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# Non-enzymatic interactions of glyoxylate with lysine, arginine, and glucosamine: A study of advanced non-enzymatic glycation like compounds

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**Abstract**

Glyoxylate is a 2 carbon aldo acid that is formed in hepatic tissue from glycolate. Once formed, the molecule can be converted to glycine by alanine–glyoxylate aminotransferase (AGAT). In defects of AGAT, glyoxylate is transformed to oxalate, resulting in high levels of oxalate in the body. The objective of this study was 2-fold. First, it was to determine, if akin to D-glucose, D-fructose or DL-glyceraldehyde, glyoxylate was susceptible to non-enzymatic attack by amino containing molecules such as lysine, arginine or glucosamine. Second, if by virtue of its molecular structure and size, glyoxylate was as reactive a reagent in non-enzymatic reactions as DL-glyceraldehyde; i.e., a glycose that we previously demonstrated to be a more effective glycating agent than D-glucose or D-fructose. Using capillary electrophoresis (CE), high performance liquid chromatography and UV and fluorescence spectroscopy, glyoxylate was found to be a highly reactive precursor of advanced glycation like end products (AGLEs) and a more effective promoter of non-enzymatic end products than D-glucose, D-fructose or DL-glyceraldehyde.


*Keywords:* AGLEs; AGEs; Glyoxylate; Glucosamine; Capillary electrophoresis

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## 1. Introduction

Reducing sugars and their metabolites can non-enzymatically interact with proteins to form a heterogeneous group of compounds commonly referred to as advanced glycation end products (AGEs). The chemistry of non-enzymatic reactions has been thoroughly investigated and the process has been shown to rely on the initial formation of an imine; i.e., a Schiff base that is brought about by the interaction of the carbonyl moiety of sugars with the free amino groups on a protein. Once formed, this imine undergoes an Amadori rearrangement to form a stable ketoamine that with time through the process of cyclization, dehydration, condensation, and oxidation converts into AGEs [1]. AGEs occur both *in vitro* and *in vivo* and their presence in diabetics has been speculated to exacerbate such conditions as renal failure, cataract formation, and Alzheimer's disease [2–5].

Glyoxylate is a 2 carbon aldo acid that is enzymatically formed in the peroxisomes from the oxidation of glycolate by glycolate oxidase. It is produced as a byproduct of the pentose phosphate pathway or from the breakdown of serine and hydroxyproline [6]. In the mammalian liver, alanine–glyoxylate aminotransferase (AGAT) converts glyoxylate to glycine. A defect in the AGAT enzyme causes glyoxylate to accumulate which subsequently leads to increased oxalate levels *in vivo* [6,7].

In earlier studies, we and others demonstrated the non-enzymatic interaction of DL-glyceraldehyde with proteins and showed that DL-glyceraldehyde was a more effective promoter of AGEs than D-glucose or D-fructose [8–10]. The increased reactivity of glyceraldehyde with proteins was speculated to be due to two factors: (i) the small size of glyceraldehyde relative to glucose or fructose (i.e., a triose carbohydrate versus hexose sugars), and (ii) the inability of the molecule to generate internal hemiacetal or hemiketal ring structures such as those formed in glucose and fructose, respectively.

To test this hypothesis, we focused attention on glyoxylate and reasoned that since it had a free aldehyde and was smaller than DL-glyceraldehyde, it too should participate in non-enzymatic reactions. We also reasoned that because it could not form an internal ring structure, it (i.e., glyoxylate) too, like DL-glyceraldehyde, should react more extensively than D-glucose or D-fructose to form advanced non-enzymatic end products. More importantly, we speculated that with it being comprised of two carbons, glyoxylate should act as a more effective agent of non-enzymatic reactions than DL-glyceraldehyde, D-glucose or D-fructose.

This report describes the formation of non-enzymatic glycation like products of glyoxylate with lysine, arginine, and glucosamine and compares the reactivity of glyoxylate (i.e., with these amino containing molecules) with DL-glyceraldehyde, D-glucose, and D-fructose. Lysine, arginine, and glucosamine were used as the model reactants in this study because each was previously shown to be vulnerable to non-enzymatic attack by different sugars [8–11].

## 2. Experimental

### 2.1. Chemicals and reagents

Analytical grade glyoxylic acid, L-lysine, L-arginine, D-glucose, D-fructose, DL-glyceraldehyde, D-glucosamine, sodium tetraborate, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma Chemical Company (St. Louis, MO,

UA). All buffers and solutions were prepared with Milli-Q purified distilled water (Millipore, Bedford, MA).

### 2.2. Preparation of reaction mixtures and buffer

Unless otherwise indicated, all controls and *in vitro* reactions were conducted in 0.2 M phosphate buffer, pH 7.2, containing 0.02% sodium azide. Reaction mixtures were comprised of 20 mM glyoxylate or 20 mM D-glucose, D-fructose or DL-glyceraldehyde in the presence of varying concentrations of lysine, arginine or glucosamine (i.e., 2, 20, and 40 mM). The final pH was maintained at pH 7.2. Control incubations included (i) glyoxylate alone (20 mM), (ii) D-glucose, D-fructose or DL-glyceraldehyde alone (i.e., each at 20 mM) or (iii) lysine, arginine or glucosamine only (i.e., each at 40 mM).

The reaction mixtures and controls were each placed in 1-ml screw cap polypropylene tubes (Sigma Chemical Co., St. Louis, MO) and incubated in the dark at 37 °C for 3 and 30 days and were then frozen until analysis. Unless otherwise indicated, all experiments were performed in duplicate.

