A high throughput screening assay for identifying glycation inhibitors on MALDI-TOF target

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ABSTRACT

The Maillard reaction plays an important role in the food industry, however, the deleterious effects generated by the advanced glycation end-products (AGEs) have been well recognized. Many efforts have been made to seek new AGE inhibitors, in particular those natural ones without adverse effect. We have developed a rapid, mass spectrometry based, on-plate screening assay for novel AGE inhibitors. The glycation reaction, inhibition feedback as well as the subsequent MALDI mass spectrometric analysis occurred on one single MALDI plate. At 1:10 M ratio of peptide to sugar, as little as 4 h incubation time allowed the screening test to be ready for analysis. DSP, inhibition and IC50 were calculated to evaluate selected inhibitors and resulting inhibition efficiencies were consistent with available references. We demonstrated that this method provide a potential high throughput screening assay to analyze and identify the anti-glycation agents.

1. Introduction

Glycation, also known as Maillard reaction, is the non-enzymatic complex formation between amino groups and carbonyl groups of reducing sugars (Van Lanker, Adams, & De Kimpe, 2011). Glycation has made great impact on food science owing to its ability to produce favorable flavor (Shibamoto & Yeo, 1994), improved functional properties (Amarowicz, 2009; Oliver, Melton, & Stanley, 2006) and physiochemical properties (Huang et al., 2012). However, with prolonged heating, glycation often results in unpleasant tasting, with the formation of advanced glycation endproducts (AGEs). Since the discovery of the glycated hemoglobin in diabetic patients, the importance of the endogenous glycation reaction and its AGEs started to be recognized (Rahbar, 1968). AGEs can generate negative effects, such as, forming carcinogenic (Mottram, Wedzicha, & Dodson, 2002; Zhang & Zhang, 2007), reducing the nutritional and digestibility (Seiquer et al., 2006). Dietary AGEs are a factor of the body's AGEs pool, leading to the development of chronic diabetic complications (Uribarri et al., 2005). AGEs have been implicated as the major pathogenesis of a number of diseases, including diabetic vascular disease (Jackson, 2004), Alzheimer's disease (Münch, Thome, Foley, Schinzel, & Riederer, 1997) and aging (Brownlee, 1995).

All these AGEs associated damaging effects have led to enormous efforts to search AGE-inhibitors that can break down or eliminate the formation of AGEs (Peng, Ma, Chen, & Wang, 2011). AGE formation can be pragmatically divided into three major stages. In the initial stage, glucose reacts with an amine to form a metastable Schiff base which rearranges to the Amadori product. In the intermediate stage, the Amadori product is fragmented to various of reactive dicarbonyl compounds such as glyoxal, methylglyoxal and deoxyglucosones. In the end stage, AGEs, the irreversible compounds are formed through a series of oxidation, dehydration and cyclization reactions. The inhibitors of AGEs can thus also be divided into three categories that execute their inhibition activity in different stages: (1) antiglycation agents that attenuate the first-step glycation reaction, (2) antioxidant agents that suppress Amadori degradation, (3) anti-AGE agents that can sequester carbonyl group, suppress glyoxidation reactions and break AGE crosslinks.

Identification and characterization of novel AGE inhibitors requires a rapid, simple and reliable biochemical assay especially if a library of agents need to be tested. Several methods have been
used to identify novel compounds with inhibitory activity. The most effective methods are immunochemical assays (Jongberg, Rasmussen, Skibsted, & Olsen, 2013; Motomura et al., 2009) and fluorescence spectrometric analysis (Derbre et al., 2010). However, all these methods are low throughput and labor intensive, usually requiring several days or weeks. To develop an effective assay for AGE inhibitors, three important aspects have to be taken into consideration. Firstly, glycation process needs to be accelerated. Since glycation speed and extent in dry-state are much higher than that in aqueous solution (Morgan et al., 1998), most of the glycation considerations. Secondly, analyzing time should be as short as possible; therefore, lengthy sample preparations like HPLC separations are not desired. Thirdly, the assay should be cost effective. Immunochemical assays, i.e., ELISA require expensive antibodies, thus are not preferred.

