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Escola Superior de Tecnologias de Saúde do Porto

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# Effect of active components from Garlic on AGEs formation

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Escola Superior de Tecnologias de Saúde do Porto

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# Effect of active components from Garlic on AGEs formation

Dissertation submitted to obtain Master degree in Bioquímica em Saúde, produced under scientific supervising from Professor Eden Tareke from Department of Food Chemistry and Applied Nutrition Lund University, Sweden, and Professors Mónica Vieira and Rúben Fernandes from Área de Ciências Químicas e Biomoléculas, Escola Superior de Tecnologias de Saúde do Porto, Portugal

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”We are an undermost grain of cosmic dust…”

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# Abstract

Advanced glycation end-products are Maillard reaction products that are found in thermal processed food. This compounds are often referred as unhealthy for human diet, namely because of their capacity to form amino-acid dimers. There is a broad range of answers to get about how these products are formed, how they interact with the organism and how these reactions can be inhibited to prevent the referred effects. Some compounds from garlic are thought to be able to inhibit these reactions. This study using spectrophotometric, High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) and Fourier transformed infrared spectroscopy (FTIR) analysis, helps to understand better not only not only the effect of some compounds obtained from garlic, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS), on these AGEs production reaction, but also helped to understand better the reaction itself.

# Index

## List of abbreviations

| | |
|---|---|
| **AGEs** | Advanced Glycation End Products |
| **CEL** | Nε-(carboxyethyl) lysine |
| **CEL-d4** | Nε-(carboxyethyl) -L-[4,4,5,5-2H4]lysine |
| **CML** | Nε-(carboxymethyl) lysine |
| **CML-d4** | Nε-(carboxymethyl) -L-[4,4,5,5-2H4]lysine |
| **DAD** | Diode array detector |
| **DADS** | Diallyl disulfide |
| **DAS** | Diallyl sulfide |
| **DATS** | Diallyl trisulfide |
| **FTIR** | Fourier transform infrared spectroscopy |
| **ELISA** | Enzyme-lynked immunosorbent assay |
| **GC** | Gas chromatography |
| **GO** | Glyoxal |
| **GOLD** | Imidazolium cross-link derived from glyoxal and lysine-lysine |
| **HPLC** | High performance liquid chromatography |
| **Lys** | Lysine |
| **MG** | Methylglyoxal |
| **MOLD** | Imidazolium cross-link derived from methylglyoxal and lysine-lysine |
| **MS** | Mass spectrometer |
| **MS/MS** | Tandem mass spectrometer |
| **PBS** | Phosphate buffered saline |
| **RAGE** | AGE receptor |

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# Introduction

## 1. AGEs

Maillard reaction is a nonenzmatic reaction that starts with the reversible formation of unstable Schiff base, by condensation of the aldehyde group of glucose with the amino group of proteins. The Schiff base undergoes Amadori rearrangement and some other partially understood reactions and result in stable compounds known as advanced glycation end-products (AGEs). Advanced glycation end-products, a heterogeneous group of macromolecules, are also formed by the non-enzymatic glycation of lipids and nucleic acids. Thermal processing which is an important part of modern food preparation, prolongs shelf-life, reduces food-borne diseases and increases palatability, however it is also responsible for the formation of both AGEs and their precursors (Nemet, Varga-Defterdarovic et al. 2006) (Semba, Nicklett et al. 2010) (Poulsen, Hedegaard et al. 2013).

Human exposure to AGEs can be either exogenous (food intake) or endogenous (produced in the human body) (Semba, Nicklett et al. 2010) leading to increased level of body pool of AGEs. AGEs interact with tissue proteins as collagens or lens proteins and it is thought that they have relevant role in development of pathologies like aging, diabetes, vascular disease, rheumatoid arthritis, chronic renal failure, different kinds of cancer and Alzheimer's disease (Thorpe and Baynes 1996), (Odani, Shinzato et al. 1998), (Baynes and Thorpe 1999). In addition to this protein structure and function alterations, AGEs can also induce oxidative stress leading to inflammation and propagation of tissue damage (Thorpe and Baynes 1996).

Preventing endogenous formation and in-take of AGEs is important, because in distinction from the Schiff base and the Amadori product, AGEs irreversibly link to proteins altering their stability and their physicochemical and biochemical properties permanently (Nemet, Varga-Defterdarovic et al. 2006).

The Maillard reaction can lead to the production of different compounds that can be organized into three main pathways, one that leads to colored compounds, other that leads to aromatic compounds and finally the AGEs pathway. A big variety of compounds can be

considered as food-derived AGEs, despite their different composition and properties (Poulsen, Hedegaard et al. 2013).

Glyoxal (GO) and methylglyoxal (MG) are abundant Maillard reaction intermediates that lead to browning and protein crosslinking. These two α-dicarbonyls' role in protein damage is considerable thus they are getting more attention because of their abundance and high reactivity. GO and MG can be obtained by various pathways both endogenously and exogenously. Glucose and Amadori products autoxidation are the main pathways for endogenously formation of GO (Chetyrkin, Mathis et al. 2011) (Lange, Wood et al. 2012), while MG is also formed endogenously in numerous reactions like glycolysis, lipid peroxidation, acetone or amino acetone metabolism or by degradation of DNA. For MG also some external sources have been identified e.g. beverages, foods such as toast, coffee or soy sauce and cigarette smoke (Murata-Kamiya and Kamiya 2001). GO and MG are being increasingly recognized as a key intermediates for AGEs formation and being widely studied so the reactions involving these two compounds are better understood (Brinkmann, Wellsknecht et al. 1995) (Nemet, Varga-Defterdarovic et al. 2006) (Poulsen, Hedegaard et al. 2013). GO and MG readily react with amino acids Lysine and Arginine, reaction with the ε side chain of Lysine leads to Nε-(1-Carboxymethyl)-L-Lysine (CML) and Nε-(1-Carboxyethyl)-L-Lysine (CEL) respectively. CML being the first AGE to be detected in food samples most of the analytical work has been done on CML and as an analogous compound CEL is also been used as a marker frequently (Ahmed, Mirshekar-Syahkal et al. 2005) (Ames 2008) (Poulsen, Hedegaard et al. 2013). CML and CEL react farther with additional amino acids, usually lysine or arginine creating a cross-link. Several cross-linkers have been identified, such as lysine dimers Glyoxal lysine dimer (GOLD) and Methylglyoxal lysine dimer (MOLD) are obtained from the reaction of lysine with respectively GO or MG (Poulsen, Hedegaard et al. 2013). GOLD and MOLD were the first imidazolium crosslinking AGEs of α-dicarbonyl origin reported in tissue proteins (Chellan and Nagaraj 1999). On Figure 1are represented some of these related compounds.

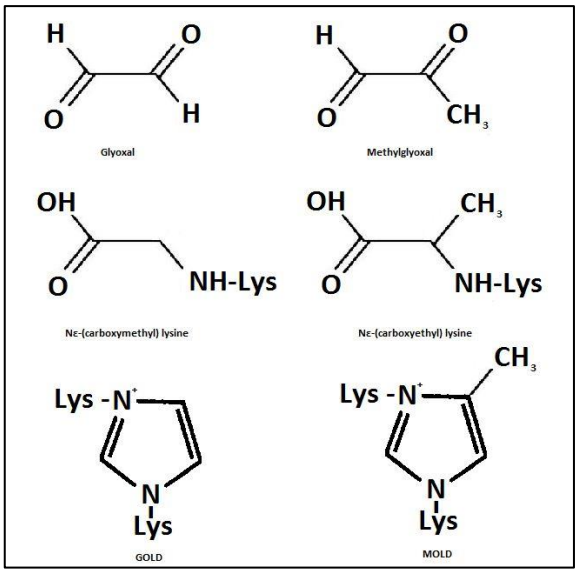



Figure 1 – Structures of analyzed AGEs and their reactive precursors.

## 1.1 Measurement methods

The measurement of AGEs is a really challenging activity due to the broad range of physical and chemical properties of AGEs, so the purification and separation can become very complex activities. There is no commonly accepted method for detecting and measuring AGEs what makes it harder getting meaningful results comparisons between different researching groups (Poulsen, Hedegaard et al. 2013).

There is a broad range of methods for qualitative and quantitative analysis of AGEs that can be classified in two main groups: Immunochemical methods and instrumental methods (Poulsen, Hedegaard et al. 2013).

The immunochemical methods are mostly based in Enzyme-Linked Immunosorbent Assay (ELISA) techniques using unspecific antibodies that allow detection of AGEs in general or specific antibodies that allow detection of some specific AGE types, like CML or CEL. The most commonly used instrumental methods are High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) and those can be coupled to different detectors like DAD, UV-Vis, fluorescence detector, Mass Spectrometry (MS) and Tandem Mass Spectrometry (MS/MS). AGEs usually show strong absorption rates for wavelengths above 350nm, but depending on the kind of compounds in solution the absorption peaks for AGEs can vary from 330nm to values above 420nm (Monnier and Cerami 1982) (Das, Sun et al.

