AGRICULTURAL UNIVERSITY OF ATHENS
SCHOOL OF FOOD AND NUTRITIONAL SCIENCES
DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
LABORATORY OF FOOD CHEMISTRY AND ANALYSIS

POSTGRADUATE STUDY

of postgraduate student STAVROPOULOU E. MARIA

Study of the formation of non-enzymatic Advanced glycation end-products (d AGEs) in food systems

Supervisor: CHRYSAVGI GARDELI

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ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

«Μελέτη σχηματισμού προϊόντων προχωρημένης μη-ενζυματικής γλυκοζυλίωσης

(d AGEs) σε συστήματα τροφίμων»

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ΠΕΡΙΛΗΨΗ

Η αντίδραση Maillard η αντίδραση μη-ενζυματικής αμαύρωσης, είναι μια χημική αντίδραση η οποία έχει μελετηθεί ευρέως και είναι συχνά εμφανής στα τρόφιμα εξαιτίας της θερμικής επεξεργασίας που υφίστανται και παίζει σημαντικό ρόλο στην τελική διατροφική οργανοληπτική αξία των προϊόντων τροφίμων. Ο γενικός μηχανισμός και τα ενδιάμεσα στάδια της αντίδρασης είναι ήδη γνωστά. Ωστόσο, μια ευρύτερη μελέτη των προϊόντων της αντίδρασης Maillard, είναι πάντα επιθυμητή, καθώς τα συγκεκριμένα προϊόντα εμπλέκονται σε διάφορα βιολογικά μονοπάτια και σχετίζονται με οξειδωτικό στρες και πληθώρα μεταβολικών και νευροεκφυλιστικών ασθενειών. Στην παρούσα μελέτη μελετήθηκε η αντιγλυκοζυλιωτική δράση του παρθένου ελαιόλαδου και βιοδραστικών συστατικών του σε σύστημα γλυκοζυλίωσης αποτελούμενο από D-Γλυκόζη και L-Λυσίνη. Ο σχηματισμός τόσο φθοριζόντων όσο και χρωστικών χρησιμοποιήθηκαν για την αξιολόγηση προόδου της αντίδρασης Maillard. Επιπλέον, τα πρώιμα προϊόντα χαμηλού μοριακού βάρους ανιχνεύθηκαν στα 280 nm, ενώ τα πιο προχωρημένα προϊόντα ανιχνεύθηκαν στα 360 nm μέσω Φασματοφωτομετρίας Ορατού – Υπεριώδους. Η εξέλιξη της αντίδρασης, συμπεριλαμβάνει το σχηματισμό προϊόντων υψηλού μοριακού βάρους, οι μελανοϊδίνες οι οποίες διαθέτουν χρωμοφόρες ομάδες με χαρακτηριστική απορρόφηση στα 420 nm. Η αντίδραση Maillard είναι ισχυρά εξαρτώμενη από τη θερμοκρασία με τη μεγαλύτερη και γρηγορότερη παραγωγή προϊόντων να σημειώνεται σε υψηλότερες θερμοκρασίες. Μερικά χαρακτηριστικά βιοδραστικά συστατικά του ελαιόλαδου, όπως η α-τοκοφερόλη και η υδρόξυτυροσόλη έδειξαν αντι-γλυκοζυλιωτική δράση δείχνοντας ανασταλτική δράση στον σχηματισμό προϊόντων προχωρημένης γλυκοζυλίωσης κατά την πρόοδο της αντίδρασης, όπως επίσης και τα δείγματα εξαιρετικά παρθένου ελαιόλαδου που εξετάστηκαν για τη δράση αυτή. Σημαντικές παρατηρήσεις συλλέχθηκαν σχετικά με την ικανή ανασταλτική δράση μεμονωμένων βιοδραστικών συστατικών (π.χ. υδροξυτυροσόλη) και του εξαιρετικά παρθένου ελαιόλαδουμε υψηλό φαινολικό περιεχόμενο. Αυτό, θα μπορούσε να αποτελέσει σημαντική πρόταση για τη βιομηχανία τροφίμων, ώστε να συμπεριλάβει το παρθένο ελαιόλαδο στην επεξεργασία τροφίμων. Ωστόσο, περισσότερη έρευνα πάνω σε διαφορετικά συστήματα τροφίμων, διαφορετικές θερμοκρασίες και ποσοτικοποίηση των προϊόντων είναι αναγκαία για την εξαγωγή εμπεριστατωμένων αποτελεσμάτων και συμπερασμάτων.

Λέξεις-κλειδιά: Αντίδραση Maillard, μη-ενζυματική αμαύρωση, προϊόντα προχωρημένης γλυκοζυλίωσης (dAGEs), D-Γλυκόζη, L-Λυσίνη, Φθορισμομετρία, εξαιρετικά παρθένο ελαιόλαδο, α-τοκοφερόλη, σκουαλένιο, υδροξυτυροσόλη, αντι-γλυκοζυλιωτική δράση, Φαματοφωτομετρία Ορατού-Υπεριώδους, Χρωματομετρία

ABSTRACT

Maillard reaction or the non-enzymatic browning reaction, is a well-studied chemical reaction that is commonly appeared during thermal process of foods and plays an important role to the subsequent nutritional and organoleptic profile of food products. The general mechanism and the subsequent steps of the reaction are well-established. However, a broader examination of Maillard products formation is always desirable, as these products interfere with many biological pathways and are related with oxidative stress and metabolic or neurodegenerative diseases. In the present study antiglycative effect of virgin olive oil and its bioactive components, were evaluated using glucose-lysine as a model food system. The formation of both fluorescence and brown pigments are used for the estimation of Maillard reaction progress. Moreover, the early low molecular weight compounds was monitored at 280 nm, and the more advanced ones at 360 nm. The progress of the reaction involves the production of high molecular weight compounds, termed melanoidins, with chromophore groups with a characteristic absorbance maximum at 420 nm. Maillard is a chemical reaction strongly related to temperature, with higher production of Maillard products in higher temperatures. Some common bioactive components, such a-tocopherol and hydroxytyrosol, of virgin olive oil show anti-glycative properties, as well as, samples of extra virgin olive oil were evaluated for their inhibitory activity. Important observations were collected regarding potential inhibitory activity by individual bioactive components (e.g. hydroxytyrosol) and extra virgin olive oil with high total phenolic content. This could be a proposal to food industry for including virgin olive oil in food process. However, further study of different model systems, different temperatures and quantification of produced products is needed for more specific results and conclusions.

Keywords: Maillard reaction, non-enzymatic glycation, Advanced Glycation Endproducts, dAGEs, D-glucose, L-lysine, Fluorescence spectrometry, extra virgin olive oil, a-tocopherol, squalene, hydroxytyrosol, anti-glycative, UV-Visual Spectrophotometry, Chromatometry

INTRODUCTION

Through centuries, the cultural and economic development of the modern world led to different nutritional patterns, customized on human's nutritional needs and culture. However, in the last decades, the need for better quality in foods is of high importance. Food quality is based on the nutritional elements and the organoleptic characteristics of the product such as appearance, aroma, taste and flavour. The good taste and colour of a food product is a result of the heating treatment, that is commonly applied for the elimination of microbiological criteria, ensuring both food safety and quality. During thermal processing of foods two reactions, the Maillard reaction and lipid peroxidation, are the main factors that determine stability and quality. The Maillard reaction or protein's glycosylation is a non-enzymatic browning reaction and was first reported by 1912, in products such as roasted coffee, baked bread, and grilled steak by the French chemist Louis-Camille Maillard. During the passage of the last century, many studies have been conducted for the characterization of each stage of the Maillard reaction and the evaluation of the compounds produced. Due to the primary reactants that participate, which are reducing sugars and the amino group of a protein, peptide or a free amino acid, Maillard reaction is also known as protein glycosylation, a term that is mainly used in biochemical studies.