In this work, we propose an on-plate high throughput screening assay for identifying glycation inhibitors. This method allows the glycation reaction to occur on a MALDI plate in a short period of time, and the inhibitory activity then be evaluated by mass spectrometry immediately after mixing with matrix. A standard 4800 TOF-TOF MALDI plate allows 384 samples to be analyzed at the same time, and this plate can be customized to accommodate more samples if necessary. MALDI-TOF mass spectrometry is a very sensitive technique that permits a fast sampling of chemical and biological compounds at abundances of sub-femtomole primarily with single charge. Importantly, MALDI-TOF MS is tolerant to the buffer solutions (phosphate buffer or Tris buffer) required for glycation reactions. Compared to another popular mass spectrometry based analytical method, liquid chromatography electrospray mass spectrometry (LC/ESI-MS), this method eliminates the need for prolonged LC prefractionation.

It should be noted that we use peptides to react with glucose in our method simply because peptides allows a much faster reaction rate than proteins. We are also mainly focused on identifying the first type of AGE-inhibitors, antiglycation agents, since MALDI-TOF MS is best suitable for detection of the glycated peptides. By comparing the glycation extent of peptides in the presence of different inhibitors, we can determine the relative activities of the inhibitors. The assay is rapid, sensitive and can analyze a large pool of samples at the same time on a single plate, rendering a real high throughput screening tool for AGE-inhibitors.

2. Materials and methods

2.1. Materials

Peptides (1. RGYVYQGL m/z 955.4996; 2. KHPGGGKVQILYVKPVDL m/z 1963.1382; 3. SELLSRPQKIRFRQWLRD m/z 2427.3368) were synthesized in the laboratory of Marcomolecular Analysis & Proteomics, Albert Einstein College of Medicine, Yeshiva University. These three peptides were chosen because of the distinct potential glycation sites available in different regions. In peptide 1, glycation can only be occurred on N-terminus, while peptide 2 contains three lysines and peptide 3 includes four arginines, one lysine and N-terminal serine that can be potentially glycated.

Glucose, carnosine, ascorbic acid, neurotensin, glutathione (GSH), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and α-Cyano-4-hydroxycinnamic acid were purchase from Sigma Chemical CO. (St. Louis, MO). Quercetin, rutin and (−)-epicatechin (EC) were purchase from Aladim Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade. Ultrapure water from a pure water purification system (Millipore, Billerica, MA) was used throughout this study.

2.2. Sample preparation

3 μL of α-glucose (100 mg/mL) was added into 300 μL of 0.2 mg/ml peptide in 6 mM Tris–HCl buffer solution at pH 8.0. The solutions were split into three aliquots to 500 μL eppendorf tube, followed by sample preparing procedure depicted in Fig. 1. The first aliquot was kept in the tube untreated. The second aliquot was frozen in liquid nitrogen and lyophilized for 12 h. 1.5 μL of the third aliquot was directly spotted on the MALDI plate at room temperature and air-dried. All of the three samples were then incubated in an oven (Thermo Fisher Scientific Inc. Waltham, MA) at 60 °C for 4 h. All three peptides in the absence of glucose were treated in the same way as the controls. 1.5 μL of samples from the first and second aliquots were spotted on the MALDI plate, followed by analysis in a 4800 TOF-TOF mass spectrometer.

2.3. Inhibition of peptide glycation

0.2 mg/ml neurotensin in 6 mM Tris–HCl buffer at pH 8.0 was mixed with 0.2 mg/ml and 2 mg/ml of glucose with a molar ratio of 1:10 and 1:50, respectively. The inhibitors with concentration ranges from 10−6 to 1 M were then added into the mixture. 1.5 μL mixture was spotted on the MALDI plate and incubated at 60 °C for 4 h. Neurotensin alone was served as the negative control and neurotensin with glucose was acted as the positive control. We
added the mixture of neurotensin, glucose and ethanol as the solvent control since ethanol was used as the solvent for the inhibitors. In addition, DPPH was tested to see whether it was an inhibitor. All the tests were performed in triplicate.