1998) (Chellan and Nagaraj 1999) (Poulsen, Hedegaard et al. 2013). A study comparing the accuracy and precision of LC-MS/MS, GC-MS and ELISA showed that LC-MS/MS had the lowest standard deviation and better accuracy (Tareke, Forslund et al. 2013).

The complexity of food matrix and biological matrix in combination with the complexity and diversity of AGEs makes AGE analysis challenging. However more Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) based method for analysis of CML and CEL are immerging (Zhang, Huang et al. 2011, Tareke, Forslund et al. 2013). There is no consensual method for AGEs analysis, not only when studying AGEs standards but particularly for research with food samples with a different matrix. These two main groups of methods complement each other as long as some group of AGEs are not easily distinguished or might suffer from a matrix effect that leads to conflicting results (Poulsen, Hedegaard et al. 2013).

Fourier transformed infrared spectroscopy (FTIR) allows accurate and precise assignment of molecular conformations, bonding types and functional groups providing an extra tool for biomolecules' composition and structure determination. (Kumar and Prasad, 2012)

## 1.2 Inhibition

The first studies concerning functional interference of advanced glycosylation was considered during research on how glucose affects the lens crystallines conformation. Acetylsalicylic acid modifies Lys-199 side chain preventing non-enzymatic glycosylation on this specific site (Walker 1976). This principle was studied *in vitro* (Huby and Harding 1988) and *in vivo* (Crompton, Rixon et al. 1985) (Bucala, Manabe et al. 1985) and were the first ones to show the protective effect of some kind of advanced glycosylation inhibitor.

In a general approach the inhibition mechanisms block sugar attachment to proteins or break down formed crosslinks, the inhibition through blocking of RAGEs, a member of the immunoglobulin superfamily that is AGE sensitive and can be found in some human cells surface working as AGE receptor has also been studied. There is a broad range of inhibitors both natural or synthetic that can be used, pharmaceutical compounds, enzymes, endogenous

scavengers or food-derived compounds, as antioxidants (Peng, Ma et al. 2011) (Poulsen, Hedegaard et al. 2013) (Ramkissoon, Mahomoodally et al. 2013).

AGE formation, and particularly the most reactive AGE precursors formation, is regulated by detoxification pathways. More than 99% of endogenously formed MG is converted, through glyoxalase system, into harmless products as lactate, for example. This enzymatic defense catalyzed by reduced glutathione prevents MG glycation. Glyoxalase activity is affected by polymorphisms, for example those related to one of its control agents, Nrf-2, one transcription factor that is also controlling phase 2 defense enzymes and apoptosis, what provides a potential way to up-regulate glyoxalase and reduce dicarbonyl formation by exogenous factors, including several plant metabolites found in diet (Poulsen, Hedegaard et al. 2013).

Garlic belongs to the plant genus Allium (*Allium sativum* L.) that also includes onion, leek, chives and shallots and is a widely consumed herb in foodstuff and medicines. There are different kinds of sulfur compounds in garlic with a wide range of biological effects, and they are responsible not only for properties like spicy aroma, pungency and lachrymatory effects but also for some ailments treatments like hypertension, high blood cholesterol, ischemia-reperfusion-induced arrhythmias or infarction (Prasad, Laxdal et al. 1996) (Wu, Sheen et al. 2002) (Bautista, Movahed et al. 2005).

Garlic and its sulfur compounds have been associated with antioxidant activity which may explain the referred therapeutic effects. Some studies showed that these compounds scavenge ·OH in a concentration-dependent way and garlic effect is not affected by heating (Prasad, Laxdal et al. 1996). Although, there is no consensus, as some other studies argument that Diallyl Dissulfide (DADS) might contribute for higher oxidative stress levels as long as it reacts with reduced glutathione (Hu, Urig et al. 2007).

There are a wide range of garlic compounds with validated pharmaceutical use, being some of them presented in figure 2, like diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS). These three compounds molecules differ only on the amount of sulfur atoms so their activity and efficiency is somehow related (Freeman and Kodera 1995) (Wu, Sheen et al. 2002).

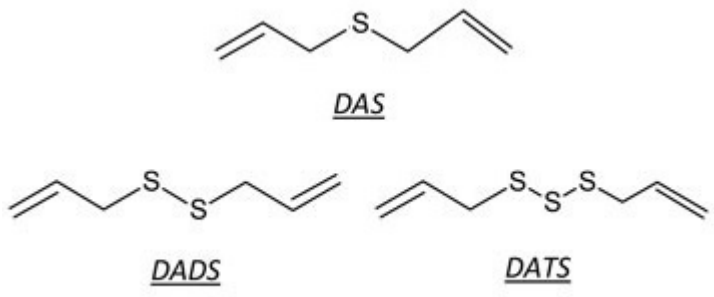



Figure 2 – Structures of selected inhibitors DAS, DADS and DATS emphasizing the number of sulfur atoms.

There are a lot of different potential inhibitors for the studied reaction. DAS, DADS and DATS had already shown some interesting perspectives in some previous research developed in our group, so these became an obvious choice not only because of the potential inhibition analysis, but also because of the potential understanding of the way the number of sulfur atoms can influence the studied reaction.

In this study the formation of reaction products MOLD, GOLD, CML and CEL of the reactive precursors GO, MG are investigated. The aim of the study is to investigate the possible way of inhibition of the formation of MOLD, GOLD, CML and CEL using three structurally similar potential inhibitors: DAS, DADS and DATS. These structurally similar potential inhibitors differ in the number of sulfur in each molecule. The specific aim is thus to understand the role of the amount of sulfur in inhibition reaction.

# Objectives

The aims of this study were:

- Understand how AGEs production is affected by three structurally related potential inhibitors: Diallyl sulfide (DAS), Diallyl disulfide (DADS) and Diallyl trissulfide (DATS);

- Optimize an experiment setting to study the reaction between lysine and glyoxal or methylglyoxal;

- Optimize a spectrophotometric method to follow the referred reaction.

# Materials and Methods

## 2. Materials

L-Lysine Monohydrate was purchased from KEBO (Germany), Glyoxal 40%, and methylglyoxal 40% (w/V), L-Lysine Monohydrochloride as well as dietary component allyl sulfide were purchased from Sigma-Aldrich (Sweden), the other dietary components allyl sulfide and diallyl trisulfide were bought from MP Biomedicals (France) and Nε-(1-Carboxymethyl)-L-Lysine d4 (CML-d4) and Nε-(1-Carboxyethyl)-L-Lysine d4 (CEL-d4) from Larodan Fine Chemicals (Malmö, Sweden). The PBS was prepared in our laboratory.

The used instrument for HPLC-MS was an Accela (Thermo Scientific) UHPLC pump with auto injector. Detection was performed by a LTQ VelosPro Orbitrap mass spectrometer run in positive electrospray ionization ion trap. The Xcalibur software (Thermo Scientific was used both for data acquisition and evaluation. The chromatographic conditions for HPLC separation were performed using Genesis column (Lightn AQ 4.6mm x 250mm, 4 micron particles; Grace Vydac, Hesperia, CA, USA).

## 2.1 Methods

A reaction already used for lysine-lysine cross-link derived from methylglyoxal study was used as basis for this study (R. H. Nagaraj et al. 1996). After further optimization the reaction consisted of adding in a tube 10mL of PBS, 70µL of glyoxal or methylglyoxal and 0.0180g of Lysine monohydrate. Then the samples were incubated at 37°C with constant rotation.

Spectrophotometric measurements were performed with a SPECTROstarNano spectrophotometer from BMG LABTECH at room temperature with the precision option activated, shaking for 5seconds at 500 rpm before plate reading and the scanning between 250nm to 650nm. The results were analyzed with the referred spectrometer software and with Microsoft Office 2010 Excel.

Different time points for collecting the samples for spectrophotometric analysis were used and after reaction optimization the time points used were:

Table 1 – Time points for collecting samples to analyze reaction with glyoxal, with methylglyoxal or both reactions in different vials.

| | untill 1st hour | after 1st hour |
|---|---|---|
| GO | 30min | 1h |
| MG | 15min | 30min |
| GO & MG | 30min | 30min |

For each time point, 150µL triplicates were collected from each reaction vial.

For the LC-MS analysis were used smaller vials than for spectrophotometric analysis, so the total volume was 10 times lower, all the reagents were proportionally adjusted. For the MS analysis were also added 100µL of CML-d4 and CEL-d4 to 1mL volume of solution.

