517nm were measured after scavenging for 30.0 min in the same condition. Using GraphPad Prism 8, the scavenging rate was calculated as following: scavenging rate (%) = (1–*A*test/*A*control) × 100. All the measurements were carried out in triplicate.

***2.7 Antibacterial assays***

The antibacterial activities were determined according to the broth microdilution method, using two strains of Gram positive bacteria (*Bacillus subtilis* ATCC 6633 and *Enterococcus faecalis* ATCC 29212) and two strain of Gram negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) (Li et al., 2019). The bacteria were cultivated on MH (Mueller-Hinton) agar broth for 24 h. Then the bacteria were cultivated at 35 °C and 120 rpm in MH liquid broth for around 5-7 h, and diluted to the final concentration of 1.0×104–1.0×105 CFU/mL. Subsequently, positive controls (emodin and streptomycin, 0.1–10.0 *μ*g/mL) were prepared and FCP solutions were diluted to 20.0–0.1 mg/mL using the stock solutions with MH liquid broth. In a bacteria-free work bench, each 100 *μ*L of FCP diluted solutions and 100 *μ*L of bacterial suspensions was added into the 96-well plates. The plated were sealed and further incubated at 37 °C for 24 h. ODs of A530nm were recorded and the tests were carried out in triplicate. The compound concentrations that reduced 50% growth of the bacteria were considered as minimum inhibitory concentration (MIC) values, which were calculated by GraphPad Prism 8.

***2.8 Statistical analysis***

All the measurements were performed in triplicate, and the results were expressed as mean values ± SD (standard deviations). The diﬀerences between individual results were considered as significant when P < 0.05. The ANOVA (one-way analysis of variance) test was carried out by STATISTICA and GraphPad Prism 8.

**3 Results** **and discussion**

***3.1 Reaction rate of FCPs***

A standard curve was constructed by the HPLC-ELSD peak areas and the concentrations of fructose solutions (Table 1 and Figure 1). Through linear regression, the standard curve was revealed as y = 604.8x ‒ 54.6, and the linear fitting coefficient (R2 = 0.998) indicated that the linear relationship was fine (Figure 1). Afterwards, the diluted FCP solutions (10.0 mg/mL) were measured by HPLC-ELSD, and the reaction rate was calculated based on the standard curve and the peak areas (Table 2).

**Table 1.** The HPLC-ELSD response values of different fructose concentrations.

|  |  |
| --- | --- |
| concentrations (mg/mL) | peak area |
| 0.1 | 17.4 |
| 0.5 | 159.4 |
| 1.0 | 487.9 |
| 2.0 | 1315.9 |
| 10.0 | 5972.2 |

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**Figure 1.** HPLC-ELSD standard curve of fructose.

As shown in Figure 2, the concentrations of remaining fructose in the FCPs were obviously decreased along with the reaction, and three reaction stages (early stage, middle stage, and late stage) could be revealed. From heat beginning to **b** (arriving of 170 °C) could be considered as the early stage (Figure 2), and the reaction rate was 0.0-20.6%. For the middle stage, it could be defined as from **b** (the arriving of 170 °C) to **e** (180 °C 4 min). Reaction rate was increased quickly to 90.1%. Following was the late stage (**e** to **f** in Figure 2), and the reaction rate was maintained at high level.

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**Figure 2.** Fructose reaction rate of FCPs. **a**, arriving140 °C; **b**, arriving 170 °C; **c**, arriving 180 °C; **d**, reacted at 180 °C for 2 min; **e**, reacted at 180 °C for 4 min; **f**, 180 °C, 4 min, 50% water supplement, reacted at 180 °C for another 2 min. The same below.

**Table 2.** The fructose concentration and reaction rate of FCPs.

|  |  |  |  |
| --- | --- | --- | --- |
| FCP | peak area | concentration (mg/mL) | reaction rate (%) |
| **a** | 5250.0 | 8.8 | 12.3 |
| **b** | 4745.2 | 7.9 | 20.6 |
| **c** | 3094.6 | 5.2 | 47.9 |
| **d** | 1936.0 | 3.3 | 67.1 |
| **e** | 544.7 | 1.0 | 90.1 |
| **f** | 123.2 | 0.3 | 97.1 |

***3.2 UV absorptions of FCPs***

As shown in Figure 3, the increased UV absorptions could be observed along with the reaction. Interestingly, two absorption peaks centered at around 290 nm and 305 nm were revealed (Figure 3). In the caramel reactions, it has been known that sugar polymerization would happen at first to form polymerization products such as caramelin and caramelen, and the polymerization products might be were associated with UV290nm (Li et al., 2020). Thus, UV290nm was quickly enhanced to a stable high level at **c** in Figure 4, which suggested that the major polymerization reaction of fructose has been performed at the arriving of 180 °C (Golon & Kuhnert, 2013). Afterwards, the exponentially growth of UV305nm suggested the caramel pigments and flavor compounds were rapidly formed (Figure 4) (Ho et al., 2007; Li et al., 2020).