### 2.3. UV and fluorescence spectroscopy

A Lambda 900 UV-NIR (Perkin-Elmer Instruments, Shelton, CT, USA) was used for scanning the UV absorbance of the reaction mixtures. The UV absorbance was measured at 273 nm for all samples. The acquisition of fluorescence readings was accomplished using an LS 55 luminescence spectrometer (Perkin-Elmer Instrumentation) at excitation and emission wavelengths of 370 and 420 nm, respectively. The UV readings were performed at 273 nm as this wavelength was found to be optimal for monitoring AGLE's. The fluorescence readings were performed at an excitation of 370 nm and an emission of 420 nm for similar reasons. These wavelengths were also found to be optimal in other glycation studies [8,14,17]. A circulating water bath set at $25 \pm 1$ °C controlled the sample cell temperature during the measurements. All spectrometric analyses were performed with disposable UV-transparent plastic cuvettes ($10 \times 35$ mm) that were purchased from Fisher Scientific (New Lawn, NJ, USA).

### 2.4. High performance liquid chromatography (HPLC)

The HPLC system (Hitachi High Technologies America Inc., San Jose, CA) consisted of a low pressure gradient pump (L-7100), a 4-channel degasser, a sequential auto sampler (L-7200) and a high sensitivity diode array detector (190–800 nm). The system was controlled by a D-700 HPLC System Manager software package. The AGE components of each mixture were separated on a Discovery C18 (Sigma–Aldrich Co., Milwaukee, WI) 5 μm column ($25 \text{ cm} \times 4$ mm). Mobile phase A consisted of 0.2% butylamine (BA), 0.5% phosphoric acid (Pi), and 1% tetrahydrofuran (THF) in water. Mobile phase B consisted of 0.1% BA, 0.25% Pi, and 0.5% THF in 60% of acetonitrile in water. A linear gradient from 5 to 100% of mobile phase B was applied in 45 min at a flow rate of 1.00 ml/min. All samples were filtered using 0.22 μm membranes (Millipore, Milford, MA). All solvents used were HPLC grade and were degassed and sonicated for 15 min before use. All HPLC experiments were performed three times to ensure that the retention times reported for the AGE's were reproducible.

### 2.5. Capillary electrophoresis

Capillary electrophoresis analyses were performed at ambient temperature using a Quanta 4000 CE unit with UV detection at 214 nm (Waters Corporation, Milford, MA). A capillary with an internal diameter of 50 μm, 600 nm total length and 500 mm effective length was cut from bulk stock purchased from Polymicro Technologies (Phoenix, AZ, USA). The separating buffer for CE consisted of 20 mM sodium tetraborate. The pH of the buffer was adjusted to 9.1 using 1 N NaOH and filtered with a 45 μm syringe filter (Millipore Corporation, Bedford, MA).

The capillary was conditioned by flushing for 15 min with 1 N NaOH, 10 min with Milli-Q water, followed by 5 min with the tetraborate buffer. It was rinsed between the runs with 1 N NaOH for 1 min followed by the separating buffer for 2 min. Buffer solution was made fresh each day and the same NaOH solution was used during every run. Acetone had a consistent migration time of 5.78 min by CE and was used as a neutral marker. All samples were applied to the capillary at the anionic end using hydrodynamic injection for 20 s. Electropherograms were monitored at 214 nm, and the data was collected on a Spectra Physics Integrator (Model SP4270, San Jose, CA).

## 3. Results and discussion

### 3.1. Spectroscopy

Tables 1 and 2 show the fluorescence and UV data of lysine, arginine, and glucosamine with glyoxylate at 37 °C for 72 h. AGE species were formed with each of the mixtures; however, the incubation solution containing glucosamine with glyoxylate yielded the highest UV and fluorescence readings. Next to glucosamine, the lysine glyoxylate mixture showed the highest rate of AGLE formation which suggested that lysine was also susceptible to non-enzymatic attack by glyoxylate. The lowest fluorescence readings were obtained with the arginine glyoxylate mixture, an observation that was supported by the UV absorbance profile. Increases in the fluorescence and UV readings with time of the test tubes containing glucosamine alone supported earlier reports [8,13,17] that glucosamine can form auto-condensation products (i.e., AGEs). No change in the fluorescence and UV absorbance readings of the other control tubes (i.e., lysine, arginine or glyoxylate

Table 1
Fluorescence data of solutions of L-lysine (40 mM), L-arginine (40 mM) or D-glucosamine (40 mM) with glyoxylate (20 mM) at 37 °C for 72 h

| Time (h) | Glucosamine (40 mM) | Arginine (40 mM) | Lysine (40 mM) | Control glucosamine alone (40 mM) |
|---|---|---|---|---|
| 0 | 17.84 | 16.83 | 25.16 | 16.48 |
| 2 | 24.42 | 17.09 | 25.17 | 28.1 |
| 5 | 39.16 | 17.1 | 28.98 | 32.1 |
| 10 | 79.93 | 17.62 | 29.71 | 69.9 |
| 24 | 156.46 | 19.02 | 32.86 | 116.66 |
| 72 | 272.88 | 20.63 | 61.67 | 180.3 |

The spectroscopic reading for every measurement was within 10% of its counterpart duplicate reading.
*Note:* Controls tubes containing L-lysine (40 mM), L-arginine (40 mM) or glyoxylate (20 mM) alone did not show any increases in fluorescence reading with time.

Table 2
UV absorbance data of solutions of L-lysine (40 mm), L-arginine (40 mm) or D-glucosamine (40 mm) with glyoxylate (20 mM) at 37 °C for 72 h

| Time (h) | Glucosamine (40 mM) | Arginine (40 mM) | Lysine (40 mM) | Control glucosamine alone (40 mM) |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 | 0 |
| 5 | 4.034 | 0 | 1.112 | 0.494 |
| 10 | 20.296 | 0 | 3.911 | 7.204 |
| 24 | 50.885 | 0 | 10.334 | 29.806 |
| 72 | 100 | 0 | 25.154 | 71.511 |

All UV readings were compared to the highest UV absorbance measurement that was set at 100%. The spectroscopic data for every measurement was within 10% of its counterpart duplicate reading.
*Note:* Controls tubes containing L-lysine (40 mM), L-arginine (40 mM) or glyoxylate (20 mM) alone did not show any increases in UV reading with time.

alone) suggested that the formation of the AGLEs was strictly due to glyoxylate non-enzymatically interacting with lysine.