The mobile phases were: eluent A, aqueous 0.1% formic acid and eluent B, acetonitrile (ACN). Eluent A (7%) was maintained for 2min followed by a linear gradient from 7% to 93% of eluent A over 5 min and finally remaining at 93% for 3min. The flow rate was 0.7mL/min and the injection volume was 20µL. (Tareke, Forslund et al. 2013)

In the first LC-MS data collect the samples were prepared in one day and kept overnight in the freezer at -20ºC. In the second LC-MS data collect the samples were introduced in the LC sampler in the different moments and kept at 7ºC so the reaction could go on in the LC sampler minimizing the reaction not stopping effect while freezing and unfreezing the sample despite losing the shaking/rotation possibility.

# Results and Discussion

## 3. Reaction

A few conditions were tested in order to understand the influence that some changes would have in the reaction progress. The following conditions were tested: Time, temperature, mass of lysine, volume of MG/GO, vial rotation and the type of lysine compound (L-lysine monohydrate and L-lysine monochloride).

- **Time –** The reaction progress is faster in the first hours until it reaches equilibrium. The MG reaction appears to occur faster than the GO reaction. This progress is influenced by temperature and amount of reagent;
- **Temperature –** The reaction is slower for lower temperatures. For temperatures over 37ºC the reaction is slightly faster. Freezing at -4ºC is not a procedure fast enough to immediately stop the reaction;
- **Mass of lysine –** Considering values between 0.0085g and 0.0350g the mass of lysine does not influence the final result of the reaction, but for lower masses the reaction progresses slower.
- **Volume of MG/GO -** Considering values between 35µL and 140µL the volume of MG or GO allow the reaction to happen but for volumes lower than 70 µL the reaction is slower.
- **Vial rotation –** The vial constant rotation, irregular rotation, irregular shaking or no movement had some evident differences both for the photometric analysis and HPLC-MS analysis but even regarding the solution observable color. Indicating that probably there is an important interaction between the studied compounds and the air inside the vial;
- **Type of lysine –** Two types of L-Lysine were tested and the differences between both were very slight being the L-lysine monohydrate reaction slightly faster.

The first prove that some reaction was occurring was optical and observable without any instrumental analysis. After mixing all the reagents neither the GO or the MG have color. At the end of the reaction not only both solutions are colored as they are distinguishable. The GO reaction goes from no color, to light yellow and after a longer period changes to dark

yellow or even an orange tone. The MG solution color change starts faster, so for equal time periods it always looks darker, quickly changing to dark yellow and after that to orange and sometimes with longer periods even to brown (Figure 3).

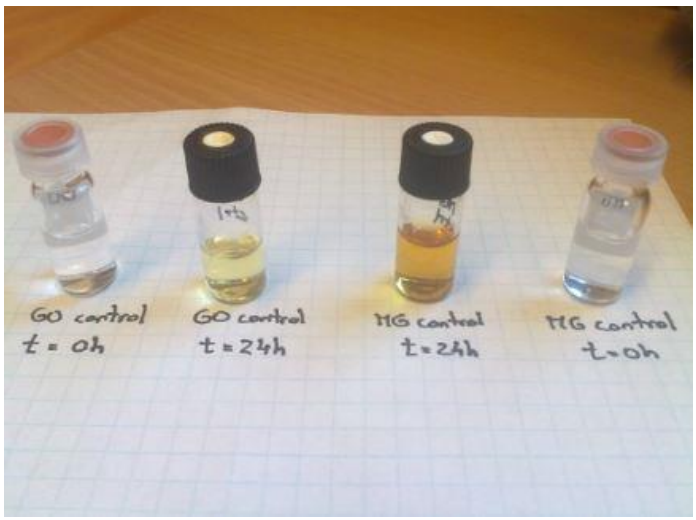


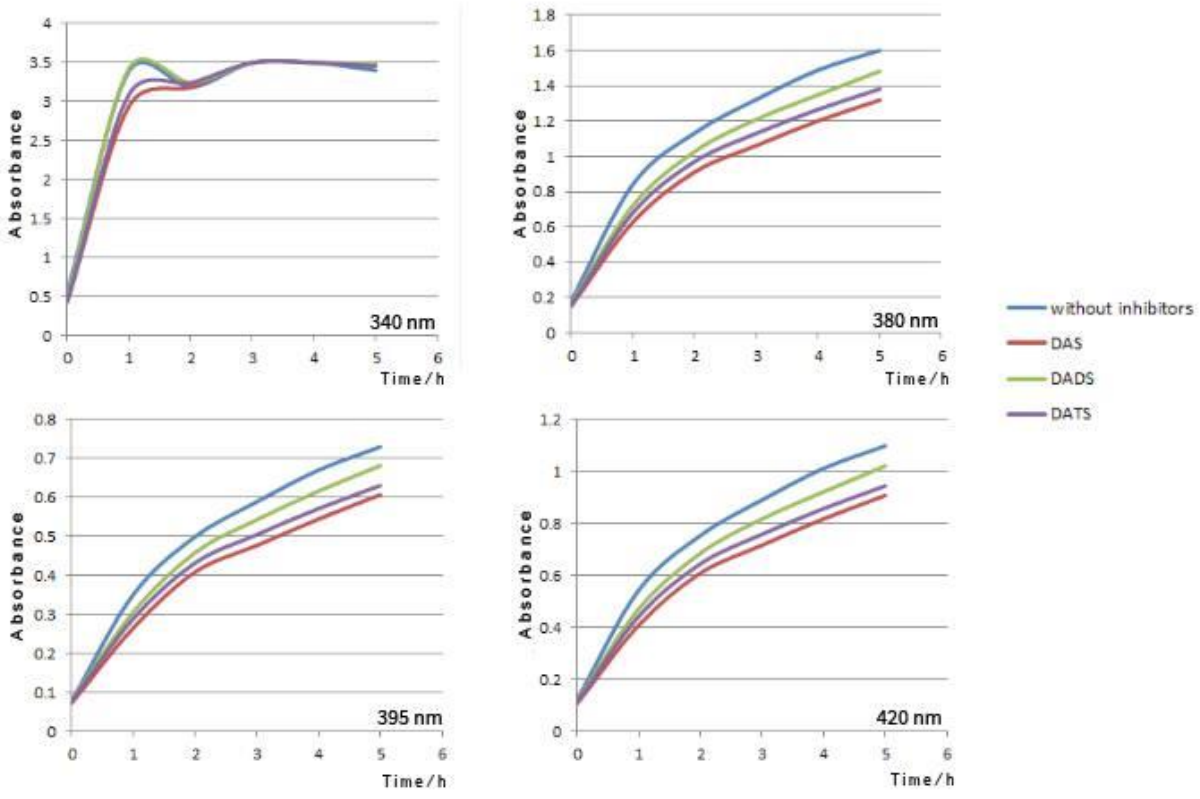

Figure 3 – Color of solutions for the studied reactions: left – GO t = 0h; center left – GO t = 24h; center right – MG t = 24h; right MG t = 0h

For solutions containing the different inhibitors the color change is the same that in the control solution without inhibitors, so it is not possible to confirm any inhibition effect from this first optical analysis (Figure 4).

Figure 4 – Color of solutions for the studied reactions: left – GO control, GO with DAS, GO with DADS and GO with DATS; right - MG control, MG with DAS, MG with DADS and MG with DATS

### 3.1 Photometric method

Four wavelengths were chosen for the photometric analysis. Based on previous publications about AGEs were chosen 380nm, 395nm and 420nm. Based on a particular behavior of the absorbance for the 336-344nm wavelength gap, noticed during the optimization process, the 340nm was chosen too (Graph 1).

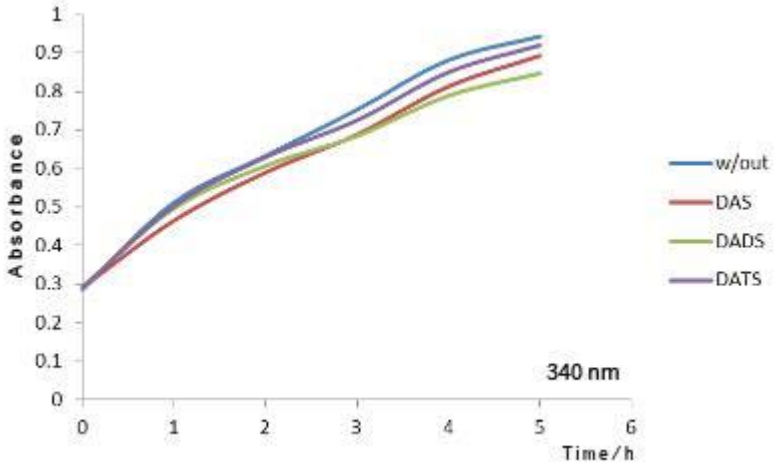



Graph 1 – Absorbance as a function of time for the reaction with MG and the wavelengths 340nm; MG 380nm; MG 395nm; MG 420

For 380nm, 395nm and 420nm, as seen on Graph 1, absorbance increases during the first 5 hours of the reaction but faster during the initial hours, but for 340nm, is easily seen not only that the absorbance values reach an equilibrium but also that at some point decrease a bit. It is also seen that the sample without inhibitor has higher absorbance values, except for 340nm where DADS values are similar, but the order for the absorbance values is not the one expected considering the number of sulfur atoms in each inhibitor, having DADS the higher absorbance values of all inhibitors and DATS and DAS lower values.