Except for the desirable characteristics that Maillard reaction contributes to final products, the need for improved nutritional choices by the consumers has turned the attention to examine Maillard reaction's impact on human health. Advanced Glycation Endproducts (AGEs) is the main category of compounds produced during thermal processing of foods containing high amounts of reducing sugars and proteins. These post-intermediate products, are not only taken when processed foods are consumed, but they are also formed biologically by endogenous protein glycosylation by the existing blood sugar in human body. The impact of these compounds on human health has been examined in the past years, and a connection has been found with oxidative stress and further with chronic diseases such as diabetes, and also with autoimmune and neurodegenerative diseases. (Uribarri, et al., 2015) The pool of endogenous AGEs is significantly enhanced by excessive consumption of dietary AGEs (dAGEs), increasing the risk of upcoming damage on human health. (Wei, et al., 2018) Important studies for the qualitive and quantitative determination of dAGEs have been conducted, in different foods produced under different thermal conditions and with different composition of protein, fat and sugar, have already been published. (Uribarri, et al., 2010) Nowadays, the knowledge of the reaction mechanism, the conditions and the parameters that affect the formation of AGEs, can help researchers to elaborate on the inhibition of their formation by functional agents, having a positive impact on human health.

Extra virgin olive oil is a nutritional element that is daily consumed in the Mediterranean diet and is already known for its high antioxidant activity (Kouka, et al., 2017). From ancient years already, olive oil has been established as a valuable nutritional and cultural element of the Mediterranean gulf. It is rich in bioactive and antioxidant components such as squalene, phenolic compounds, α-tocopherol and unsaturated fatty acids. For these reasons extra virgin olive oil and its bioactive components were chosen, for the evaluation of their effect on dAGEs formation in food model systems and on the reaction kinetics. Important results and observations were reported and the anti-glycative effect of olive oil was determined.

Chapter 1

1.1 The Maillard Reaction

One of the most common reactions that takes place in food systems during their thermal processing, is non-enzymatic browning, also known as Maillard reaction (MR). It is a complex reaction that consists of many stages and a vast variety of products are formed. Non-enzymatic browning reaction can happen between reducing sugars, such as glucose, and molecules with free amino groups, such as proteins, peptides or free amino acids. The reaction's path can be divided into three basic stages, the initial, the intermediate and the final stage. Each one of them results in the formation of intermediate products, that can further react and therefore lead to the following stages. The browning products of this reaction are either desirable during thermal processing due to the improved final flavour and colour, or undesirable products in the case of inappropriate storage conditions or intense heating temperatures. The reaction occurs faster during processing under high temperatures, such as frying, roasting or baking, where the rate of the chemical reaction is much higher, with higher production of subsequent products (Nie, et al., 2013). The water is also an important factor for the evolution of the reaction, as in higher water activity where the reactants can move in better way, the rate of browning is increased (Eichner & Karel, 1972). The stages of the reaction are described below:

1.1.1 Initial stage

In the beginning of MR , the reducing sugar reacts with the free amino group of an amino acid or a protein and as a result Schiff bases are created. Schiff imines, can turn to cyclized products, called glycosylamines. One of the main products of this stage is fructosamine. However, these non-stable products can quickly be altered through Amadori and Heyns rearrangements that promote the first relatively stable compounds. The initial condensation of free D-glucose and amino acids leads to the formation of a labile *N*-substituted D-glycosylamine which may undergo the Amadori rearrangement to form the respective *N*-substituted D-fructosamine, a 1-amino-1-deoxyketose. The specular mechanism takes place in presence of D-fructose: in this case the formation of an unstable *N*-substituted D-fructosylamine occurs, subsequently, upon the Heyns rearrangement to the *N*-substituted D-glycosylamine, a 2-amino-2-deoxyaldose is formed. Amadori and Heyns compounds are characterised as early intermediate Maillard products. (BeMiller & Huber, 2008)

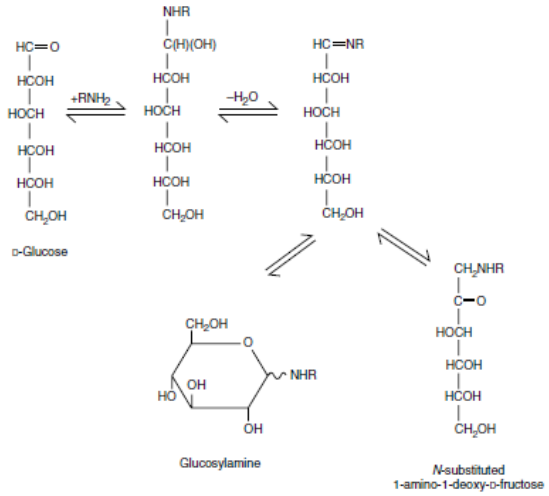


Figure 1: Initial stage of Maillard Reaction (BeMiller & Huber, 2008)

1.1.2 Intermediate stage

In the next stage, the Amadori (ketosamine) and Heyns (aldosamine) compounds, produced in the initial stage, are turned into a complex of numerous intermediates. Amadori compounds undergo transformation via four known pathways starting with four different intermediates formed from them. The result is a complex mixture of intermediates and products. Three of the four intermediates formed by rearrangements and eliminations are 1-, 3-, and 4-deoxydicarbonyl compounds, usually known by their common names that are 1-, 3-, and 4-deoxyosones. Formation of these intermediates occurs most readily at pH 4–7.The most prevalent of these intermediates is usually the 3-deoxyosone (more properly called a 3-deoxyhexosulose). The enolization is relevant to pH value. In neutral or acidic environment 1,2-enolization is preferred. On the other hand, alkaline pH leads to 2,3-enolization, with further formation of a-dicarbonyl compounds which undergo Strecker degradation. (Troise, 2018) In a pH value ≤ 5, hydroxymethylfurfural (HMF) is formed from hexoses or a furfural (furaldehyde) is formed from pentoses. Enolization, elimination of water, retroaldol cleavage, substitution of an amino function for a hydroxyl function are the reaction steps occurring on deoxyosones that lead to the formation of the stable secondary products of the MR.

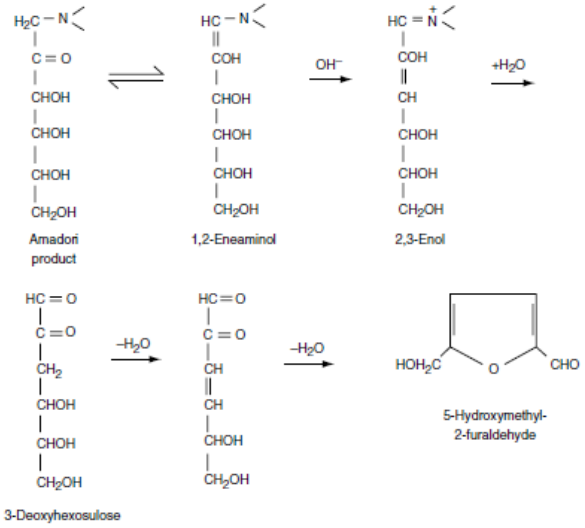


Figure 2: Intermediate stage of Maillard Reaction (BeMiller & Huber, 2008)