Fig. 1A and B demonstrate the fluorescence and UV absorbance profiles of increasing concentrations of lysine with constant glyoxylate concentration at 37 °C for 3 days. Fig. 2A and B show the respective fluorescence and UV spectral profiles for incubation mixtures containing increasing concentrations of glucosamine with a set concentration of glyoxylate under identical incubation conditions as those that were described for lysine. The data from UV and fluorescence analysis demonstrated that the increased trend in the formation of AGE species was directly relatable to lysine or glucosamine concentrations. In other words, as lysine and glucosamine concentrations were increased so did the UV and fluorescence readings. It must be pointed out, however, that the rate of increase in AGLE species as well as the amount of the AGLEs that were formed appeared higher for the glucosamine glyoxylate mixtures than the lysine glyoxylate solutions.

The formation of the AGLEs in the test tubes containing glucosamine and glyoxylate were attributed to two types of non-enzymatic reactions, reactions that we speculate were occurring concurrently. The first of these reactions was ascribed to the self polymerization of glucosamine, a finding that was confirmed by a close scrutiny of the UV and fluorescence profiles of the control glucosamine solution. The second reaction was attributed to glyoxylate non-enzymatically interacting with glucosamine, a speculation that was supported by a direct comparison of the UV and fluorescence profiles of the control glyoxylate solution with mixtures that contained glyoxylate and varying concentrations of glucosamine.

Similar to the AGLEs of lysine, the rate and the extent of the formation of glucosamine AGLEs appeared to be influenced by reactant concentration. For example, with glyoxylate concentrations being kept constant, the increases in UV and fluorescence readings were found to be directly relatable to the amounts of glucosamine that were reacted with glyoxylate (see Fig. 2A and B). Fig. 3A and B compare the reactivity of glyoxylate and of other sugars such as DL-glyceraldehyde, D-glucose or D-fructose with D-glucosamine. The fluorescence and UV data show that glyoxylate was 60% more reactive than D-glucose or D-fructose and 20% more reactive than DL-glyceraldehyde, a finding that was consistent with our speculation that being a smaller molecule than D-glucose, D-fructose or DL-glyceraldehyde, glyoxylate was more likely to yield higher amounts of advanced non-enzymatic end products [8,15,16].

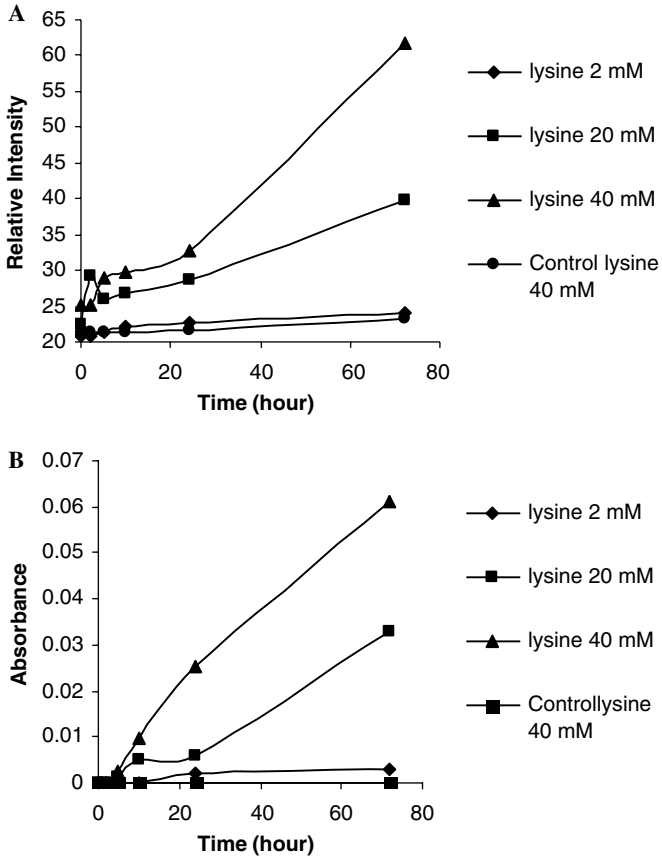


Fig. 1. (A and B) Respective fluorescence and UV time course profiles of reaction mixtures containing varying lysine concentration and a fixed glyoxylate concentration (20 mM) at 37 °C for 3 days. The spectroscopic data for every measurement was within 10% of its counterpart duplicate reading. Control tubes included solutions of either lysine alone (40 mM) or glyoxylate alone (20 mM) that were incubated at 37 °C for up to 3 days.

### 3.2. HPLC analysis

Fig. 4A and B show the respective HPLC profiles for glucosamine alone (control) and for mixtures of glyoxylate (20 mM) with glucosamine (20 mM) that were incubated at 37 °C for 30 days. The control glucosamine solution was resolved by reverse phase HPLC into three peaks with retention times ($R_t$) of 2.32, 2.59, and 2.85 min, respectively (see Fig. 4A). The addition of glyoxylate to glucosamine caused a sharp decrease in the peak at 2.32 min and allowed the formation of two new AGLEs at 2.83 and 3.60 min (Fig. 4B).

Chromatographic analysis of a freshly prepared solution of glucosamine (20 mM) by reverse phase HPLC yielded no detectable UV absorbing peaks indicating that the formation of glucosamine AGLEs was a time dependent process. It is important to point out also that no UV absorbing peaks were observed with a freshly prepared solution of glyoxylate alone or a solution of glyoxlate that was incubated by itself only at 37 °C for 30 days.