To better understand the influence of the different inhibitors the comparison between them was made not for each assay but considering average values between various assays (Graphs 2 and 3).

As the GO reaction is slower it was chosen to analyze the inhibitors effect until the equilibrium.



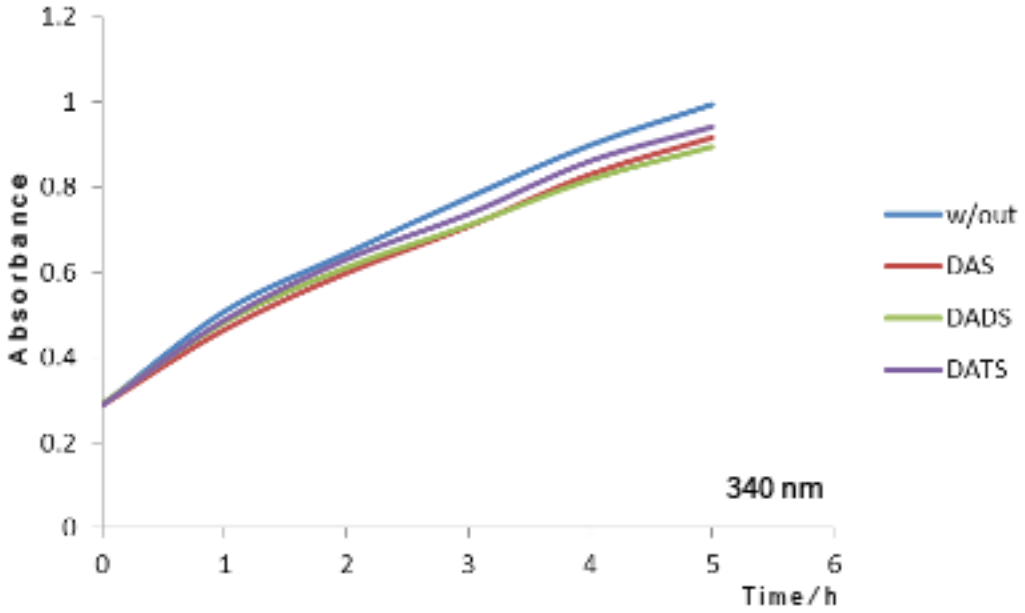

Graph 2 – Absorbance as a function of time, average absorbance values for 2 different GO samples from the same day.

Regarding time and space limitations it was only possible to try two assays per day. Figure 6 allows us to understand the average results for the inhibition for those two parallel assays considering the 340nm wavelength.

The highest curve is the one for the sample without inhibitor, so it is possible to understand that there is a slight inhibition for the compounds detected at this wavelength but once more the inhibitor effect does not seem related to the number of sulfur atoms.

To increase the relevance of these results were made three different assays in three consecutive days. Figure 7 shows the final result considering the average absorbance values for 340nm.

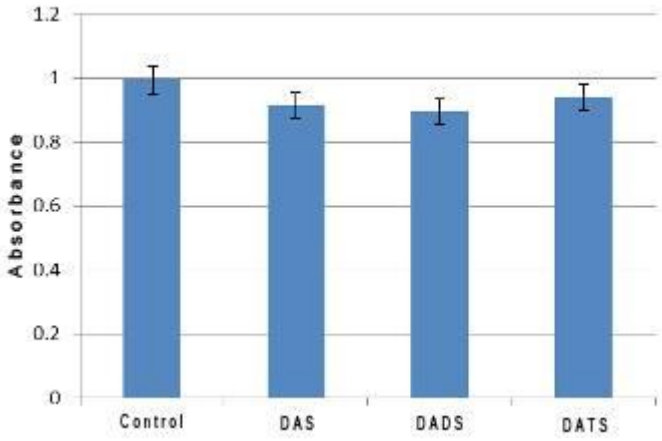
The relative position for all the four curves is the same considering the tendency of the previous results. The sample without inhibitor reaches higher absorbance values and the inhibitors order is not related to the number of sulfur atoms.




Graph 3 – Absorbance as a function of time, average absorbance values for 3 different GO samples from different days.

To conclude about the significance of these results were considered the values for the 5$^{th}$ reaction hour and analyzed with Microsoft Office Excel 2010 software. The results are presented in Graph 4 and it is possible to conclude that some of these differences have statistical significance.

These previous results show that somehow the inhibitor influences the reaction progress, despite that it is not possible to fully understand how that is happening and if this influence is really an inhibition or is just a change in the reaction equilibrium.




Graph 4 - 340nm absorbance after 5 hours

## 3.2 HPLC-MS method

### 3.2.1 – Assay number 1 – Methylglyoxal

As the HPLC-MS method is even more limited than the photometric method concerning the number of samples and the time spent analyzing each one, the analysis was made only between a sample without inhibitor and a sample with DADS.

At this point, the knowledge about the products of this reaction was limited, so the method was chosen aiming for an efficient qualitative analysis. The first limitation was that the samples could not be analyzed immediately after coming out from the hoven like it happened for the photometric method so the reaction took place in one day, then at each time-point the samples were frozen and then analyzed on HPLC-MS on next day. Eight samples were chosen as shown on table 2, but only seven are considered as valid results in this dissertation as one of the intermediate time points without inhibitor had an obvious misrepresented chromatogram.

Table 2 –HPLC-MS assay 1 scheme

| | inhibitor | time/h | validation |
|---|---|---|---|
| 1c | without | 0 | v |
| 1d | DADS | 0 | v |
| 5c | without | 1 | X |
| 5d | DADS | 1 | v |
| 9c | without | 3 | v |
| 9d | DADS | 3 | v |
| 13c | without | 5 | v |
| 13d | DADS | 5 | v |

From the analysis of the seven valid chromatograms and mass spectrum the first evident result is that the freezing at -4ºC was not completely efficient as the sample that was frozen before the 37ºC exposure had relevant peaks for some of the reactions products.

From all the analyzed samples we can choose 9 main common peaks. The expected peak for CEL with value 218 or CEL-d4 with value 222 was not found. But a very relevant peak for value 341.22 was evident, revealing that MOLD was one of the main products of this reaction.

Another relevant value that appears in more than one peak revealing the presence of lysine residues (molecular weight 146), is 147.11. It is also important to note that DADS molecular weight is also 146, so it was important to check if it appeared only in the sample with the inhibitor or if it appears also in the sample without inhibitor, in this method, all the 147.11 values appear both for the sample without inhibitor and the one with DADS).

For this first HPLC-MS assay, nine relevant peaks can be chosen for a detailed analysis (Table 3).

Table 3 – Relevant peaks from first HPLC analysis, time gap when they can be detected and number of conditions that each peak was registered

| Peak | Time Gap | No. of results |
|---|---|---|
| 1 | 4.08 – 4.22 | 3x |
| 2 | 4.32 – 4.41 | 7x |
| 3 | 4.55 – 4.67 | 7x |
| 4 | 6.90 – 6.94 | 4x |
| 5 | 7.07 – 7.10 | 6x |
| 6 | 7.32 – 7.35 | 2x |
| 7 | 7.78 – 7.87 | 7x |
| 8 | 7.99 – 8.08 | 6x |
| 9 | 8.35 – 8.41 | 5x |

Three of these nine peaks appear to be more relevant to understand which kind of products is being formed with this reaction.