1.1.3 Final stage

The products from the previous stage, can react with compounds containing primary amino groups, such as amino acids, peptides or proteins with lysine residues, and lead to dark-colored polymers containing nitrogen, known as melanoidins. These polymeric compounds are responsible for the characteristic colour of the Maillard reaction. 1-deoxyosones are responsible for the formation of different intermediate compounds such as reductones, with high antioxidant capacity and due to their reducing activity can lead to the formation of other products. (Wang, et al., 2011)

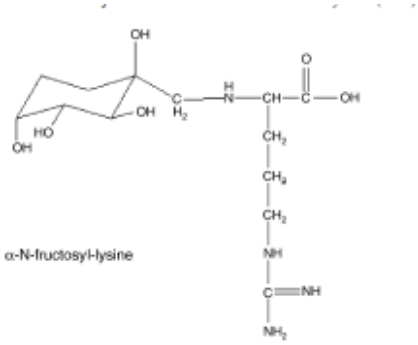
During these stages of the reaction, apart from the formation of the main products, there are numerous pathways and reactions that can result to the formation of secondary yet very important products. One of these reactions of osones and deoxyosones is the Strecker degradation. Firstly, the interaction of these compounds with an a-amino acid results to the formation of a Schiff base and after decarboxylation, dehydration and elimination an aldehyde with a shorter chain than the initial amino acid is produced. The structure of the formed aldehyde is based on the amino acid that interacts with the ozone or deoxyosone and are responsible for the aroma produced by Maillard reaction. Some of the main compounds in this category are 3-methylthiopropanal from L-methionine, phenylacetaldehyde from L-phenylalanine and 2-methylbutanol from L-isoleucine.

Another important category of the Maillard reaction products is modified proteins. Modified proteins are formed during the reaction of proteins and carbonyl compounds such as reducing sugars, pyrrole derivatives, osones, furfural and HMF. The amino acids' side chains of L-lysine and L-arginine residues are highly reactive. An example of such product is N-fructofuranosyl-lysine which is formed when a residue of a protein's L-lysine reacts with a carbonyl compound and is subsequently altered to furan

or pyrrole rings. However, this path can be destructive for the amino acid, and consequently, as L-lysine is a primary amino acid the nutritional value of foods is reduced. (BeMiller & Huber, 2008)

1.2 Important Maillard Reaction Products in food systems

1.2.1 Amadori Products



In the beginning of MR, there is a quick production of numerous compounds. The first stable products that are formed from the early stage of MR known as Amadori compounds. The interaction between carbonyl compounds such as reducing sugars and the amino group of a protein, peptide or amino acid results in condensation, a subsequent rearrangement and a hexosyl-amino acid is formed. The primary compound that is produced by heat treatment of foods containing proteins with lysine residues and glucose is ε-fructosyl-lysine. The amino acid and the type of reducing carbohydrate contribute to the formation of similar compounds. Amadori compounds are the precursors of subsequent products displaying an important role to the formation of aroma, flavour and colour. (Silvan, et al., 2006)

1.2.2 1,2-dicarbonyl compounds and reductones

The heating process of carbohydrates, can lead to an open form of the chain following by tautomerism reactions. As a result, unsaturated 1,2-diols are created via a reversible reaction and glucose can be tautomerized to fructose and mannose. Subsequent dehydration of 1,2-enediol has as a result the formation of 1,2-dicarbonyl compounds, specifically 3-deoxyglucosone. Successive removal of water molecules can lead to the formation of unsaturated osones and heterocyclic compounds, such as 5-hydroxymethylfurfural. In a similar pathway, dehydration of 2,3-enedioles results in 1-deoxydiuloses and specifically 1-deoxyglucodiulose. This last compound is known as reductone, that has a high antioxidant activity due to its α-hydroxy diketo structure and can be considered as an important intermediate compound of the final stage of the MR. It can be noted that dicarbonyl compounds are connected to the formation of heterocyclic compounds that contribute to the aroma of food. Reactive dicarbonyls interact with amino groups of proteins and peptides especially with lysine or arginine residues, can lead to the formation of **advanced glycation end products (AGEs)** which will be analysed below. (Hellwig, et al., 2017)

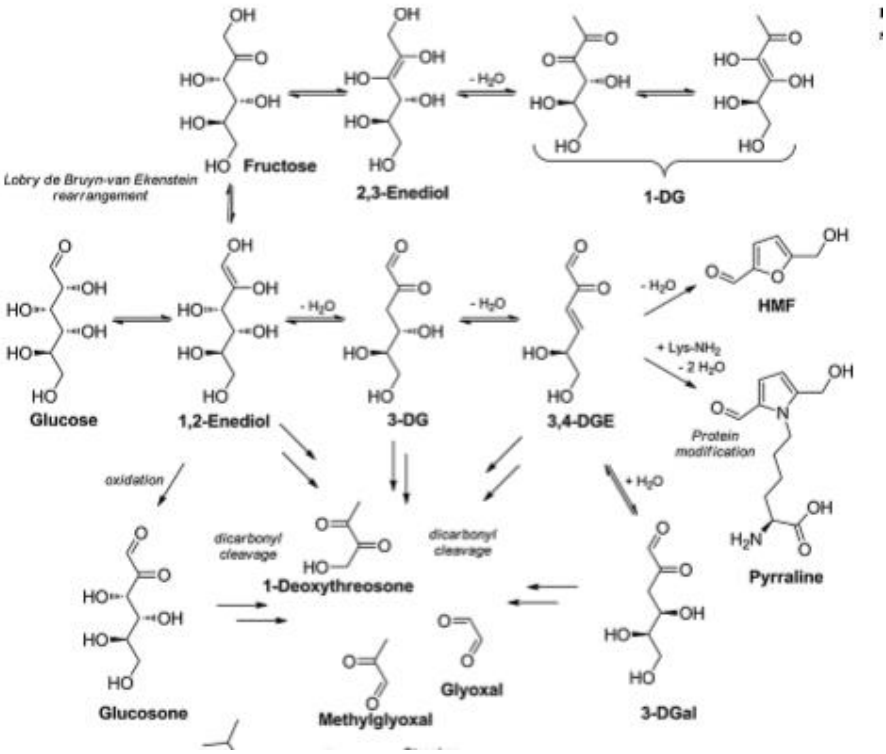


Figure 3: Schematic plan of formation of 1,2-dicarbonyl compunds and their following transformation (Hellwig, et al., 2017)

1.2.3 Advanced Glycation Endproducts – AGEs

The purpose of this research is the study of the formation of Dietary Advanced Glycation Endproducts (dAGEs) that are produced during heat treatment of foods, as part of non-enzymatic browning reaction products. As it was mentioned above, from the reaction between the carbonyl group and the amino group and a series of subsequent reactions (such as degradations, dehydrations and rearrangements), numerous compounds can be formed. Advanced Glycation Endproducts are derived from the reaction of 1,2-dicarbonyl compounds, formed in the stage of enolization, with amino acid residues of proteins or peptides, especially with the ε-amino group of lysine or the guanidino group of arginine. Primary compounds that can be classified as AGEs are CML (N-ε-carboxymethyllysine) that is formed from the reaction of GO (glyoxal) and lysine residues, CEL (N-ε-carboxyethyllysine) from MGO (methylglyoxal) and pyrraline that is derived from 3-DG (3-deoxyglucosone). (Hellwig, et al., 2017) The mechanism of the formation of AGEs is a series of subsequent chemical reactions including, condensation, enolization, dehydration, rearrangements, cyclization, fragmentation and oxidation. (Abbas, et al., 2015)