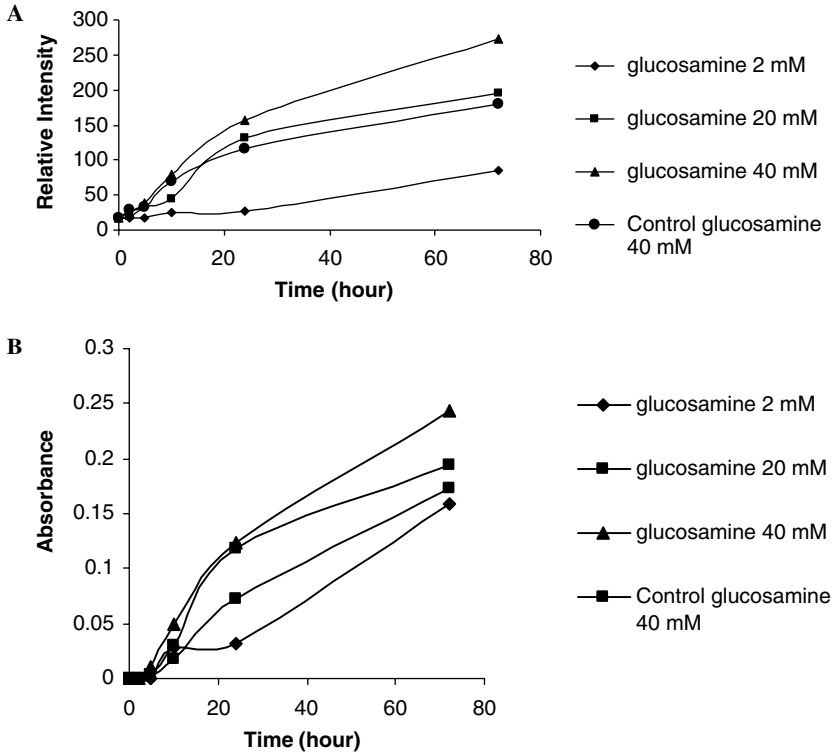


Fig. 2. (A and B) Respective fluorescence and UV time course profiles of reaction mixtures containing varying D-glucosamine concentration and a fixed glyoxylate concentration (20 mM) at 37 °C for 3 days. The spectroscopic data for every measurement was within 10% of its counterpart duplicate reading. Control tubes included solutions of either D-glucosamine alone (40 mM) or glyoxylate alone (20 mM) that were incubated at 37 °C for up to 3 days.

Fig. 5A and B show the HPLC analysis of mixtures of glyoxylate with lysine and glyoxylate with arginine, respectively. The glyoxylate solution containing lysine was resolved into nine distinct AGE species (i.e., with $R_t$ of 2.29, 2.40, 2.53, 2.61, 2.83, 4.83, 5.17, 5.44, and 6.59 min) whereas that with arginine and glyoxylate was resolved into three AGE peaks only (i.e., with $R_t$ at 2.32, 2.83, and 4.8 min). HPLC analysis of the control lysine and arginine solutions yielded no detectable AGLE species (data not shown).

### 3.3. Capillary electrophoresis analysis

Fig. 6 shows the electropherogram of mixtures of glucosamine with glyoxylate after immediate mixing and after 3 and 30 days of incubation. The CE results confirmed the earlier HPLC findings that the formation of AGLEs ($tr_4 = 11.10$, $tr_5 = 16.46$, and $tr_6 = 18.42$) was a time dependent process. The CE data also substantiated the earlier speculation that the presence of glyoxylate was contributory to the inhibition of glucosamine auto-condensation products ($tr_1 = 12.26$, $tr_2 = 10.58$, and $tr_3 = 15.88$).

Fig. 7 shows the electropherogram of the AGLEs that were formed at 37 °C for 3 days with increasing concentrations of glucosamine and in the presence of a set concentration