2.4. Mass spectrometric analysis

Mass spectrometry analysis was carried out using MALDI TOF-TOF mass spectrometer (AB Science, ABI 4800 PROTEOMICS TOF/TOF ANALYZER, Foster City, CA) in positive ion mode. α-Cyano-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% TFA (5 mg/mL) was used as the matrix. A matrix droplet was applied to the top of the sample analyte and air dried. We applied this two layer sample preparation method to avoid the matrix effect occurring in glycation process. External calibration was performed by use of a standard peptide mixture. Each sample was spotted on at least three individual target positions and 60 individual spectra of each spot were averaged to produce a mass spectrum.

To evaluate glycation extent of each peptide, the average degree of substitution per peptide molecule (DSP) was calculated according to the following formulation (Kislinger et al., 2003; Wang et al., 2013).

\[
DSP = \frac{\sum_{i=1}^{n} I_i \times (\text{peptide } i + \text{glucose})}{\sum_{i=1}^{n} I_i \times (\text{peptide } i + \text{glucose})}
\]

where \(I\) is the sum of the intensities of glycated peptide, and \(i\) is the number of glucose units attached to the peptide in each glycated form.

2.5. Statistical analysis

The data are expressed as mean ± SD. The analysis was done here with logistic curve fitting using Origin8.0 (OriginLab Corp., Northampton, MA). IC50 values were calculated by fitting the data points to a logistic curve.

3. Results and discussion

3.1. On-plate glycation vs dry-state and aqueous-state glycation

The first step of the assay is to validate on-plate glycation. In our proposed assay, all reactions including glycation reaction, glycation inhibition and detection of the glycated products occurs on a single plate, therefore, it is necessary to compare the results of the on-plate heating with those obtained by traditional dry-state as well as aqueous-state glyations. The MALDI-TOF/MS spectra of peptide 1 (RGVYVYQGL) glycated under different conditions were shown in Fig. 2. As expected, in aqueous solution, very limited glycation was observed with a small peak at \(m/z\) 1117.55 representing the mono-glycated form of peptide 1 (\(\Delta m = 162\)). When the reaction was moved to dry-state, apparent higher degree of glycation occurred. The monoglycated peak at \(m/z\) 1117.55 exhibited a much higher intensity than that in aqueous solution. In addition, the diglycated peak was detected, as represented by the peak with \(m/z\) of 1279.59 (\(\Delta m = 324\), Fig. 2C & D). During this nonenzymatic glycation process, the functional groups which could be glycated by glucose are α-amino group of the N-terminus, the ε-amino groups of the lysine residues and the guanidine groups of the arginine residues (Kislinger, Humeny, & Pischetsrieder, 2004). Since Arg is located at the N-terminus of peptide 1, this peptide can be diglycated if extensively heated and the glycation sites should be the α-amino group and the guanidine group of the arginine residue. This is actually confirmed by MS/MS as shown in Supplementary Fig. 1s. When the peak 1117.55 and 1279.59 were isolated for tandem mass spectrometry, a series of y- and b-ions indicated that both of the glycation occurred on Arg residue in peptide 1.

Compared to traditional dry-state glycation, our on-plate glycation also exhibited comparable extent of glycation as shown in peptide 2 and 3 (Supplementary Figs. 2s & 3s), suggesting that the on-plate heating is as effective as dry-state heating. Figs. 2s & 3s also show the DSP values and the glycated sites of these peptides under different preheating conditions. The DSP values of on-plate heating are 0.613, 1.909, and 1.389 for peptide 1–3, while the corresponding DSP values of dry-state heating are 0.709, 1.454 and 1.287, respectively. Both of these values were much larger than those obtained from aqueous-state glycation. Consistent with DSP values, the number of glycated sites obtained by on-plate heating was the same as that by dry-state heating, while significantly more than that by aqueous-state heating. These results are consistent with our previous observation on the glycation of the protein, ovalbumin (Huang et al., 2013). In dry-state, the energy transfer is more efficient and reactant concentration is much higher, therefore the reactions are much higher efficient than those in aqueous-state (Oliver, 2011). It should be noted that our on-plate method does not need time-consuming lyophilization process prior to heating. The total time consumed for on-plate glycation can be shortened by 10–20 h, practically with only the incubation time needed.