## **Peak 7** – 7.78 – 7.87

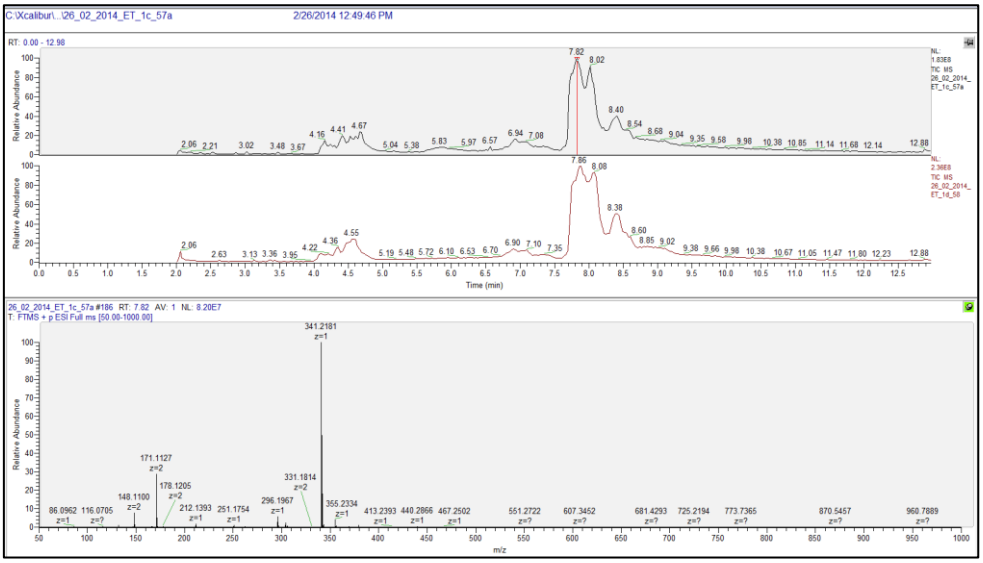


Figure 5 – HPLC-MS analysis, assay number 1, peak number 7. HPLC chromatograms for the sample without inhibitor (top), sample with DADS (middle) and MS peaks (bottom)

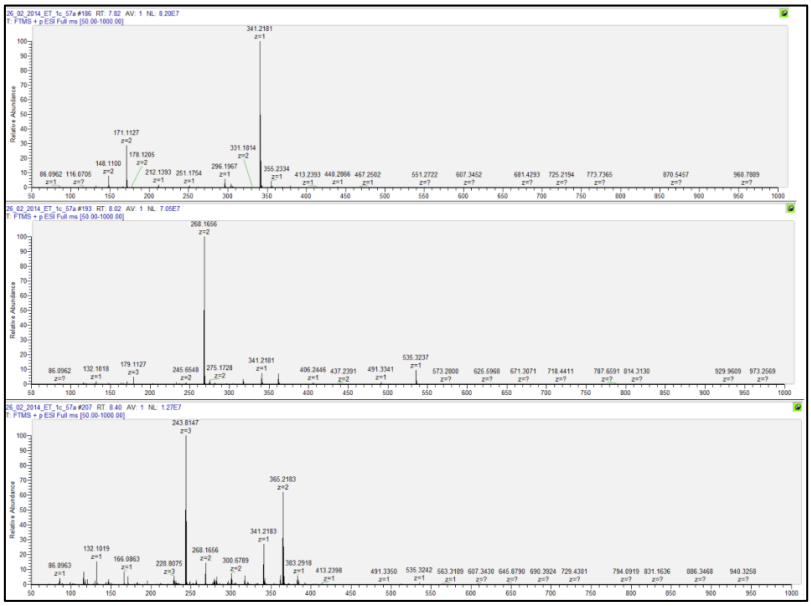


Figure 6 – HPLC-MS analysis, assay number 1, peaks number 7 (top), number 8 (middle) and number 9 (bottom). HPLC chromatograms for the sample without inhibitor (up), sample with DADS (middle) and MS peaks (down)

Table 4 – HPLC-MS analysis, assay number 1, peak number 7. Relative abundance gap for each pair mass-charge in all 7 assays considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1c | 1d | 5d | 9c | 9d | 13c | 13d |
| 148.11 | 2 | 0-10 | 0-10 | 0-10 | 0-5 | 0-10 | 0-10 | 0-10 |
| 171.11 | 2 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 |
| 212.14 | 1 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 251.17 | 1 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 296.20 | 1 | 0-10 | 0-5 | 0-10 | 0-10 | 0-5 | 0-10 | 0-10 |
| 341.22 | 1 | **100** | **100** | **100** | **100** | **100** | **100** | **100** |
| 355.23 | 1 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 369.21 | 1 | 0-5 | - | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |

## **Peak 8** – 7.99 – 8.08

Table 5 – HPLC-MS analysis, assay number 1, peak number 8. Relative abundance gap for each pair mass-charge in all 7 assays considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1c | 1d | 5d | 9c | 9d | 13c | 13d |
| 132.10 | 1 | 0-5 | 0-5 | - | X | 0-5 | 0-5 | 0-5 |
| 179.11 | 3 | 0-5 | 0-10 | 0-5 | X | 0-5 | 10-20 | 10-20 |
| 233.18 | 1 | 0-5 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 |
| 245.65 | 2 | 0-5 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 |
| 268.16 | 2 | **100** | **100** | **100** | X | **100** | **100** | **100** |
| 275.17 | 2 | 0-5 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 |
| 282.16 | 2 | 0-5 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 |
| 310-320 | - | 0-5 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 |
| 331.22 | 2 | - | - | 0-5 | X | 0-5 | 0-10 | 0-5 |
| 341.22 | 1 | 0-10 | 0-10 | 0-10 | X | 10-20 | 10-20 | 10-20 |
| 362.25 | 1 | 0-10 | 0-10 | 0-10 | X | 0-10 | 0-10 | 0-10 |
| 369.21 | 1 | - | - | - | X | 0-5 | 0-5 | 0-5 |
| 535.32 | 1 | **10-20** | **10-20** | **0-10** | X | **0-10** | **0-10** | **0-10** |

Peak no. 7 presents a very high relative abundance of 341.22 compound. This is the mass for MOLD, so this confirms the expected result of lysine-lysine dimer as one product obtained from this reaction.

Peak no. 8 presents a relevant relative abundance for the value of the product between mass and charge that is 535.32, not only with charge +1, but also with charge +2 and +3. Although this mass would not be an expected result.

Peak no. 9 presents also an unexpected mass that is 729.43. Not seen for this exact value, but obtained from the values with charge +2 and +3.

## **Peak 9** – 8.35 – 8.41

Table 6 – HPLC-MS analysis, assay number 1, peak number 9. Relative abundance gap for each pair mass-charge in all 7 assays considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1c | 1d | 5d | 9c | 9d | 13c | 13d |
| 86.10 | 1 | 0-5 | 0-5 | 0-5 | 0-10 | 0-5 | X | X |
| 116.07 | 1 | 0-10 | 0-5 | 0-5 | 10-20 | 0-10 | X | X |
| 132.10 | 1 | 10-20 | 0-10 | 0-5 | 20-30 | 20-30 | X | X |
| 148.11 | 1 | 0-5 | 0-5 | 0-5 | 0-10 | 0-10 | X | X |
| 166.09 | 1 | 0-10 | 0-5 | 0-5 | 0-10 | 0-10 | X | X |
| 171.11 | 2 | 0-10 | 0-5 | 0-5 | 10-20 | 10-20 | X | X |
| 195.11 | 2 | 0-5 | 0-10 | 0-10 | 0-5 | 30-40 | X | X |
| 228.81 | 3 | 0-10 | 0-10 | 0-5 | 0-5 | 0-5 | X | X |
| 243.81 | 3 | **100** | **100** | **100** | **100** | **100** | X | X |
| 268.16 | 2 | 10-20 | 0-10 | 10-20 | 20-30 | 20-30 | X | X |
| 285.82 | 3 | 0-5 | 0-5 | 0-10 | 10-20 | 10-20 | X | X |
| 300.68 | 2 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | X | X |
| 341.22 | 1 | 20-30 | 10-20 | 10-20 | 50-60 | 60-70 | X | X |
| 365.22 | 2 | **60-70** | **50-60** | **40-50** | **50-60** | **50-60** | X | X |
| 383.29 | 1 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | X | X |
| 729.43 | 1 | **0** | **0** | **0** | **0** | **0** | X | X |

Despite it is not possible to fully understand what this compound is, it is possible to predict that it is somehow related to MOLD. The compound 535 should be something like X-MOLD, where X is a radical with mass 194. The compound 729 should be something like X-MOLD-X, where X is the same radical with mass 194. (Figure 7)

The data about inhibitors effect collected during this analysis was not conclusive about the quantity of product formed during the reaction, despite it indicates that despite DADS is not forbidding the formation of MOLD, and the two other referred compounds.

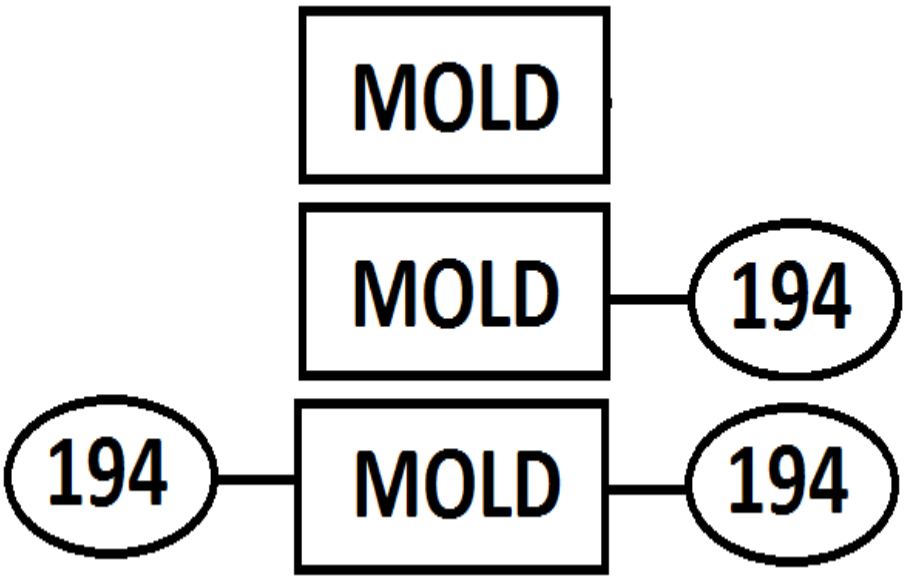



Figure 7 – HPLC-MS analysis, assay number 1, draw form the compounds detected on peak 7 (mass 341.22), peak 8 (mass 535.32) and peak 9 (mass 729.43).