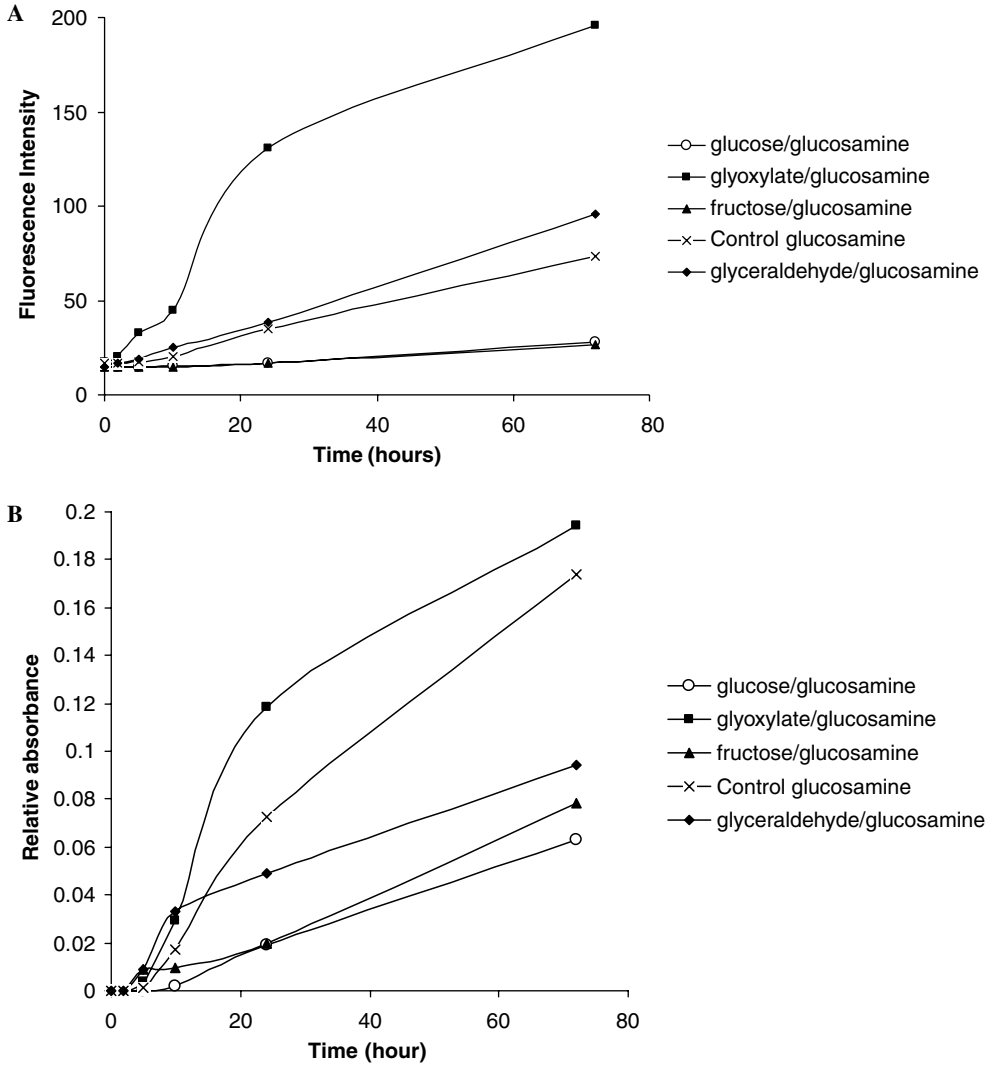


Fig. 3. (A and B) Respective fluorescence and UV time course profiles of glyoxylate, DL-glyceraldehyde, D-glucose or D-fructose (i.e., each at 20 mM) with D-glucosamine (40 mM) at 37 °C for 3 days. The spectroscopic data for every measurement was within 10% of its counterpart duplicate reading. Control solutions included D-glucosamine (40 mM) alone in phosphate buffer, pH 7.2 at 37 °C for up to 3 days.

of glyoxylate (20 mM). At 2 mM glucosamine, no prominent auto-condensation products were observed and the decrease in the height of the CE peaks suggested that the presence of glyoxylate in the reaction mixture adversely affected the non-enzymatic self polymerization of glucosamine. As the glucosamine concentration was increased to 20 mM and above, new AGLEs were formed; i.e., in addition to the auto-condensation products of glucosamine.

The electropherograms in Fig. 8 shows the AGLEs of lysine and arginine with glyoxylate at two periods of incubation, i.e., at 3 days and 30 days. With increasing incubation

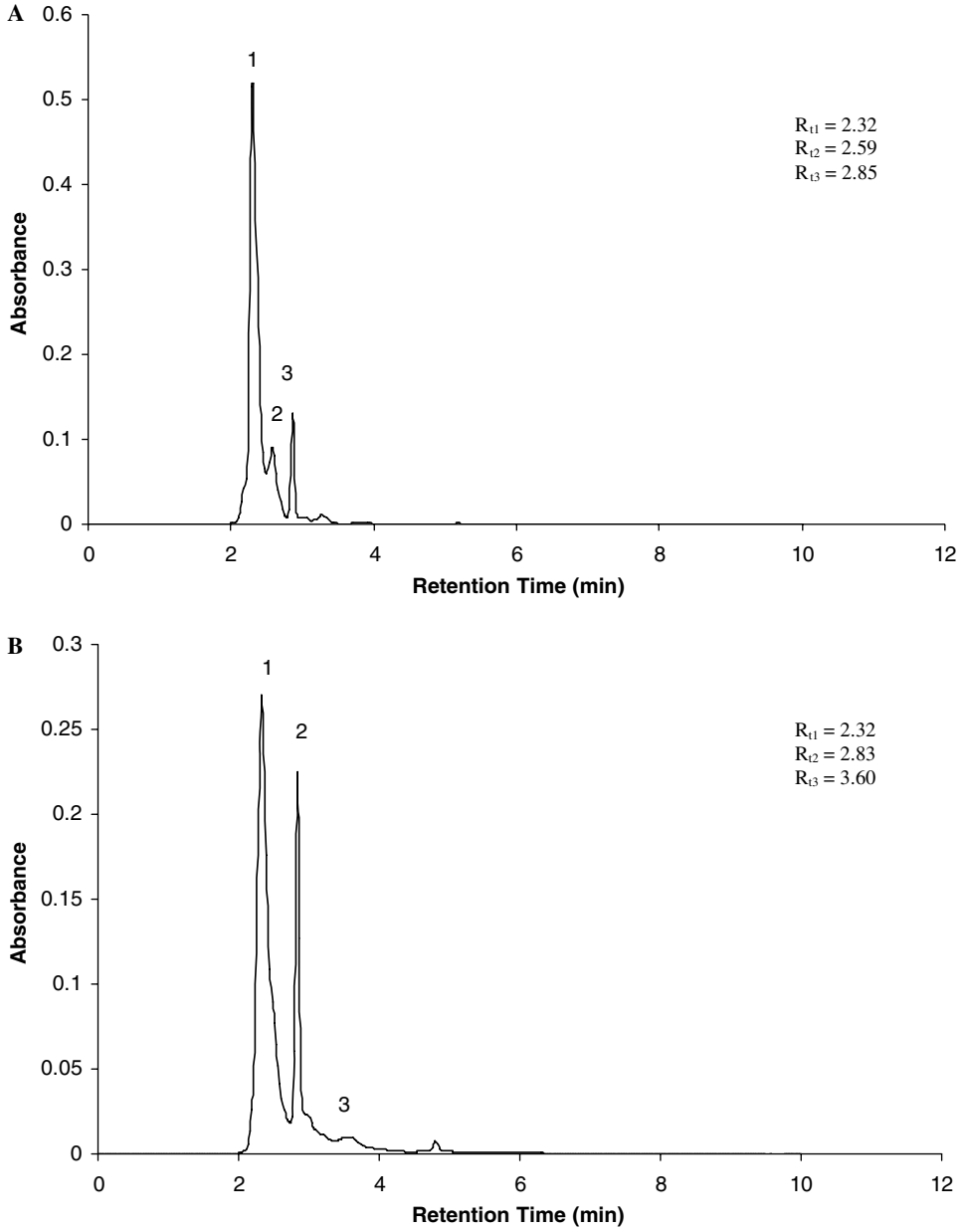


Fig. 4. (A and B) A comparison of the HPLC profiles for a solution that contained D-glucosamine alone (20 mM) and a solution that contained glyoxylate (20 mM) with D-glucosamine (20 mM), respectively. Each sample was incubated at 37 °C for 30 days. *Note:* Freshly prepared glucosamine showed no HPLC peaks.

time, both lysine and arginine glyoxylate mixtures showed an enhanced formation of AGLEs. Finally, it needs to be pointed out that the analysis of samples by CE was performed in triplicates and in each instance, the results were reproducible and no significant run-to-run and day-to-day differences were observed.