Moreover when the amine group is attached to a protein, the reaction can lead to cross-linking or unchangeable deformation of the protein, which in turn leads to functional alteration. It is confirmed that pentosidine is a cross-linker produced by reacting pentose with lysine (Lys) and arginine (Arg) residues of proteins. The compounds formed are known as cross-linking products (two amino acid residues can react together through a

cross-linker).The 1,2-dicarbonyl compounds, that are referred above, are an example of cross-linker. As a result GO (Glyoxal), MGO (Methylglyoxal) and 3-DG (3-Deoxyglucose), can contribute to the formation of amino acid dimers. For example, two lysine residues can form GOLD (Glyoxal-derived lysine dimer), MOLD (Methylglyoxal-derived lysine dimer) and DOLD (3-Deoxyglucosone-derived lysine dimer) respectively. Similar compounds can be formed by the reaction between lysine and arginine residues creating common dimer structures. The structure of AGE's (as it is shown in Figure 4) is responsible for the different chemical properties, that differentiate the ways of their analysis and quantification. Due to the heterocyclic structure of some AGEs, they indicate fluorescent properties. (Wei, et al., 2018) making them easily detected by fluorometric spectroscopy. Lipid peroxidation is also an alternative path for the creation of AGEs, as the peroxidation of polyunsaturated fatty acids can lead to the generation of Glyoxal which is a precursor of CML. Finally, the reaction between ascorbic acid and proteins is another route of dietary AGEs creation. As a conclusion, during heat treatments of foods, AGEs can be formed following one of the pathways, described above. (Nguyen, et al., 2014)

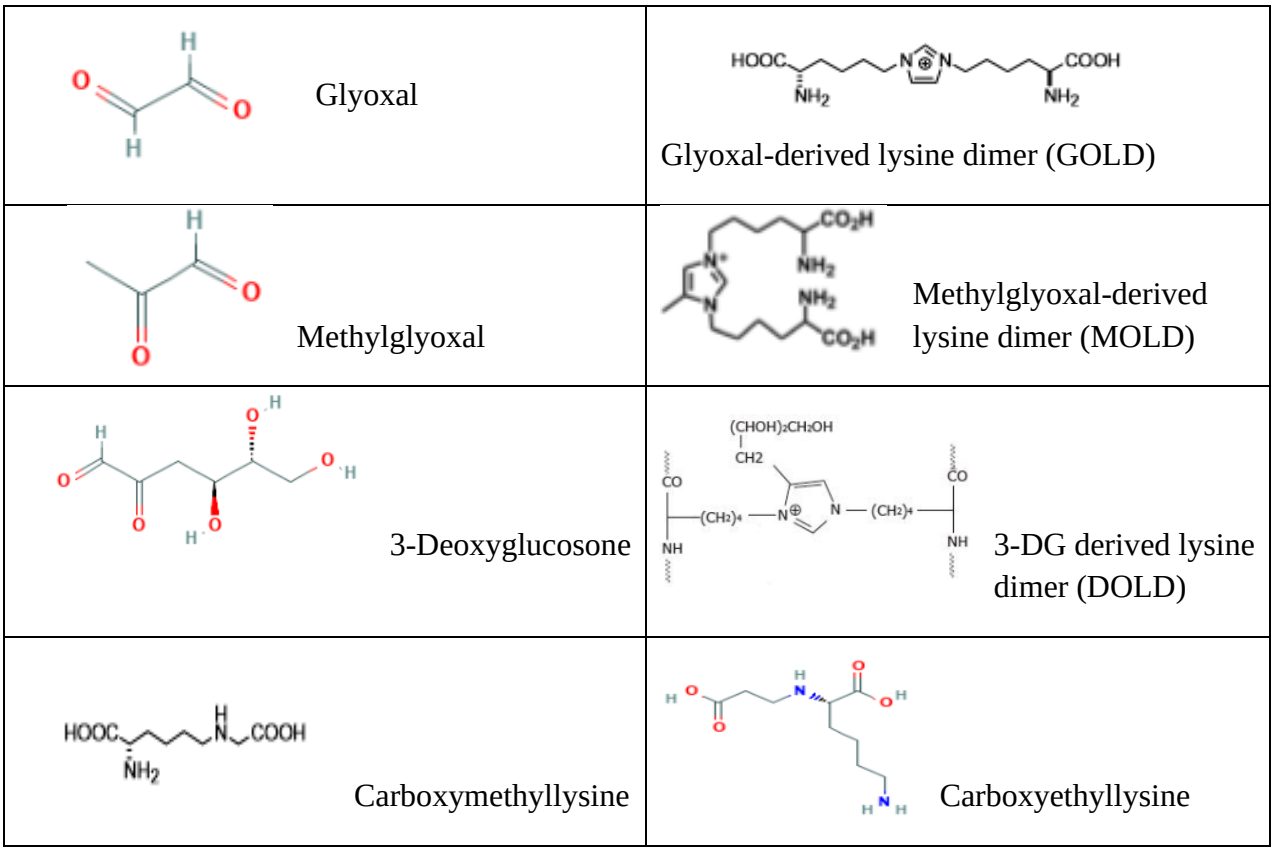


Figure 4: Chemical structure of important AGEs and their precursors (https://pubchem.ncbi.nlm.nih.gov/, n.d.)

1.2.4 Melanoidins

At the final stage of glycosylation, through the complex path of multiple subsequent chemical reactions, brown coloured polymers are created. These compounds are responsible for the brown colour in foodstuff, giving the impression of high-quality products with unique taste and flavour. There are a lot of studies concenring the formation, the chemical structure and the health impact of melanoidins through heating treatments of foods and their consumption. The reaction path that is followed for the formation of these chromophore polymers, includes successive reactions of cyclizations, dehydrations, rearrangements, retroaldolizations, isomerizations and condensations and as result two types of melanoidins are formed. High Molecular Weight melanoidins (HMW) and Low Molecular Weight melanoidins (LMW) depending on the conditions. In aqueous and neutral environment, the reaction between reducing sugars and amino acids results in LMW melanoidins and coloured compounds including furans, pyrroles and their derivatives. On the other hand, anhydrous conditions can lead to HMW melanoidins formation. One path to melanoidins formation, starts from the degradation of products derived from the reaction between sugars and amino compounds. (Wang, et al., 2011)

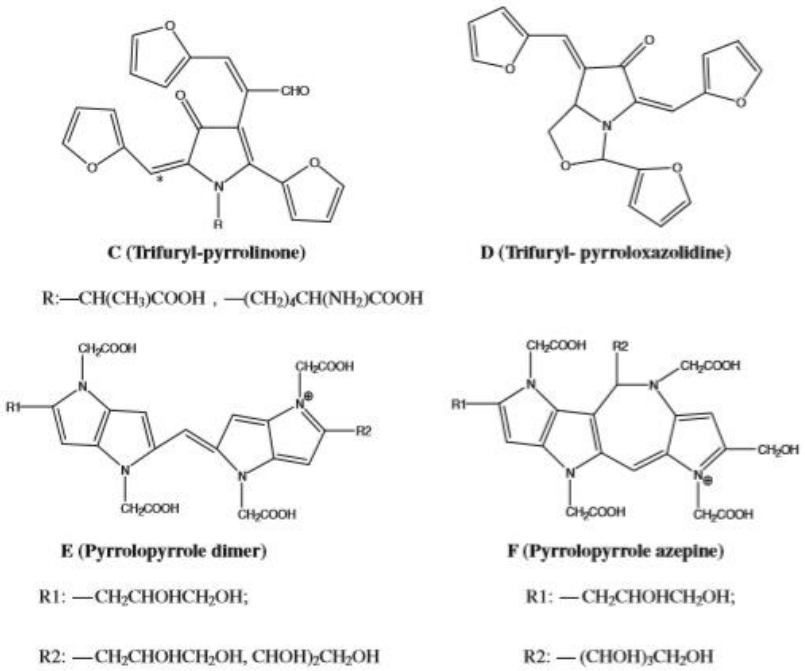


Figure 5: Some examples of LMW melanoidins formed through Maillard reaction (Wang, et al., 2011)