3.2. Peptide selection

It is important to know whether on-plate glycation can be universally applied to all types of peptides. We performed our on-plate glycation on two other peptides (peptide 2&3, Materials and Methods) containing more arginine or lysine residues scattering at various positions. The results (Figs. 2s, 2s & 3s) indicated that on-plate glycation was comparable to dry-state glyation for all of the tested peptides. Because of this feature, any commercial available peptides containing lysine or arginine residues can serve as the substrate for the inhibitor test. In the following experiment, we selected neurotensin (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), an inexpensive and easily available peptide, to carry out the screening test for inhibitors. It should be noted that the glycation extent for any given lysine or arginine residues is dependent on the surrounding environment. For example, the presence of a histidine or lysine residue close to a lysine in either the primary or the 3D structure has been reported to promote the glycation of lysines (Acosta et al., 2000; Baynes et al., 1989; Shilton, Campbell, & Walton, 1993). In our screening assay, it is preferred to select those peptides that can be relatively easier to be glycated, so that the incubation time can be shortened. The number of available glycation sites also plays an important role in this assay. More glycation sites allow higher glycation extent and subsequently permit extended dynamic range of the detection of inhibitors.

3.3. Evaluation of inhibition efficiency

Average degree of substitution per peptide molecule (DSP) by glucose has been widely used to evaluate the extent of glycation of a peptide (Thomsen et al., 2012). Since DSP (equation 1) takes into account not only glycation degree of every glycated site, but also the number of the glycated sites, it actually reflects the glycation level of the substrate. This provides a basis to evaluate the glycation degree mathematically. To judge whether a novel agent is effective at inhibiting glycation, a simple calculable value is proposed. Inhibitory effect is evaluated by comparing the DSP values before and after adding the inhibitor, it is necessary to convert the DSP values to inhibition by the following equation:
Inhibition(%) = 100 * \frac{(DSP_{control} - DSP_{inh})}{DSP_{control}} \tag{2}

where DSP_{inh} and DSP_{control} are the DSP values with and without inhibitor, respectively.

Figs. 3 and 4 show the MALDI-TOF mass spectra of the mixture of neurotensin and glucose at molecular ratio of 1:10 and 1:50 in the presence and absence of various inhibitors. As shown in Fig. 3 and Table 1, when quercetin, rutin, EC, ascorbic acid, carnosine and GSH were present during glycation of neurotensin with glucose (~1:10), the DSP value was decreased from 0.502 to 0.412, 0.348, 0.212, 0.266, 0.421, and 0.398, respectively. When the peptide was mixed with more glucose at molar ratio of 1:50, the glycation rate was increased and the DSP value was increased.
to 0.739 in the absence of inhibitor (Fig. 4 and Table 1). In the presence of inhibitors, the DSP value was declined from 0.739 to 0.574, 0.496, 0.364, 0.223 and 0.522 for quercetin, rutin, EC, ascorbic acid and carnosine, respectively. Under both conditions, it is clear that all the tested compounds executed their inhibitory activity on peptide glycation as suggested by the significantly decreased DSP values. An important issue in evaluating the inhibitory activity is the sensitivity. At 1:10 M ratio, the sensitivity is much higher than that at 1:50 M ratio as the required amount of inhibitor is much smaller ($1 \times 10^{-3} \text{ M}$ vs $1 \times 10^{-1} \text{ M}$). Therefore, lower molar ratio of peptide to sugar is suggested for the inhibitor screening assay. However, the molar ratio need to be maintained at a certain level to ensure the glycation rate is relatively fast to suit the basic requirement of high throughput screening. We found that a ratio of $\sim 1:10$ allowed a fast glycation reaction with DSP reaching 0.5. It should be noted that higher ratio of peptide to sugar is still be valid in this type of assay, particularly when shorter incubation time is desired.