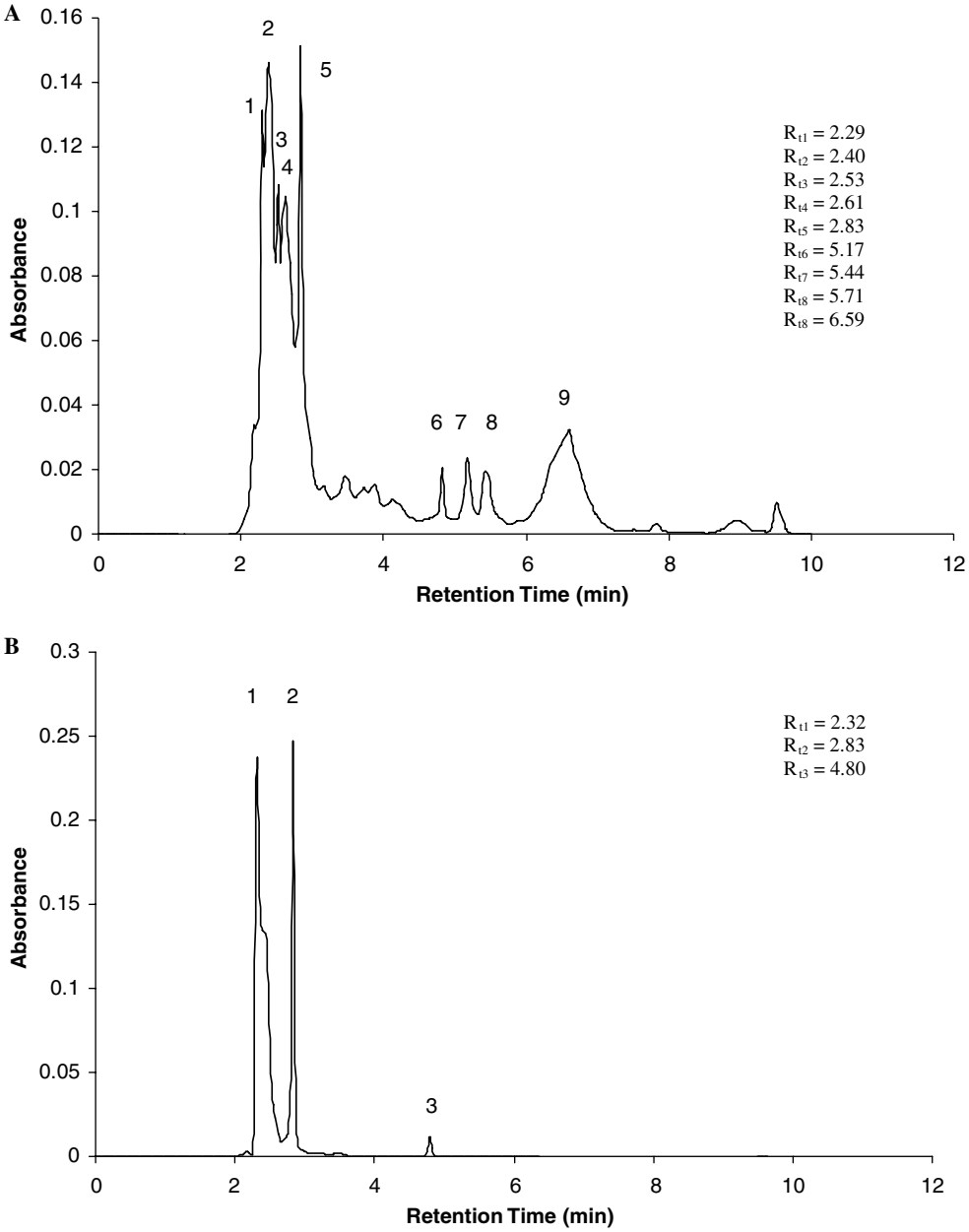


Fig. 5. (A and B) Respective HPLC profiles of a glyoxylate (20 mM) lysine (20 mM) and a glyoxylate (20 mM) arginine (20 mM) mixture that were each incubated at 37 °C for 30 days.

### *3.4. Reactivity of glyoxylate with modified lysine*

To determine the contribution of the amino groups on lysine to the formation of glyoxylate AGLEs, in separate experiments glyoxylate was reacted with either *N*α-acetyl