Chapter 2

AGEs in foods

As it is already mentioned, the formation of AGEs is a result of the general reaction between carbonyl compounds such as reducing sugars and amino groups of proteins, peptides and amino acids, and are the result of the reaction stage of dicarbonyl compounds with amino acids. More specifically, methylglyoxal is a main precursor for the formation of fluorescent AGEs during the thermal process. (Ho, et al., 2014) Foods with high amounts of proteins and reducing sugars such as glucose and proteins, peptides or free amino acids with high percentage of lysine or arginine residues, are prone to the Maillard reaction, when they are thermally processed. The mechanism of AGEs' formation is described in Chapter 1. Lipid oxidation is also a potent starting mechanism for lipoxidative byproducts of thermally processed foods, with formation of Advanced Lipoxidation Endproducts. Lipid peroxidation is the starting part of the mechanism which leads to the formation of reactive carbonyl species with further formation of ALEs. As a result, the amount of AGEs and ALEs is mainly analogous to protein, fat, sugars content. Studies have shown that high protein and high fat levels in foods can be correlated with high AGE levels, being an additional factor in correlation with the heating treatment of foods. (Wei, et al., 2018) (Uribarri, et al., 2010) have conducted a study on the AGE content, expressed in MG and CML units, in different kinds of foods and under various ways of thermal processing, and created a useful table with the AGE content of 549 foods. Process methods of foods, moisture content and the desirable temperature are primary factors for glycation or lipoxidation products. As a conclusion, higher consumption of foods with high-fat content or treated in high temperatures in the presence of low moisture can lead to higher amounts of AGEs, and on the other hand, foods such as fish, legumes, fruits, whole grains with lower amounts of fat and higher content of bioactive components such as, dietary fibres, polyphenols and carotenoids with anti-glycative properties result in limited formation and as a further result lower consumption of AGEs. (Uribarri, et al., 2015)

Impact of AGEs on human health

2.1 General impact

Glycation products, generated by non-enzymatic browning reactions, also known as Maillard reaction have been connected to high-risk of chronic diseases, as they are responsible for the oxidative stress in the human body. There are two possible ways that AGEs affect human health. In the first case, AGEs are responsible for proteins'

alteration by cross-linking that results in alteration of their properties and their functionality. The second way is based on their interaction with AGEs' receptors on the cell's surface. By the first path, the acceleration of AGEs formation by protein cross-linking can increase the risk of diabetes evolution. On the other hand, the second pathway, conducts an activation of AGEs receptor on cellular surface and a circular inflammatory response follows. As a consequence, the following abnormal degeneration of human cells can be correlated with chronic diseases such as, diabetes, cardiovascular diseases and neurodegenerative diseases. When the pool of endogenous AGEs is fortified by the extreme consumption and absorption of dietary AGEs, the risk for development of chronic diseases is increased rapidly. (Wei, et al., 2018)

There is high concern about the effect of the consumption of dietary AGEs on chronic diseases and especially diabetes, a disease that affects millions of patients all over the world. Apart from endogenous protein glycation that takes place in the human body, excessive consumption of dAGEs can promote protein glycation that plays an important role in the chronic disease of diabetes. Significant results are presented by (Vlassopoulos, A.; Lean, M.E.J.; Combet, E.;, 2013) on the effect of in vitro oxidative treatment of albumin on its glycation path. An interesting observation that can be conducted is that oxidation conditions and high blood glucose concentrations show synergistic action, promoting higher glycosylation levels. As a result, higher blood glucose levels interacting with oxidative conditions can show higher glycoxidation values. A higher consumption of dAGEs in diabetic cases enhances the glycoxidation products with higher risk for following oxidative stress.

2.2 Inhibition of AGEs formation

Many studies on the inhibition of AGEs formation have been conducted, and many compounds of natural origin, are already known to have this effect. Among these compounds, a primary role to the inhibition of glycosylation seems to have many antioxidant components, and above all polyphenols and flavonoids, as **(Uribarri, et al., 2015)** have mentioned. Many mechanisms are involved in the inhibition of AGEs formation, such as, antioxidant activity, trapping of reactive dicarbonyl compounds and inhibition of sugar autoxidation. Additionally, many natural products contain high amounts of polyphenols and in combination with other antioxidant compounds such as vitamin C and E can result in high inhibitory role. **(Sadowska-Bartosz & Bartosz, 2015)** However, studies on the inhibition of AGE formation are still conducted examining different types of inhibitory agents. For example, **(Bodiga, et al., 2013)** showed the anti-glycative properties of β-carotene on models consisted of Glucose and BSA. More specifically incubation of this model system with β-carotene in different concentrations had as a result a significant lower production of glycation products. Additionally, **(Chawla & Sahu, 2007)** on their study refer to anti-glycative properties of addition of essential oils on early stages of Maillard reaction of glucose-glycine model systems studying that by HPLC procedure. **(Ho, et al., 2014)** mentioned the anti-glycative properties of herbal infusions on BSA and methylglyoxal model systems, showing the suppression of methylglyoxal-mediated fluorescent AGEs formation by herbal infusions, in comparison with anti-glycative capacity of aminoguanidine which inhibited the formation of fluorescent AGEs by 86-107%. One part of this study is based on the inhibitory effect of virgin olive oil as a whole and its bioactive compounds such as, squalene, α-tocopherol, hydroxytyrosol, on the AGEs formation in model food systems, showing a potent anti-glycative role.

The model systems for Maillard reaction that were tested, were constructed in two molar ratios according to existing bibliography. For example, (Brands & van Boekel, 2001) in their study, used a model system of Maillard reaction consisted of caseinate salts and sugar in a final molar ratio of sugar to lysine residues of 10:1. Additionally, (Chen & Kitts, 2011) studied the formation of α-dicarbonyl compounds in model systems consisted of monosaccharides such as, glucose, fructose and ribose and amino acids such as, L-lysine and glycine, in a molar ratio of 1:1.

Chapter 3
Determination of AGEs and analytical techniques

3.1 Fluorescence

3.1.1 About Molecular Luminescence Spectrometry

Photoluminescence is a term that describes two different phenomena, fluorescence and phosphorescene. These two phenomena are the result of absorption of electromagnetic radiation. However, there is a difference between fluorescence and phosphorescence in the mechanism of excitation of electrons. During fluorescence, the electron is excited after radiation's absorption but without a spin-change and a short-lived excited state is succeeded. On the other hand, during phosphorescence the excited state of electrons is accompanied by a spin-change and a longer lifetime of this state is recorded. It should be noted that the photoluminescence phenomena happens in longer wavelengths than excitation.