### 3.2.2 – Assay number 2 - Methylglyoxal and glyoxal

In this assay the vials were placed in the HPLC sampler during the reaction at 37ºC so the analysis took place instantaneously, but this created some chronological limitations. The chronological optimization for this activity allowed to analyze four time points in each of the four MG samples (control, DAS, DADS and DATS), and eight time-points for each of the four GO samples (control, DAS, DADS and DATS).

### 3.2.2.1 – Methylglyoxal results

Table 7 explains the organization of this assay for the samples containing MG and which assays were validated.

Table 7 –HPLC-MS assay 2 scheme for MG samples

| | inhibitor | time/h | validation |
|---|---|---|---|
| 1 | without | 0 | X |
| 1 | DAS | 0 | v |
| 1 | DADS | 0 | v |
| 1 | DATS | 0 | v |
| 2 | without | 1 | v |
| 2 | DAS | 1 | v |
| 2 | DADS | 1 | v |
| 2 | DATS | 1 | v |
| 3 | without | 2 | v |
| 3 | DAS | 2 | v |
| 3 | DADS | 2 | v |
| 3 | DATS | 2 | v |
| 5 | without | 4 | v |
| 5 | DAS | 4 | v |
| 5 | DADS | 4 | v |
| 5 | DATS | 4 | v |
| 10 | without | 9 | X |
| 10 | DAS | 9 | X |
| 10 | DADS | 9 | X |
| 10 | DATS | 9 | X |

For the samples with MG, six relevant peaks can be chosen for a detailed analysis (Table 8).

Table 8 – Relevant peaks from second HPLC analysis for MG analysis, time gap when they can be detected and number of conditions that each peak was registered

| Peak | Time Gap | No. of results |
|---|---|---|
| 1 | **4.51 – 4.56** | 3x |
| 2 | **4.78 – 4.79** | 4x |
| 3 | **5.71 – 5.71** | 1x |
| 4 | **7.64 – 7.71** | 4x |
| 5 | **7.70 – 7.79** | 4x |
| 6 | **7.78 – 7.91** | 4x |

From these six peaks mass values analysis three compounds seem to gather some relevance for better understanding of this reaction.

## **Peak 1** – 4.51 – 4.56

Table 9 – HPLC-MS analysis, MG analysis assay number 2, peak number 1. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1ctrl | 1DAS | 1DADS | 1DATS | 5ctrl | 5DAS | 5DADS | 5DATS |
| **84.08** | **1** | X | 0-10 | 0-10 | 0-10 | 0-5 | 0-5 | 0-5 | 0-5 |
| **118.08** | **1** | X | 0-10 | 10-20 | 20-30 | 0-10 | 0-5 | 0-5 | 0-10 |
| **128.07** | **1** | X | 10-20 | 10-20 | 20-30 | 0-5 | 0-5 | 0-5 | 0-5 |
| **138.09** | **1** | X | 10-20 | 10-20 | 20-30 | 0-5 | 0-5 | 0-10 | 0-5 |
| **158.08** | **1** | X | 20-30 | 10-20 | 40-50 | 0-5 | 0-5 | 0-5 | 0-5 |
| **174.11** | **1** | X | 0-10 | 0-10 | 10-20 | 0-5 | 0-5 | 0-5 | 0-5 |
| **193.13** | **1** | X | 0-5 | 0-10 | 0-10 | 0-5 | 0-5 | 0-5 | 0-5 |
| **210.11** | **1** | X | 10-20 | 20-30 | 20-30 | 0-10 | 0-10 | 0-10 | 0-10 |
| **212.09** | **1** | X | 20-30 | 0-10 | 20-30 | 0-5 | 0-5 | 0-5 | 0-5 |
| **228.12** | **1** | X | 90-100 | **100** | **100** | 20-30 | 10-20 | 40-50 | 20-30 |
| **230.10** | **1** | X | **100** | 30-40 | 70-80 | 0-10 | 10-20 | 0-10 | 10-20 |
| **248.11** | **1** | X | 40-50 | 10-20 | 30-40 | 0-10 | 0-10 | 0-5 | 0-10 |
| **260.11** | **1** | X | 0-10 | 0-10 | 10-20 | 0-10 | 0-10 | 0-10 | 0-10 |
| **273.14** | **1** | X | 20-30 | 30-40 | 50-60 | 0-5 | 0-5 | 0-5 | 0-5 |
| **282.13** | **1** | X | 10-20 | 0-10 | 0-10 | 40-50 | 40-50 | 30-40 | 40-50 |
| **300.14** | **1** | X | 20-30 | 0-10 | 10-20 | 20-30 | 30-40 | 30-40 | 30-40 |
| **302.12** | **1** | X | 60-70 | 20-30 | 30-40 | 20-30 | 20-30 | 10-20 | 20-30 |
| **310-320** | **?** | X | 0-10 | 0-5 | 0-5 | 10-20 | 10-20 | 10-20 | 10-20 |
| **327.15** | **1** | X | 20-30 | 10-20 | 10-20 | 0-5 | 40-50 | 0-10 | 40-50 |
| **329.08** | **1** | X | 20-30 | 10-20 | 0-10 | **100** | **100** | **100** | **100** |
| **345.16** | **1** | X | 40-50 | 0-5 | 30-40 | 0-5 | 10-20 | 0-10 | 0-10 |
| **354.15** | **1** | X | 0-10 | 0-5 | 0-5 | 10-20 | 20-30 | 10-20 | 20-30 |
| **374.14** | **1** | X | 0-10 | 0-5 | 0-5 | 10-20 | 10-20 | 10-20 | 10-20 |
| **383.09** | **1** | X | 10-20 | 0-5 | 0-5 | 70-80 | 40-50 | 60-70 | 80-90 |
| **401.10** | **1** | X | 10-20 | 0-5 | 0-5 | 70-80 | 60-70 | 70-80 | 70-80 |
| **417.19** | **1** | X | 0-10 | 0-5 | 0-5 | 0-10 | 10-20 | 0-10 | 0-10 |
| **426.17** | **1** | X | 0-5 | 0-5 | 0 | 0-10 | 0-10 | 0-10 | 0-10 |
| **455.11** | **1** | X | 0-5 | 0-5 | 0 | 10-20 | 10-20 | 10-20 | 20-30 |
| **473.13** | **1** | X | 0-10 | 0-5 | 0-5 | 10-20 | 10-20 | 10-20 | 10-20 |

Despite the first time-point for the control was not considered due to evident background noise, peak number 1 (Table 9) is interesting for analyzing the development of the reaction, as the first time-point has a different result than the time-point after five hours, also we can notice some differences for DAS, but it is not possible to conclude about them as only one assay took place and this might be just a precision error for example related to the timing that the vials were put in the HPLC sampler. In this peak it's also possible to clearly understand that some of the masses are related to the deuterium presence.

In the first time-point we see masses of 228 (DADS and DATS) and 230 (DAS), despite they are a bit higher that CEL and CEL-d4 masses they appear to be somehow related to them. After 5 hours the masses detected are 327 and 329, being the 327 the mass for GOLD. The explanation for this result is probably related to MG methyl group, as it might lose its methyl group and become GO and this way react directly with lysine forming GOLD, other option is that the presence of CML-d4 might also influence this formation of GOLD in the MG vials.

## **Peak 5 – 7.70 – 7.79**

Table 10 – HPLC-MS analysis, MG analysis assay number 2, peak number 5. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1ctrl | 1DAS | 1DADS | 1DATS | 5ctrl | 5DAS | 5DADS | 5DATS |
| **84.08** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **128** | | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 |
| **130.09** | **1** | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 20-30 | 20-30 | 30-40 |
| **147.11** | **1** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |
| **148** | | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **185.07** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **223.02** | **1** | 0-5 | 0-5 | 0-10 | 0-10 | 0-10 | 0-5 | 0-10 | 0-5 |
| **246.18** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-5 | 0-10 |
| **284.14** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 | 0-5 | 0-5 |
| **322.09** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **359.23** | **1** | 0 | 0-5 | 0 | 0 | 0-5 | 0-5 | 0-5 | 0-5 |
| **407.08** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |

The second reference goes to peak number five (Table 10) that is the highest peak in more than half of these assays. It is a well-known mass already detected in the first HPLC-MS analysis, 147, the mass for Lysine. As one of the reagents this mass is common in all the MS analysis but assumes particular relevance for this peak.