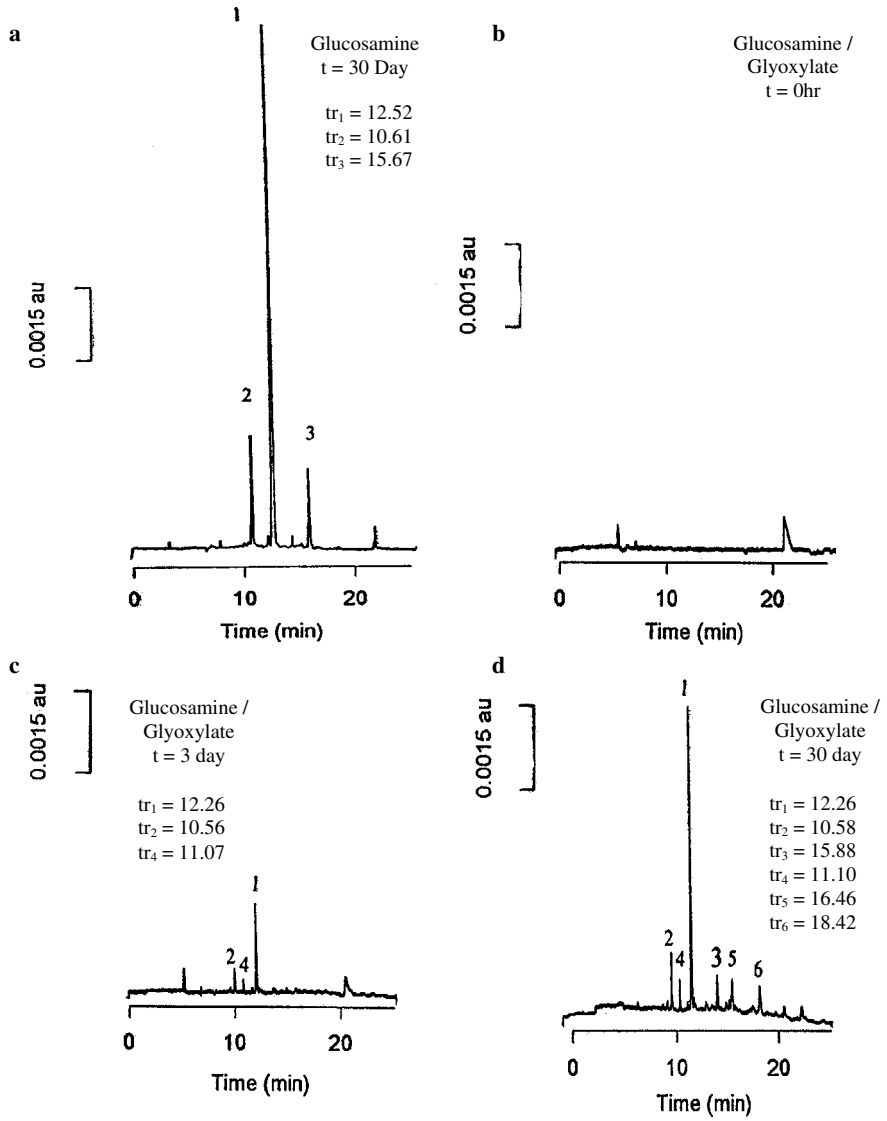


Fig. 6. CE anlyses of a freshly prepared solution of (a) glucosamine alone (20 mM) that was incubated at 37 °C for 30 days and D-glucosamine (20 mM) with glyoxylate (20 mM) after immediate mixing (b) and after incubation at 37 °C for (c) 3 days and (d) 30 days.

lysine or *N*ε-acetyl lysine. In the absence of any AGLEs with each of the lysine derivates, the conclusion was reached that both amino groups on lysine play a vital role in the formation of the glyoxylate AGLEs. In contrast to glyoxylate, DL-glyceraldehyde reacted with the *N*ε-acetyl substituted lysine [9]. Additional findings revealed that glyoxylate did not react with guanidine chloride, perhaps, indicating that both the guanidine group and the α-amino groups of arginine are required for the formation of glyoxylate AGLEs. In view of these results, we speculate that glyoxylate may be more reactive with free

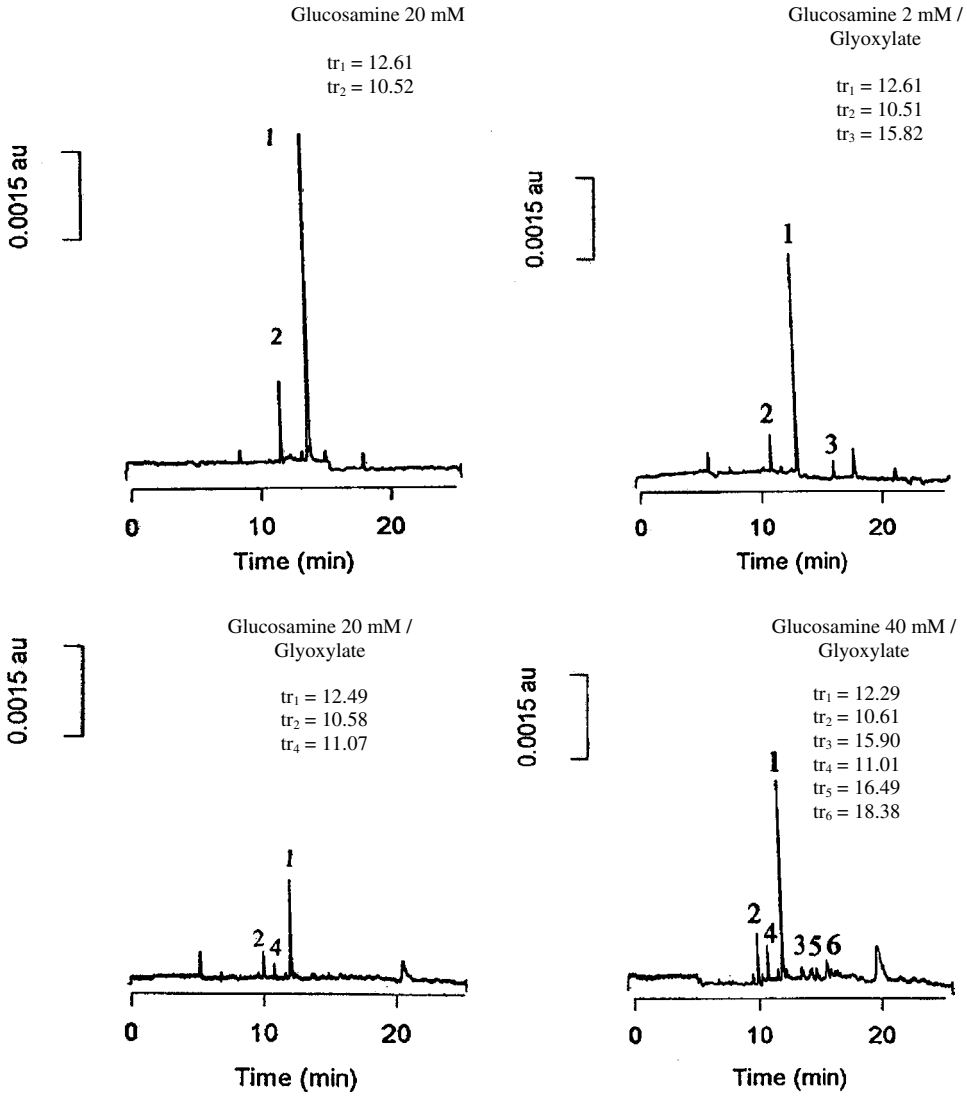


Fig. 7. Monitoring the AGLE formation of D-glucosamine (2, 20, and 40 mM) with glyoxylate (20 mM) after 3 day incubation time period using CE.