Although the mechanism of inhibition is currently still unknown, all of the substances presenting inhibitory behavior are found to be those can bind to either sugars or proteins and are proven antioxidants. Binding to the sugar or proteins can block the sugar attachment to proteins, preventing the formation of Amadori product and subsequent AGE formation. It should be noted that under our experimental conditions, the glucose used was in 10 M excess to ensure fast and sufficient glycation. When equal amount of anti-glycation agents were added, the inhibition efficiency will exhibit differently for different type of anti-glycation agents. For anti-glycation agents binding to protein/peptide, the inhibition efficiency will be prominent since the relative smaller portion of protein/peptide will be consumed faster than the larger portion of sugar. By contrast, the inhibition efficiency of sugar-binding agents will be less prominent due to the larger portion of sugar used. In this point of view, it is fairer to divide the inhibitors to two categories, (1) protein/peptide binding agents and (2) sugar binding agents.

EC, ascorbic acid, quercetin and rutin belong to protein/peptide binding agents. All of these compounds presented to be more potent inhibitors than sugar binding agents, carnosine as suggested by their inhibition values (Table 1). Based on these values, the five inhibitor activity can be ranked as the order: EC > ascorbic acid > rutin > quercetin > carnosine. Quercetin, rutin and EC are members of the flavonoid family ubiquitously present in food, including vegetables, fruits, tea, and red wine (Boots, Haenen, & Bast, 2008). These compounds have been previously established
as potent AGE-inhibitors (Aldini et al., 2013). As a protein/peptide binding antiglycation agents, the number of hydroxide group in the inhibitor plays very important role in the glycation. As the hydrolytic product of rutin, quercetin contains 5 less hydroxide groups. Compared to rutin, the inhibition value of quercetin is much lower. This also suggests that the effect of steric hindrance is far less than the number of hydroxide groups in this type of the inhibitors. The structure of EC is also quite similar to quercetin, with one extra hydroxyl group. However, EC exhibited a much higher inhibition effect, suggesting the importance role of this group on the antiglycation reaction. This could be the reason why the other inhibitor, ascorbic acid (also contains a hydroxyl group), is also a potent inhibitor. Ascorbic acid is a powerful antioxidant that can neutralize free radicals generated during the early stage of glycation. In an investigation of roles of various nutrients in glycation and AGE formation conducted by (Vinson & Howard, 1996), ascorbic acid was shown to possess anti-AGE capability at physiological concentrations. In addition, the inhibition efficiency of ascorbic acid was found to be significantly higher than carnosine, which is consistent with our quick assay of these two compounds.

Carnosine can be considered the direct scavenging (trapping) of the reactive carbonyls during the glycation. Carnosine is a dipeptide (β-alanyl-L-histidine) highly concentrated in brain and muscles, which is a heterocycle-based natural compound (Hipkiss, Michaelis, & Syrris, 1995). Carnosine can inhibit protein glycation by preventing the crosslinking between protein and reducing sugars, and it can also inhibit the carbonyl formation (Pepper, Farrell, Nord, & Finkel, 2010; Vistoli, Carini, & Aldini, 2012). As mentioned earlier, carnosine showed inhibitory effect on glycation, however, its inhibitory effect is relatively weaker than protein/peptide binding agents. We contribute the weaker inhibitory effect to its relatively lower portion applied on the glycation mixture (1:10 M of AGE-inhibitors (Aldini et al., 2013)).