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**Figure 3.** UV absorptions of FCPs in water.

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**Figure 4.** UV absorptions of FCPs at 290 and 305 nm.

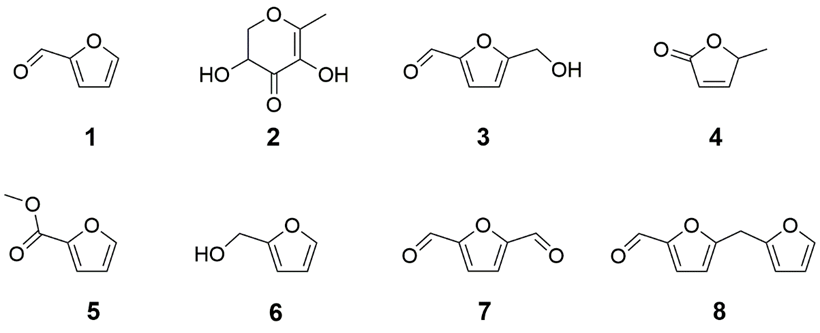
***3.3 Flavor compounds of FCPs***

Through GC-MS analysis, eight caramel flavor compounds were disclosed as shown in Figure 5 and Table 3, which could be possibly distinguished as three classes (Golon & Kuhnert, 2013; Li et al., 2020). Class I was the early stage caramel flavors including furfural (**1**), 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP, **2**), and 5-HMF (**3**), since they were all found in the early stage FCP **b**. These flavor compounds could also be considered as the main flavor compounds due to their relatively high concentrations in FCPs. Following was the middle stage compounds consisting of 5-methyl-2(5*H*)-furanone (**4**) and methyl 2-furoate (**5**). The remaining compounds **6**–**8** (class III) were the late stage flavors because they were only found in FCP **f**.

**Table 3.** Concentrations of flavor compounds in FCPs (*μ*g/mL).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| No. | flavor compounds | *t*R(min) | **a** | **b** | **c** | **d** | **e** | **f** |
| **1** | furfural | 9.75 | –# | 2.00 | 2.62 | 2.54 | 3.02 | 3.20 |
| **2** | 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one | 17.85 | – | 0.08 | 1.42 | 1.51 | 1.53 | 1.64 |
| **3** | 5-hydroxymethylfurfural | 19.38 | – | 45.02 | 54.95 | 50.22 | 44.12 | 33.93 |
| **4** | 5-methyl-2(5*H*)-furanone | 12.58 | – | – | 0.50 | 0.52 | 0.65 | 0.79 |
| **5** | methyl 2-furoate | 16.48 | – | – | 0.85 | 1.02 | 1.29 | 2.70 |
| **6** | 2-furanmethanol | 14.25 | – | – | – | – | – | 0.30 |
| **7** | 2,5-furandicarboxaldehyde | 16.34 | – | – | – | – | – | 0.35 |
| **8** | 5-(2-furanylmethyl)-2-furancarboxaldehyde | 22.80 | – | – | – | – | – | 0.21 |
|  | total |  | – | 47.10 | 60.34 | 55.81 | 50.61 | 43.12 |

#–, not detected.

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**Figure 5.** Structures of flavor compounds identified by GC-MS.

Among all the flavor compounds, the content of 5-HMF (**3**) was the highest. The concentrations of total flavor compounds and 5-HMF were first increased and then decreased, and the highest point was found in FCP **c** (Figure 6 and Table 3). Thus, a preferred condition was suggested at arriving of 180 °C for the fructose–water caramel reaction.

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**Figure 6.** Concentrations of 5-HMF and DDMP in FCPs.

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**Figure 7.** Speculated formation pathway of flavor compounds in FCPs.

In comparison with glucose, fructose is more active and the fructose caramel reaction needs the condition that is more moderate. It is interesting to note that five forms of fructose will be found when it is dissolved in water, namely *β*-D-furan fructose, *α*-D-furan fructose, *β*-D-pyran fructose, *α*-D-pyran fructose, and the chain fructose (Figure 7) (Chen et al., 2019; Ho et al., 2007). The easily transformational forms of fructose indicated it was more active.

For the flavor compound formation (Figure 7), furan fructose would be first polymerized, and then dehydrated and oxidized to obtain 5-HMF (**3**) (Chen et al., 2019). The cleavage of 5-HMF would give furfural (**1**), and the oxidation of 5-HMF brought 2,5-furandicarboxaldehyde (**7**). Subsequently, the oxidation and esterification of furfural would form methyl 2-furoate (**5**). The reduction of furfural would give 2-furanmethanol (**6**), which was further reduced and oxidized to form 5-methyl-2(5*H*)-furanone (**4**). On the other hand, the polymerization of furfural would yield **8**. Interestingly, DDMP (**2**) could be yielded by pyran fructose (Figure 7) (Moon & Shibamoto, 2011).