The mechanism of photoluminescence is based on the excitation procedure of electrons due to their magnetic characteristics. Due to spin pairing, diamagnetic molecules do not show magnetic field. On the other hand, paramagnetic molecules which contain unpaired electrons can be attracted by magnetic field. Describing the general mechanism of photoluminescence, the conclusion can be drawn that it is a two-step procedure. When a doublet state of a free radical receives electromagnetic radiation, an excitation to higher energy levels of singlet or triplet state happens. The first state is described by paired electrons and no electronic energy splitting exists, whereas the triplet state is described by separated spins, with parallel orientation. Therefore, there is a difference between the excited singlet and triplet state as on the first case the molecule is diamagnetic and in the triplet state one molecule is paramagnetic. Subsequently, radiation absorption or emission follows. As it is described above, a different lifetime is expected for the two excited states and transitions between them are possible. Singlet-to-singlet transition is more possible to happen than singlet-to-triplet transition. The lifetime of the excited triplet state is about 10^{-4} to several seconds, compared to the shorter lifetime of the excited singlet state which is almost 10^{-8} seconds. As a conclusion the excited singlet or triplet state can be followed by emission and transmission back to a ground singlet state. The singlet-to-singlet transmission is described as the fluorescence phenomena and the triplet-to-singlet transmission as the phosphorescence phenomena respectively.

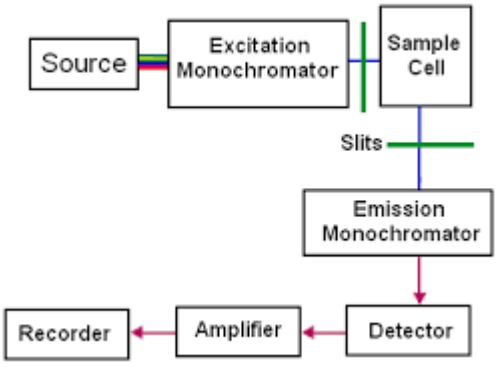
3.1.2 Effect of chemical structure to Fluorescence

The most common structures that are responsible for the fluorescence or phosphorescence phenomena, are chemical compounds with low-energy electron transition of π to π^*. Such structures contain aromatic, aliphatic or alicyclic carbonyl

functional groups, with highly-conjugated double bonds. It can be noted that the existance of condensed cyclic compounds with conjugated double bonds can increase the fluorescence signal. On the other hand, simple heterocyclic compounds do not fluoresce in solutions. Other parameters that affect fluorescence or phosphorescence are the quantum yield, which is the ratio of luminescent molecules to the total excited molecules. A high fluorescent molecule's value approaches 1 and on the other hand a low-fluorescent molecule has a very low quantum yield. The type of transition can affect the fluorescence emission. Lower energetic processes such as π^* to π and π^* to n are most frequently observed during fluorescence.

It should be noted that the excitation and emission phenomena can happen in different wavelengths. The first step of excitation can be reached by absorption of radiation under same conditions. On the other hand, the fluorescence or phosphorescence emission can happen on fixed wavelength different from the excitation's one. For every type of fluorescent compound of interest there is an excitation – emission band.

3.1.3 Instrumentation



The instruments that are used for fluorescence or phosphorescence measurement have common characteristics to spectrophotometers. These instruments have a light source that emits electromagnetic radiation and the main difference between spectrophotometers is the filters for the selection of both excitation and emission wavelengths. Transducers, cell compartments and data recorder are also main parts of a fluorometer.

Specifically, the most common light source for filter fluorometers is a low-pressure mercury lamp with a fused silica window. From this source exciting fluorescence can be produced at a range of wavelength between 250 nm and 780 nm. The most important part of a fluorometer is the filters or the monochromators. This part is responsible for the selection of the wavelength of excitation and the fluorescence beam respectively. The emitted fluorescence signals can be reinforced by sensitive transducers. Finally, the samples can be examined into cells, and especially in modern types of fluorescent

instruments, special micro-plate readers with multiple spaces, can provide simultaneous examination of multiple samples using small sample volume.

3.1.4 Fluorescence spectroscopy for AGEs determination in food samples

One of the methods that are commonly used for the measurement of Advanced Glycation End Products is fluorescence spectroscopy, as these products can emit fluorescence when irradiated by UV-Vis radiation. The radiation is produced by a low-pressure mercury lamp and passes through excitation and emission filters. The wavelength of excitation is recorded at 370 nm and the emission one at 440 nm, so the corresponding filters are chosen. The sample's molecules are excited by absorbing the radiation and the emission of fluorescence is achieved at 440 nm. The signal is reinforced, detected and recorded in digital form translated to Units. The measurement of blank solution is required for the expression of results to Arbitary Units. This is an easy-to-use and rapid method and has shown significant findings on Advanced Glycation End Products research in food samples. Cross-linking AGEs such as, GOLD, MOLD and pentosidine are the most common fluorescent products that are measured by fluorescence spectroscopy in excitation/emission wavelength at 370/440 nm respectively. Although is a very sensitive and selective method, is a non-specific method as it can determine the total of fluorescent compounds the time of analysis. (Wei, et al., 2018)

3.2 Ultraviolet-Visual Molecular Absorption Spectrometry

3.2.1 About UV-Vis Molecular Absorption Spectrometry

UV-Vis spectrophotometry is a commonly used analytical technique when quick results are needed. UV-Visible spectrophotometry is also frequently used in food analysis due to the easy application and the low cost of the method. Molecular Absorption Spectroscopy is based on the interaction between electromagnetic radiation and the molecules of a chemical compound when the latter is examined in the form of solution. 1The absorption of radiation, expressed as absorbance by the analyte, can easily be used for the calculation of the analyte's concetration as there is a linear relationship between these two units.

Specifically, the examined solution has to be transferred into a transparent cell, called cuvette, which is constructed of special material for each type of measurement parameters (Ultraviolet or Visible region). The length of the cuvette is the path length of the electromagnetic radiation through the examined solution. A beam of light emitted by a light source (Deuterium lamp for Ultraviolet region and Tungsten lamp for Visible

region), which consists of photons, passes through the solution. The initial power P_o of the electromagnetic radiation decreases due to absorption of radiation by the molecules of the analyte. The mathematical equation expressing the linear relationship between absorbance and concentration is known as Beer's Law and it is the one shown below:

$$\boldsymbol{A = \varepsilon bc}$$

A stands for absorbance, ε stands for molecular absorptivity ($M^{-1}cm^{-1}$), b is the centimeters od the path length (cm) and c is the concentration of the analyte (M)

6.2.2 Beer's Law

The equation described above is the well-known Lambert-Beer's Law and is the basic principle of Molecular Absorption Spectroscopy. According to this principle, there is a linear relation between analyte's concentration and absorbance signal that is recorded. From this equation concentration can be easily calculated knowing the absorbance value. However, for the application of Beer's law there are deviations that need to be considered otherwise the law cannot be used.

One of the most significant deviation is the analyte's concentration. The concentration of the chemical compound that is responsible for the absorption of the radiation has to be in low levels. Higher concentrations lead to lower absorptivity at the given wavelength. This alteration is based on different inetractions between solvent and solute, or between solute's molecules. Beer's law can be applied in solutions with concentrations lower or equal to 0,01 M.

Other deviations that affect the applivation of Beer's law are instrumental due to the type of radiation or the length of the cell. Beer's law it can be strictly applied when a monochromatic source of radiation is chosen. A polychromatic source of radiation can affect the Beer's law as the molecular absorptivities of each wavelength differ and the relationship between absorbance and analyte's concentration is no longer linear. The different length between cells could be an important deviation, while an intercept will be added to the equation but this can be avoided by the selection of equal cells.

3.2.3 Instrumentation

There are plenty of different types of instruments used for Molecular Absorption measurement, called spectrometers, either simple or more complex systems, but the majority of them have the same basic structure which consists of the following parts: a light source, a wavelength selector, the sample container, radiation transducers, signal processors and a signal recorder.