Fig. 4. The MALDI-TOF mass spectra of the mixture of neurotensin (0.2 mg/ml) and glucose (2 mg/ml) (molar ratio of ~1:50) in the presence and absence of various inhibitors. (A) Control (no inhibitor), (B) quercetin, (C) rutin, (D) EC, (E) negative control (no glucose), (F) ethanol, (G) ascorbic acid and (H) carnosine. The peaks represent the peptide and the glycated peptides are shown in bold. The peak with m/z of 1543.6 corresponds to the truncated peptide with the loss of N-terminal amino acid residue, E.

Table 1

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<thead>
<tr>
<th>Inhibitor</th>
<th>1:10</th>
<th>1:50</th>
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<tbody>
<tr>
<td>DSP</td>
<td>Inhibition (%)</td>
<td>IC50 (M)</td>
</tr>
<tr>
<td>Control</td>
<td>0.502 ± 0.002</td>
<td>–</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.412 ± 0.003</td>
<td>17.6 ± 0.6</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.348 ± 0.013</td>
<td>30.5 ± 2.7</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>0.212 ± 0.028</td>
<td>57.6 ± 5.5</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.266 ± 0.032</td>
<td>46.9 ± 6.4</td>
</tr>
<tr>
<td>Carnosine</td>
<td>0.421 ± 0.007</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>GSH</td>
<td>0.398 ± 0.015</td>
<td>20.6 ± 1.8</td>
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ratio of peptide to sugar). To verify this, we applied the other potent inhibitor, GSH, to the glycation reaction. GSH is the endogenously reducing tripeptide (\(\gamma\)-L-glutamyl-L-cysteinylglycine) with a thiol group, which is the polyreactive molecule in carbonyls quencher (Aldini et al., 2013). The \(\alpha\)-amino group and the thiol of GSH can spontaneously react with glycation reactant (sugars) and the reactive carbonyls generated by the glycation. Therefore, this agents should show relatively stronger inhibition effect on the peptide-sugar glycation. In agreement with the notion, the inhibition value of GSH was 20.6 (Table 1), indicating a stronger inhibitory effect than carnosine (15.8).

It has been reported that free radicals can be formed in glycated proteins by an electron exchange occurring between the sugar moiety of glycated proteins and molecular oxygen (Monboisse, Gillery, Maquart, & Borel, 1990). Also, highly reactive oxidants have been found in glycated peptides and proteins (Trznadel et al., 1990; Wolff, Jiang, & Hunt, 1991). Free radicals may stimulate the AGE formation by autooxidation of sugars (Wolff et al., 1991). Oxidants were found to play a critical role in the AGE formation (Le Guen, Jones, Barnett, & Lunec, 1992). In addition, a linear correlation between plasma glycation and AGE formation has been established (Hunt, Skamarovskas, & Mitchinson, 1994; Vinson & Howard, 1996). The antioxidants can react with free radicals and reduce the concentration of free radicals produced in the process of glycation resulting in decreased AGE crosslinks (Wolff et al., 1991). Because the important role that free radicals may play in the glycation reaction, we selected DPPH as a control to test whether it is able to alter the glycation rate. DPPH is a well-known radical which is able to scavenge other radicals. Therefore, the reducing rate of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. As shown in Fig. 3J, when DPPH was introduced in the glycation reaction, there was almost no change in the glycation extent of the peptide, suggesting that DPPH does not affect the glycation reaction, therefore, is not an inhibitor. DPPH can actually be served as a negative control in this type of assay. It should be noted that in this experiment, ethanol was the solvent we used for dissolving the anti-glycation agents including EC, ascorbic acid, quercetin and rutin. It is also important to know that whether or not it will affect the glycation rate. As suggested in Figs. 3C and 4F, ethanol did not significantly change the glycation rate of the peptide-sugar, therefore, containing negligible effect in our assay.