## **Peak 6** – 7.78 – 7.91

Table 11 – HPLC-MS analysis, MG analysis assay number 6, peak number 6. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1ctrl | 1DAS | 1DADS | 1DATS | 5ctrl | 5DAS | 5DADS | 5DATS |
| **84.08** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **116.07** | **1** | 0-10 | 0-5 | 0-10 | 0-10 | 0 | 0 | 0 | 0 |
| **130.08** | **1** | 10-20 | 0-10 | 20-30 | 20-30 | 0-10 | 0-10 | 0-5 | 0-10 |
| **147.11** | **1** | 60-70 | 30-40 | 70-80 | 70-80 | 20-30 | 20-30 | 10-20 | 20-30 |
| **171.11** | **2** | 0-10 | 20-30 | 10-20 | 0-10 | 10-20 | 0-10 | 10-20 | 10-20 |
| **207.12** | **2** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **234.13** | **2** | 0-5 | 0-5 | 0-5 | 0-5 | 10-20 | 0-5 | 0-10 | 20-30 |
| **249.16** | **1** | 0-10 | 0-10 | 10-20 | 10-20 | 0-5 | 0-5 | 0-5 | 0-5 |
| **262.19** | **1** | 30-40 | 20-30 | 50-60 | 60-70 | 0-10 | 0-10 | 0-5 | 10-20 |
| **279.19** | **1** | 20-30 | 0-10 | 20-30 | 20-30 | 0-5 | 0-5 | 0-5 | 0-5 |
| **296.20** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 | 0-10 | 0-10 | 0-5 |
| **306.15** | **1** | **100** | 0-5 | **100** | **100** | 0-5 | 0-5 | 0-5 | 0-5 |
| **341.22** | **1** | 20-30 | **100** | 40-50 | 20-30 | **100** | **100** | **100** | **100** |
| **355.23** | **1** | 0-5 | 0-10 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **377.25** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **379.17** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 | 0-5 | 0-5 | 0-5 |
| **413.24** | **1** | 0-5 | 10-20 | 0-5 | 0-5 | 10-20 | 20-30 | 0-10 | 10-20 |
| **467.25** | **1** | 0 | 0-5 | 0 | 0 | 0-10 | 0-10 | 0-5 | 10-20 |

Peak number 6 (Table 11) is easily identified as the MOLD peak. It starts with a low signal for this compound (except for the sample with DAS) and it has a very high signal for the 341 mass in all the posterior time-points. There is also an interesting signal for 413 that should be something MOLD + MG, indicating that MOLD can interact with one MG molecule.

### 3.2.2.2 –Glyoxal results

Table 12 explains the organization of this assay for the samples containing GO and which assays were validated.

Table 12 –HPLC-MS assay 2 scheme for GO samples

| | inhibitor | time/h | validation |
|---|---|---|---|
| 4 | without | 0 | V |
| 4 | DAS | 0 | V |
| 4 | DADS | 0 | V |
| 4 | DATS | 0 | V |
| 6 | without | 2 | V |
| 6 | DAS | 2 | V |
| 6 | DADS | 2 | V |
| 6 | DATS | 2 | V |
| 7 | without | 3 | V |
| 7 | DAS | 3 | V |
| 7 | DADS | 3 | V |
| 7 | DATS | 3 | V |
| 8 | without | 4 | V |
| 8 | DAS | 4 | V |
| 8 | DADS | 4 | V |
| 8 | DATS | 4 | V |
| 9 | without | 5 | V |
| 9 | DAS | 5 | V |
| 9 | DADS | 5 | V |
| 9 | DATS | 5 | V |
| 11 | without | 7 | V |
| 11 | DAS | 7 | V |
| 11 | DADS | 7 | V |
| 11 | DATS | 7 | V |
| 12 | without | 8 | V |
| 12 | DAS | 8 | V |
| 12 | DADS | 8 | V |
| 12 | DATS | 8 | V |
| 13 | without | 9 | V |
| 13 | DAS | 9 | V |
| 13 | DADS | 9 | V |
| 13 | DATS | 9 | V |

For the samples with GO, six relevant peaks can be chosen for a detailed analysis (Table 13).

Table 13 – Relevant peaks from second HPLC analysis for GO samples, time gap when they can be detected and number of conditions that each peak was registered

| Peak | Time Gap | No. of results |
|---|---|---|
| 1 | **4.46 – 4.57** | 4x |
| 2 | **6.91 – 6.92** | 4x |
| 3 | **7.13 – 7.15** | 4x |
| 4 | **7.49 – 7.54** | 4x |
| 5 | **7.71 – 7.74** | 4x |
| 6 | **7.86 – 7.97** | 3x |

From these six peaks mass values analysis three compounds seem to gather some relevance for better understanding of this reaction.

## **Peak 1** – 4.46 – 4.57

Table 14 – HPLC-MS analysis, GO analysis assay number 2, peak number 1. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 4ctrl | 4DAS | 4DADS | 4DATS | 13ctrl | 13DAS | 13DADS | 13DATS |
| **84.08** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **118.09** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 10-20 | 0-10 | 0-10 | 0-10 |
| **126.09** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 10-20 | 0-10 | 0-10 |
| **120-130** | **?** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **160-170** | **?** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **183.11** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **187.11** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **200-210** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **227.10** | **1** | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 |
| **245.11** | **1** | 40-50 | 40-50 | 40-50 | 40-50 | 40-50 | 40-50 | 40-50 | 40-50 |
| **263.12** | **1** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |
| **277.14** | **1** | 0-10 | 0-10 | 0-10 | 0-10 | 0-5 | 0-10 | 0-10 | 0-5 |
| **321.13** | **1** | 0-5 | 0-10 | 0-5 | 0-10 | 0-5 | 0-10 | 0-5 | 0-10 |

Peak number 1 (Table 14) seems to have a relevant intensity, but the analysis does not seem similar for the equivalent peak for MG analysis, being the mass for this peak higher than the one detected for MG and not lower as it would be expected considering GO as a smaller molecule than MG. This shows that the GO and MG might not follow exactly the same mechanisms, despite this result is not conclusive.

## **Peak 5 – 7.71 – 7.74**

Table 15 – HPLC-MS analysis, GO analysis assay number 2, peak number 5. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 4ctrl | 4DAS | 4DADS | 4DATS | 13ctrl | 13DAS | 13DADS | 13DATS |
| **84.08** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **130.09** | **1** | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 | 30-40 |
| **147.11** | **1** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |
| **185.07** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 | 0-10 | 0-5 |
| **200-210** | | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| **223.02** | **1** | 0-5 | 10-20 | 0-10 | 0-10 | 0-5 | 10-20 | 0-10 | 0-5 |
| **246.18** | **1** | 0-5 | 0-10 | 0-5 | 0-10 | 0-10 | 0-10 | 0-10 | 0-10 |
| **284.14** | **1** | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 | 0-10 | 0-10 | 0-10 |
| **355.20** | **1** | 0 | 0 | 0-5 | 0 | 0-5 | 0-5 | 0-5 | 0-5 |

Although for peak 5 (Table 15) for example, the result is very similar to the one from the MG analysis, having an equivalent relative position in the chromatogram, a similar retention time and detecting a relevant mass of 147 that should be lysine.

For peak 6 (Table 16) despite the different mass, we have the expected peak when comparing to MG analysis. This 327 result represents the GOLD formation, instead of MOLD formation, despite with a smaller relative abundance than the one registered for MG analysis. This result is concordant with the prediction made while optimizing the reaction that with MG the reaction should develop faster than with GO.