Light source: The light source is made of different material related to the region of the spectrum that is going to be measured. The majority of spectrometers contain two types of light sources so as either ultraviolet or visible region can be measured. For the ultraviolet region (190-400 nm) a deuterium lamp is the most frequently used for the emission of radiation to these wavelengths, due to electrical excitation of deuterium at low pressure. The continuum spectrum is produced by initial excitation of molecules, leading to two atomic species by dissociation of the excited molecule and the production of an ultraviolet photon. Tungsten filament lamps are commonly used for measurements in the visible region. The energy of this kind of source is temperature dependent and a common temperature of 2870 K is used.

Sample container: The container of the sample or the blank solution is also known as cell or cuvette. The cell must be made of inert material, depending on the range of the spectrum that is doing to be measured, so that there is no interaction of the radiation with it. Cells made of quartz are chosen commonly for measurements in ultraviolet spectra region and cells made of glass are commonly used for measurements in visible spectra region. Plastic cuvettes can also be used for the visible region spectra. The most important characteristic of the sample container is the dimensions as they determine the path length that the beam of radiation has to pass through the sample solution. The cuvettes for the blank and sample solution are required to have the same path length so as the Beer's law to be applied. The most common path length for absorption measurements is 1 cm and calibrated cells of the same size can be found from different commercial sources. It should be noted that the proper cleaning and maintenance of the sample containers play a significant role to the absorbance measurement as grease, scratches or fingerprints can modify the transmission characteristics of the cell. For this reason, a thorough cleaning before and after the use of the cuvette is essential.

Instruments: The types of instruments that are used for the measurement of absorbance are categorised according to the number of beams that are emitted. There are four main categories of instruments: single beam, double beam in space, double beam in time and multichannel spectrometers. In every type, a light source emits electromagnetic radiation with a number of photons in a specific spectra region, which passes through the monochromator for the wavelength determination. The difference between single beam and double beam spectrophotometers is that the latter contains a beam splitter for the creation of two separate beams for simultaneous permeability of the beam through the reference cell and the sample cell. Double beam spectrophotometers are classified into two categories of double-beam-in-space and double-beam-in-time respectively. The first category has two matched photodetectors before the readout machine and the second category alternately shares the beam through reference and sample cell and has a unique photodetector for the detection of the absorbance signal. Modern spectrophotometers contain a photodiode array detector with a general design of a single-beam instrument. The produced radiation of a tungsten or deuterium lamp is reduced by a lens and a diaphragm and then passes through the sample cell. There is no

selection of a specific wavelength, but the radiation passes through the sample cell as a spectrograph. (Skoog , et al., 2007)

3.2.4 Application of UV-Vis Molecular Absorption Spectrometry

The mechanism of molecular absorption spectrometry is a two – step process. The radiation that is produced by the light source is a sum of photons and passes through the sample container. The analyte of interest can absorb a quantity of visible or ultraviolet radiation in a specific chosen wavelength. At first, a molecule of the analyte is electronically excited absorbing radiation and results in an excited species of molecule. Subsequently, the deexcitation of the excited species happens and the excitation energy is transformed to thermal energy.

The mechanism of absorption is a result of excitation of bonding electrons by visible or ultraviolet radiation. There is a relation between wavelength bands and the type of bonds in the compounds of interest, making UV-Vis Spectrometry a useful technique for identification of functional groups of molecules.

The most common compounds of interest that are examined by molecular absorption spectrometry are organic compounds, which contain valence electrons and as a result they can absorb electromagnetic radiation. An excitation of valence bond electrons to higher energy state takes place. The application of molecular absorption spectrometry in organic compounds is related to excitation of n or π electrons to π^* excited state, and the energy for this transition leads the absorption bands to ultraviolet-visible region spectra (200-700 nm). This type of electrons are responsible for unsaturated functional groups and are known as chromophores.

For the spectrophotometric absorbance measurement, the criteria for the selection of wavelength is based on the recorded maximum absorption level of the analyte and the adherence of Beer's law is succeded. The absorbance measurement is based on taking into account all the parameters that can affect the measurement such as temperature, solution's pH, presence of interference substances, or inappropriate cleaning and handling of the cells.

As it was described above, following Beer's law there is a linear relation between absorbance and concentration of the analyte. By measuring the percentage of absorption of electromagnetic radiation, due to unsaturated bonds in its structure, the concentration of the compound of interest can be easily calculated. For this reason, the creation of the calibration curve is essential, using the external standard method.. The measurement of absorbance in solutions of known concentration for the analyte will lead to the construction of a linear curve against concentration . The curve's equation can be used for the calculation of concentration in the unknown sample. (Skoog, et al., 2007)

3.2.5 UV-Vis Molecular Absorption Spectrometry in AGEs determination

Food products are complicated systems, consisting of various components with different functional properties. There is a difficulty in examination of food products due to their complex structure and the sample preparation that is necessary. For this reason, the Maillard reaction can be more easily examined using model systems rather that foods, that include the primary reactants such as free amino acids, containing free amino-group and reducing sugars such as glucose. After the heating process that is required, the progress of the reaction can be studied using different methods.

The UV-Vis molecular absorption spectrometry is a valuable method for the determination of different products that are produced in different stages of the Maillard reaction. The chosen wavelength for the measurement of absorbance is strictly connected to the functional groups of the products in each stage of the reaction. The most common application of UV-Vis molecular absorption spectrometry when examining the Maillard reaction is the measurement of samples in three main wavelengths, related to the products of the three main stages of the reaction. Firstly, the radiation at 280 nm is absorbed by the initial stage's products such as Schiff bases and Amadori rearrangement products.. Intermediate products such as unsaturated carbonyls, dicarbonyls, ROS and furans that come from the dehydration, oxidation and fragmentation of initial products are measured at 360 nm. and Finally the final products of non-enzymatic browning such as cross-linked products, aromatic compounds and coloured polymers are determined at 420 nm. The absorbance signal in association with the time of heating treatment can generate a curve describing the progress of Maillard reaction during heating time. As a conclusion UV-Vis molecular absorption spectrometry is a valuable technique that can give significant results for the reaction's progress according to temperature and heating time. However it is not a selective method, because one or more compounds with similar structures can absorb radiation of the same wavelength.

3.3 Colour Development – Colour Measurement

3.3.1 About Colourimetry

Colourimetry is another method that can give useful results, when colour products are produced during a chemical reaction. With this method, the colour that is observed can be analysed by specific instruments, the colorimeters. The colour observation is based on parameters such as the illumination, the object under observation, the optical properties and the response of the human eye. The colorimetric method is based on the reflection of the illumination produced by a light source, on the surface of the observed object and the intensity of this reflection that reaches the human eye. There are different types of illumination sources but the most common is the D65, which represents

average daylight with a colour temperature of 6500 K. The colour observation can be held by standard observers which are known as 2-degree and 10-degree respectively. The one that is used commonly in modern colorimeters is the 10-degree observer and the degree value is connected to the eye region which contain the most colour sensitive cells, responsible for the colour observation. (Gilchrist & Nobbs, 1999)

*3.3.2 L*a*b* system*

The most commonly used colour description system in modern colorimetric methods is the L*a*b* colour space. L*a*b* system can be presented as a three-dimensional colour space with three axes. Each one of these axes, represent a climax between white and black, yellow and blue and red and green respectively. These colours are unique and can be used for the description of any observed colour. Every axis has two extremities where two opposite colours exist. The observed colour can be described by three parameters, that represent one of these scales, L*, a* and b*. More specifically, the vertical axis L*, can describe the lightness, taking values from 0 to 100. 100 represents a totally white sample and on the other hand 0 represents a totally black sample. The a* axis stands for the greenness-redness quality of the observed colour +a* values shows redness whereas -a* values show green notes. Finally, the b* axis represents yellow-blue notes of the colour. In a similar way with the a* values, +b* values stand for yellow notes and -b* values represent blueness in the sample. It should be noted that the measured L*, a* and b* values have to be counted under the same agents of illuminant and observer. Two additional values can complete the colour description. Chroma C*, which is a combination of a* and b* values and is calculated by these two values, and hue angle h° that is associated with the direction of each primary hue in accordance with the three axes and is measured anticlockwise from the positive a* axis. All of these different parameters can be combined into the equation for the calculation of the total chromatic difference between an unknown and a standard sample. (Gilchrist & Nobbs, 2000)