IC\(_{50}\) is commonly used as a measure of drug/inhibitor effectiveness. IC\(_{50}\) can be readily calculated in our screening assay when glycation reaction with a series of concentration of inhibitory agents was performed. The concentration response curves of the five inhibitors are shown in Fig. 5A-F. At the peptide/glucose ratio 1:10, with the concentration ranging from 1.0 \(\times\) \(10^{-6}\) to 1.0 \(\times\) \(10^{-1}\) M, all five inhibitors exhibited typical S-shape curve that permitted IC\(_{50}\) determination. The correlation coefficients \((R^2)\) are also indicated in Fig. 5. All the coefficients are close to 1, allowing accurate estimation of IC\(_{50}\) values. IC\(_{50}\) values of quercetin (1.87 mM), rutin (0.487 mM), EC (0.224 mM), ascorbic acid (0.375 mM), carnosine (0.419 mM) and GSH (1.95 mM) were obtained by fitting the curve to logistic function. It should be noted that the IC\(_{50}\) was heavily dependent on the molar ratio of peptide and glucose. As demonstrated in Table 1 and Fig. 5, when the molar ratio was increased to 1:50, the concentration response curves of the five inhibitors are shown in Fig. 5A-F, the IC\(_{50}\) was increased as much as two orders of magnitude. As mentioned earlier, lower

![Fig. 5.](https://example.com/fig5.png)

**Fig. 5.** The inhibition (%) as a function of concentration for five inhibitors. Left panel: neurotensin (0.2 mg/ml) was incubated with glucose (0.2 mg/ml) (molar ratio of \~1:10) and the five tested inhibitors \((10^{-6} - 10^{-3}\) M). (A) Quercetin, (B) rutin, (C) EC, (D) ascorbic acid, (E) Carnosine, (F) GSH. Right panel: neurotensin (0.2 mg/ml) was incubated with glucose (2 mg/ml) (molar ratio of \~1:50) and the five tested inhibitors \((10^{-5} - 1\) M). (A') Quercetin, (B') rutin, (C') EC, (D') ascorbic acid, (E') carnosine, (F') GSH. The curve is fitted with logistic function to determine the IC\(_{50}\) for each inhibitor. The correlation coefficients \((R^2)\) are indicated.
molar ratio would be helpful to increase the sensitivity of the assay; however, longer incubation time would be required. In this work, we aim to develop a fast and straightforward screening assay that can determine the inhibitory activity of novel anti-AGE agents.

It is crucial to keep the assay time as short as possible; therefore, 1:10 peptide to glucose ratio was applied. If higher sensitivity is demanded, lower peptide to glucose ratio will be necessary.

Table 1 also lists the standard deviation of DSP under all the conditions with and without inhibitors. The small standard deviation suggests that the variation of each measurement is very minimal. The 4800 MALDI TOF/TOF mass spectrometer is equipped with on-axis laser irradiation, enabling sensitivity in the attomole range. In addition, proprietary source modeling algorithms enable low ppm mass detection across the MALDI plate. The day to day and plate to plate measurements are very stable, therefore, making it a reliable analytical tool for high throughput screening assay.

Zhang, Chung, and Oldenburg (1999) suggest that a positive Z-factor value between 0.5 and 1 is associated with a high quality assay. As we have known, the glycated form of the tested peptide displays a mass shift (Δm = 162 for monoglycation) in the mass spectrum, the unglycated form, however, does not show any mass shift. This unique feature determines that the DSP value of the positive control (no glycation) is zero with no standard deviation. The Z-factor for the inhibition assay window was calculated as 0.99 and 0.90 for the mixture with molar ratio of 1:10 and 1:50, respectively, indicating that this is a robust assay for detection of inhibitors.

Another important feature of this method is that the MALDI plate can be readily automated by connecting Probot MALDI spotter (Thermo Scientific, CA). The spotter provides small volume of spotting with excellent precision and accuracy; therefore, resulting in the results with minimized the standard deviation. Ideally, a library of compounds should be tested to examine the robustness of the method. The limited source of anti-AGE agents did not allow us to perform our assay on a large set of data. Nevertheless, all five tested anti-AGE agents exhibited inhibitory activities consistent with previously published data, validating the effectiveness of the method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.08.063.

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