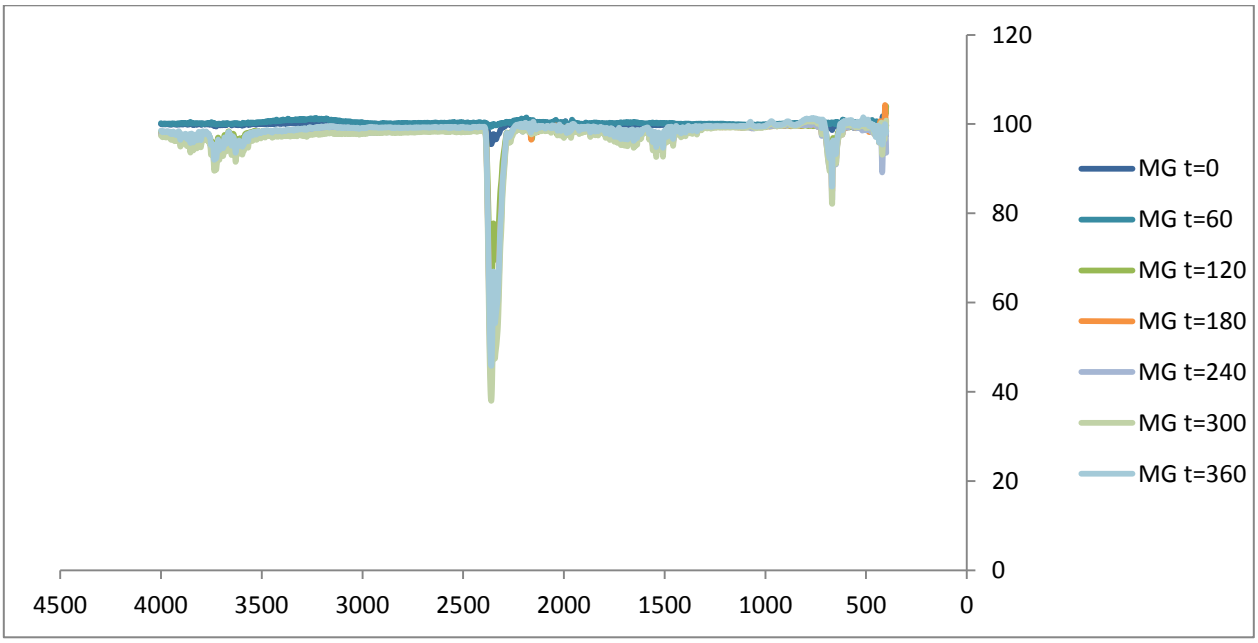
## Peak 6 – 7.86 – 7.97

Table 16 – HPLC-MS analysis, GO analysis assay number 2, peak number 6. Relative abundance gap for each pair mass-charge in 2 time-points for all 4 assays, considering the masses with the strongest signal.

| Mass | Charge | Relative abundance (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 4ctrl | 4DAS | 4DADS | 4DATS | 13ctrl | 13DAS | 13DADS | 13DATS |
| 84.08 | 1 | 0-5 | 0-10 | X | 0-10 | 0-10 | 10-20 | 10-20 | 0-10 |
| 100-110 | ? | 0 | 0 | X | 0 | 0-10 | 0-5 | 0-10 | 0-5 |
| 118.09 | 1 | 0-10 | 0-5 | X | 0-5 | 0-5 | 0-10 | 0-5 | 0-5 |
| 128 | ? | 0-10 | 0-10 | X | 10-20 | 0-10 | 0-10 | 10-20 | 0-5 |
| 130.09 | 1 | 10-20 | 20-30 | X | 10-20 | 20-30 | 20-30 | 20-30 | 20-30 |
| 147.11 | ? | 60-70 | 70-80 | X | 60-70 | 50-60 | 60-70 | 60-70 | 70-80 |
| 148 | ? | 0-10 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 158.10 | ? | 0-5 | 0-5 | X | 0-5 | 10-20 | 10-20 | 10-20 | 10-20 |
| 164.10 | 2 | 0-10 | 0-10 | X | 20-30 | 10-20 | 10-20 | 10-20 | 10-20 |
| 160-170 | ? | 0-5 | 0-5 | X | 0-5 | 0-10 | 0-10 | 0-10 | 0-5 |
| 170-180 | ? | 0-5 | 0-5 | X | 0-5 | 0-10 | 0-10 | 0-10 | 0-5 |
| 180-190 | ? | 0-5 | 0-5 | X | 0-5 | 0-10 | 10-20 | 0-10 | 10-20 |
| 198.12 | ? | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 10-20 |
| 200-210 | ? | 0-5 | 0-5 | X | 0-5 | 0-10 | 10-20 | 10-20 | 10-20 |
| 210 | ? | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 |
| 221.13 | 1 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-10 | 0-5 | 0-5 |
| 228.13 | ? | 0-5 | 0-5 | X | 0-5 | 0-10 | 0-10 | 0-10 | 0-10 |
| 234.20 | ? | 0-5 | 0-5 | X | 0-10 | 0-5 | 0-5 | 0-5 | 0-5 |
| 249.16 | 1 | 0-10 | 10-20 | X | 10-20 | 10-20 | 20-30 | 10-20 | 10-20 |
| 262.19 | 1 | 10-20 | 40-50 | X | 40-50 | 40-50 | 40-50 | 40-50 | 40-50 |
| 260-270 | ? | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 0-10 |
| 278.19 | 1 | 0-10 | 10-20 | X | 10-20 | 10-20 | 10-20 | 10-20 | 10-20 |
| 290-300 | ? | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-10 | 0-10 | 0-10 |
| 290-300 | ? | 0-10 | 0-5 | X | 0-5 | 0-5 | 0-10 | 0-10 | 0-10 |
| 306.18 | 1 | **100** | **100** | X | 50-60 | **100** | **100** | **100** | 0-10 |
| 315.20 | 1 | 0 | 0-5 | X | 0-5 | 0-10 | 0-10 | 0-10 | 0-10 |
| 327.20 | 1 | 20-30 | 30-40 | X | **100** | 40-50 | 60-70 | 30-40 | **100** |
| 328 | ? | 0-5 | 0-10 | X | 10-20 | 0-10 | 0-10 | 0-10 | 10-20 |
| 344.14 | 1 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 355.20 | 1 | 0-5 | 0-5 | X | 0-5 | 30-40 | 30-40 | 20-30 | 40-50 |
| 365.16 | 1 | 0-5 | 0-5 | X | 0-10 | 0-5 | 0-5 | 0-5 | 0-5 |
| 373.21 | 1 | 0-5 | 0-5 | X | 0-5 | 10-20 | 10-20 | 10-20 | 30-40 |
| 377.25 | 1 | 0-5 | 0-5 | X | 0-5 | 0-5 | 0-5 | 0-5 | 0-5 |
| 385.21 | 1 | 0-5 | 0-5 | X | 0 | 0-5 | 0-5 | 0-5 | 0-5 |
| 413.20 | 1 | 0 | 0-5 | X | 0 | 0-5 | 0-5 | 0-5 | 0-10 |

### 3.3 – FTIR analysis

For better understanding of these reaction mechanisms a FTIR detector was used trying to get extra information about which functional groups are there in solution during this reaction and how their detection changes over time.




Graph 5 – FTIR analysis between 400nm and 4000nm from 0min to 360min

It was possible to understand that there were some peaks growing across time. The highest peak was detected at a wavelength between 2250nm and 2500nm and some smaller ones between 500nm and 1000nm. Consulting some online information it was possible to conclude that this result was happening because $CO_2$ was being solved while the vial was shaken. (http://www.colby.edu/chemistry/PChem/notes/NormalModesText.pdf, Accessed 26 September 2014 and http://www.d.umn.edu/~psiders/courses/chem4644/labinstructions/CO2CS2gamess.pdf Accessed 26 September 2014).

## Conclusions

The studied reaction leads to a solution color change from non-colored to yellow, orange or brown, confirming the reaction occurrence. HPLC-MS results clearly confirm that the reaction occurred and some of the formed products. The spectrophotometric and FTIR analysis also help to understand how the reaction evolves despite the results are not conclusive about the products formed.

AGEs formation was confirmed, including cross-links like GOLD and MOLD.

MOLD formation was associated with the MG reaction and GOLD with the GO reaction, although we can detect the GOLD mass value in the MG assay, suggesting that it can be formed there too.

Spectrophotometric and FTIR assays show some signs that exposure to air (in particular oxygen and carbon dioxide) interfere in a very important way with the reaction progress.

The spectrophotometric analysis suggests that the presence of inhibitors like DAS, DADS or DATS influences the reaction, although the other used techniques do not allow to fully understand how these compounds interfere with the reaction progress and what effect exactly they have in the reaction.

The HPLC-MS analysis does not confirm the inhibitors influence, but this might be a limitation of focusing on a qualitative scanning, and maybe an analysis focusing on quantitative scanning would help to understand better if the inhibitors really have an effect in one or more products formation and how these inhibitors affect each product formation.

Comparing the MG and GO reaction seems that some reaction mechanisms might be different and that some products do not differ only on the methyl group.

This study that initially was aiming for the inhibition of the AGEs formation reaction with inhibitors obtained from garlic lead to a big amount of new information about the reaction itself. Understanding that this reaction has a lot of nuances made it prior a better comprehension of the formation mechanisms before understanding the inhibition widely. These results should not be seen as a final step of this research, but as a “door opening” for

future researches, not only about the AGEs formation inhibition, but also about the AGEs formation products, mechanisms and kinetics adding a new reference for AGEs research.

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