***3.4 Antioxidant effects***

As shown in Table 4, the antioxidant effects of FCPs were reinforced quickly along with the caramel reaction, since the IC50 values of FCPs decreased from 42.61 to 0.75 mg/mL and from 24.15 to 0.28 mg/mL for DPPH and ABTS assays respectively. DDMP and its derivatives were known to exhibit antioxidant effects, which were possibly associated with olefinic alcohol functional groups in their structures (Yu et al., 2013). Therefore, the concentrations of DDMP (Table 3 and Figure 6) were increased along with the caramel reaction, and the radical scavenging effects were also enhanced.

**Table 4.** IC50 values of FCPs in the antioxidant assays (mg/mL).

|  |  |  |
| --- | --- | --- |
| FCP | DPPH | ABTS |
| **a** | 42.61 ± 3.02 | 24.15 ± 2.33 |
| **b** | 9.74 ± 0.87 | 8.90 ± 0.78 |
| **c** | 4.14 ± 0.49 | 1.52 ± 0.27 |
| **d** | 3.75 ± 0.41 | 1.18 ± 0.23 |
| **e** | 2.75 ± 0.23 | 0.61 ± 0.09 |
| **f** | 0.75 ± 0.06 | 0.28 ± 0.04 |
| 5-HMF | > 2.00 | > 2.00 |
| Trolox# | 0.04 ± 0.01 | 0.04 ± 0.01 |

#Positive control. Fructose was considered as inactive since its IC50 > 50.0 mg/mL. 5-HMF was inactive since its IC50 > 2.00 mg/mL. Data are expressed as mean of three measurements of triplicate tests ± standard deviation.

***3.5 Antibacterial activities of FCPs***

The antibacterial activities of FCPs against two strains of Gram negative and two strains of Gram positive bacteria were evaluated (Table 5). Interestingly, all the FCPs were inactive against Gram negative bacteria, and they showed better inhibitory effects on *B. subtilis* than those of *E. faecalis*. Overall, the antibacterial activities of FCPs were enhanced along with the caramel reaction, which was possibly associated with the formed flavor components in the reactions (Li et al., 2020). This result was also proved by the antibacterial effects of 5-HMF (Table 5).

**Table 5.** MIC values of FCPs against four strains of bacteria (mg/mL).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Products | *Ef*\* | *Bs*\* | *Ec*\* | *Pa*\* |
| **a** | > 10.0 | > 10.0 | > 10.0 | > 10.0 |
| **b** | 8.9 ± 1.8 | 7.6 ± 1.4 | > 10.0 | > 10.0 |
| **c** | 6.6 ± 1.5 | 5.2 ± 1.0 | > 10.0 | > 10.0 |
| **d** | 6.2 ± 1.2 | 4.9 ± 1.2 | > 10.0 | > 10.0 |
| **e** | 5.3 ± 1.0 | 3.7 ± 0.9 | > 10.0 | > 10.0 |
| **f** | 4.2 ± 0.9 | 2.6 ± 0.6 | > 10.0 | > 10.0 |
| 5-HMF | 1.2 ± 0.2 | 0.9 ± 0.1 | > 10.0 | > 10.0 |
| Emodin# | 0.01 ± 0.002 | 0.01 ± 0.003 |  |  |
| Streptomycin# |  |  | 0.001 ± 0.0002 | 0.001 ± 0.0003 |

\**Ef*, *Enterococcus* *faecalis* ATCC 29212; *Bs*, *Bacillus subtilis* ATCC 6633; *Ec, Escherichia coli* ATCC 25922; *Pa*, *Pseudomonas aeruginosa* ATCC 27853. #Positive control. The products were inactive when MICs > 10.0 mg/mL. Data are expressed as mean of three measurements of triplicate tests ± standard deviation.

It has been known that type I caramel products could give rich flavors, which was also proved by our study that the total flavor compounds of FCPs could be more than 50 *μ*g/mL (Li et al., 2020; Wei et al., 2019). Overall eight characteristic flavor compounds were revealed for the fructose-water caramel system and the content of 5-HMF was the highest. The concentrations of 5-HMF and total flavor compounds were suggested as a kind of indicator for fructose-water caramel system not only in experiment research but also in industrial preparation. Additionally, the fructose caramel reaction needs some more moderate condition than those of other sugars such as glucose and sucrose.

With the participation of accessory chemicals like ammonia and ammonium salts, type III and type IV caramels are obtained with dark color but poor flavor. Thus, due to the rich and natural flavor and the safety, these type I caramels could be used for baked food, flavored drink, and flavor and fragrance production. Moreover, the antioxidant and antibacterial activities of this kind of caramels could extend the storage period of foods and enhance their application as food additive.

**4 Conclusion**

For the fructose-water caramel reactions, an optimized condition was suggested at arriving of 180 °C, and the type I FCP under this condition was revealed to have the highest concentrations of total flavor compounds and 5-HMF Moreover, the FCP under this condition also exhibited potent antioxidant and antibacterial activities. All these results suggested that such kind of type I caramel products could be used as food additive in food industry for the further development.

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