L-lysine or L-arginine than with the same amino acids when they are engaged in peptide bonds as part of a protein. Evidence to support this speculation comes from the study of Schmitt et al. who demonstrated that glyoxylate exhibited a negligible reactivity to human serum albumin [18].

## 4. Conclusion

This study focused on the non-enzymatic interaction of glyoxylate with lysine, arginine, and glucosamine and demonstrated that glyoxylate was a highly reactive AGLE precursor.

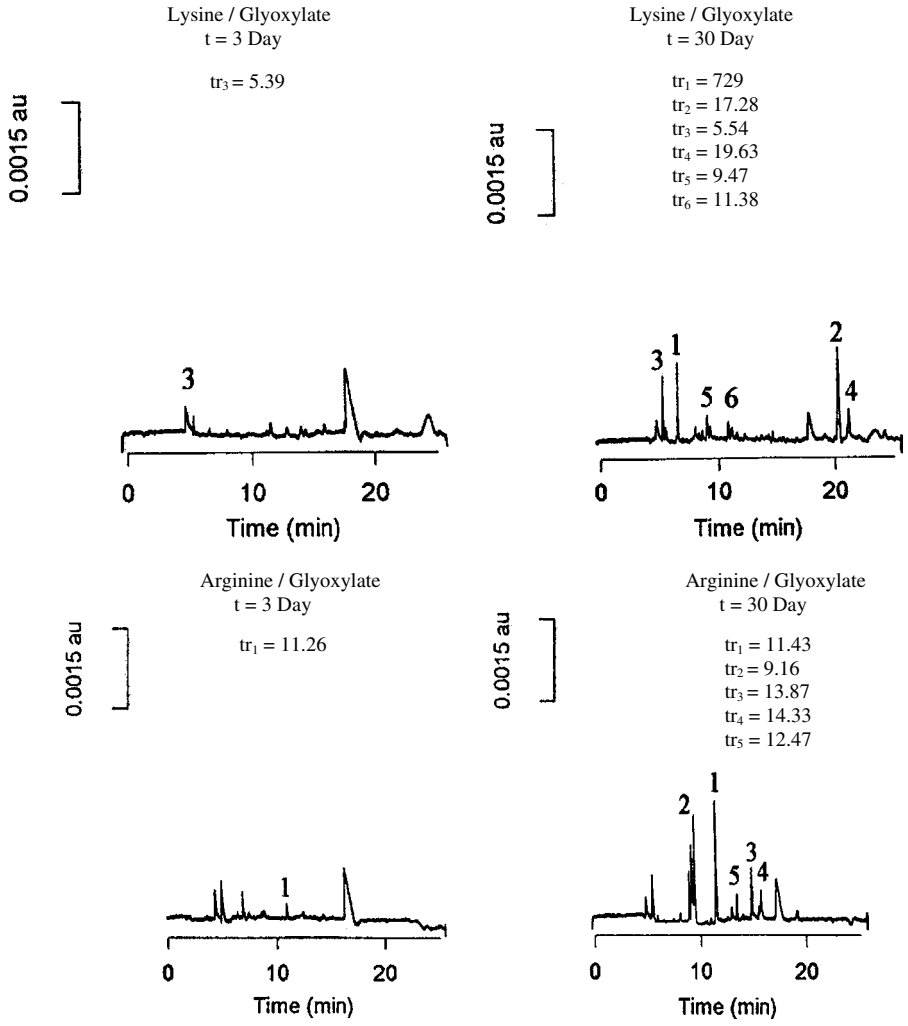


Fig. 8. Monitorng the AGLE formation of lysine (40 mM)–glyoxylate (20 mM) and arginine (40 mM)–glyoxylate (20 mM) after 3 and 30 day incubation period using CE.

Lysine and arginine were used as the model amino acids because each was previously shown to be highly vulnerable to non-enzymatic attack by D-glucose, D-fructose, and DL-glyceraldehyde [8–11]. Glucosamine was employed as a model for glycation because (i) it is a commodity item for the treatment of osteoarthritis and (ii) it can auto-condense and form AGEs with different carbohydrates, a finding that was demonstrated previously by us and others [12–14,17]. Additionally, no studies to date have identified whether the therapeutic effects of glucosamine result from glucosamine itself or from a glucosamine AGE [12].

The HPLC and CE data confirmed the UV and fluorescence findings that glyoxylate was a more reactive molecule in its interaction with lysine, arginine or glucosamine than DL-glyceraldehyde, D-glucose or D-fructose (Fig. 5A and B). The two chromatographic

techniques also demonstrated that glyoxylate formed different AGLE intermediates with lysine and arginine and that the number of the AGLE species that were formed depended on the amino acid structure. Additional studies revealed that glyoxylate did not react with any of the amino substituted lysines such as *N*α-acetyl lysine or *N*ε-acetyl lysine. The lack of reactivity of glyoxylate with these compounds suggests that the lysyl groups in a protein may not be highly reactive with glyoxylate. This speculation supports our finding and the studies of Schmitt et al. who demonstrated negligible reactivity of glyoxylate with human serum albumin [18]. The glucosamine glyoxylate AGLE profile showed inhibition of glucosamine auto-condensation AGEs, an effect that was reported earlier with other reducing sugars and glucosamine [8].

In short, capillary electrophoresis proved to be an effective and a powerful technique for separating the glyoxylate related AGE species, a technique that we found to be also rapid and simple to perform. Additionally, since no sample preparation was required, this method provided a convenient approach for the monitoring of AGLEs.

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