3.3.3 Determination of Colour Development in the Maillard Reaction

Maillard reaction has plenty of products, many of them displaying colour characteristics. Final products of Maillard reaction, such as melanoidins are coloured polymers with a characteristic orange to brown colour. Colour measurement is a valuable method for the examination of the colour development during thermal processing of food model systems. The intensity of colour and the rate of colour development can be examined according to heating time and can be related to the heating temperature applied. (Echavarria, et al., 2016) By measuring the L*, a* and b* values the colour difference ΔE can be calculated as a function of time, and a kinetic model of the colour development of the Maillard reaction can be evaluated.

3.3.4 Other Instrumental analysis

3.3.4.1 High Performance Liquid Chromatography (HPLC)

HPLC is a widely used instrumental chromatographical technique for the separation and determination of different chemical compounds, adjustingthe conditions of the technique according to the chemical and the physical properties of the compounds of interest. Different mobile and stationary phases, and different detectors are chosen. In the case of AGEs determination, a polar mobile phase and non-polar stationary phase are chosen, as have described.

A DAD detector is chosen for the detection of the compounds, such as glyoxal and methylglyoxal that can absorb UV-Vis radiation after derivatization procedure with 1,2-diaminobenzene (Daglia, et al., 2007), and a fluorescence detector (FLD) is chosen when fluorescent compounds such as pentosidine, and AGEs precursors, are the compounds of interest. CML, CEL, pyrraline are not fluorescent compounds and a previous pre-treatment with derivatization reagents is needed. (Lin, et al., 2018)

3.3.4.2 Gas Chromatography (GC)

Volatile compounds are not easily determined. Gas Chromatography is a chromatographic technique specifically designed for the determination of volatile compounds. This technique can also be performed for the determination of AGEs, such as CML after derivatization procedure, or other volatile intermediates of Maillard reaction, such as pyrazines, pyridines, furans, thiophenes and thiazoles with even higher efficiency when coupled to Mass Spectrometer (GC-MS). (Milkovska-Stamenova, et al., 2015) have proposed a GC-MS technique for the determination of carbohydrate intermediates in glycation systems, after derivatization with MSTFA.

3.3.5 Immunoassay Analysis

Specific assays for the determination of AGEs with higher selectivity, are immunoassay techniques, based on the interaction of antigens and specific antibodies. There used for the determination of AGEs, such as CML, CEL or MG, for example immunosorbent assay or ELISA, which is the most commonly used. (Wei, et al., 2018) This determination in foods and food systems can be performed by interaction of CML monoclonal antibodies as (Goldberg, et al., 2004) have proposed on their study. Although this is a selective and specific assay, the cost for the performance is high and is not selected frequently.

Chapter 4: Olive Oil

4.1 About Olive Oil

For this study, important antioxidant compounds of olive oil were chosen to be examined as inhibitory factors of AGEs formation. Olive oil is the most common used media of cooking in the Mediterranean cuisine from ancient years. Olive oil is mainly produced in Mediterranean countries, such as, Italy, Spain, Greece, Turkey and Morocco, with higher production in Greece and Italy. Its significant health effects of olive oil have made it really popular to international trade. (Tsimidou, et al., 2003) The extraction of olive oil from olive fruit is taken place under specific conditions and cold pressure which is described by legislation. Olive oil is categorized according to its acidity, expressed as oleic acid % wt and through national standards into I. Virgin Olive oil containing i) extra virgin olive oil (1% wt oleic acid) , ii) virgin olive oil (2% wt), iii) lampante olive oil (3,3% wt), II. refined olive oil (0,5% wt), III. olive oil composed of refined and virgin olive oil (1,5% wt), IV. Olive-pomace oil containing i) crude olive-pomace oil, ii) refined olive-pomace oil, iii) olive-pomace oil. (COMISSION, 1991) This valuable product is known for its high content in bioactive compounds and its beneficial effects on human health, preventing from the onset of many diseases. Olive oil consists mainly of triacyloglycerols and free fatty acids. Also high levels of α-tocopherol are determined in olive oil, responsible for its dietary benefits. Hydroxytyrosol (3,4-dihydroxyphenethyl alcohol)and tyrosol (4-dihydroxyphenethyl alcohol) are the primary phenolic compounds of olive oil. Squalene, sterols, carotenoids, chlorophyls and volatile compounds are also important components of olive oil (Tsimidou, et al., 2003). The main characteristics of some of these components will be described below.

4.2 Important antioxidant components of Olive Oil

4.2.1 Squalene

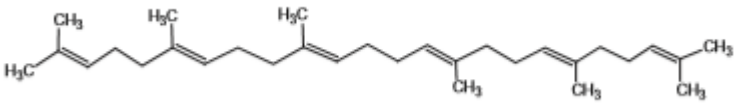


Figure 6: Squalene's chemical structure (Secmeler & Galanakis, 2019)

The 90% of the hydrocarbon fraction consists of squalene ($C_{30}H_{50}$). (Tsimidou, 2010) This polyunsaturated hydrocarbon is a triterpene and can be easily affected by oxidation risks, due to its hydrophobic and unsaturated structure. Therefore it can also prevent polyunsaturated fatty acids from temperature-based autoxidation, acting as a peroxyl radical scavenger. (Secmeler & Galanakis, 2019) Its antioxidant properties have been correlated with the prevention of many chronic diseases and many types of cancer. (Huang, et al., 2009) The high amount of squalene found in olive oil, makes it a good

source for human consumption and in combination with phenolic compounds in olive oil, the antioxidant effect of olive oil is fortified. Squalene levels in virgin olive oil range from 200 to 7500 mg kg^{-1}, and can reach the upper level of 12000 mg kg^{-1}. Inappropriate storage conditions, or high temperatures can lead to significant loss of squalene. (Tsimidou, 2010)

4.2.2 a-tocopherol

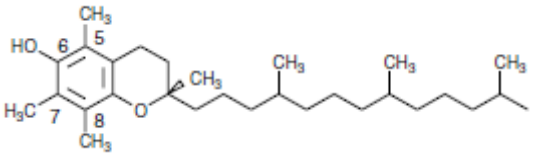


Figure 7: α-tocopherol's structure (McClements & Decker, 2008)

Another component of olive oil is tocopherols. This is a group of compounds with high antioxidant properties, preventing unsaturated fatty acids from autoxidation. The tocopherols group consists of four chemical structures called α-, β-, γ-, and δ-tocopherol. Their structure is characterized by a hydroxylated ring system (chromanol ring) with a phytol chain. The structural difference between the four compounds, is the methylation pattern of the chromanol ring, with α-tocopherol being trimethylated as it is shown on Figure 7, β- and γ-tocopherol being demethylated and the monomethylated δ-tocopherol. Alpha-tocopherol in olive oil comprises 90-95% of the total tocopherol content (Kalogeropoulos & Tsimidou, 2014) and it also has a higher Vitamin E activity and higher antioxidant capacity. (McClements & Decker, 2008) The high amounts of α-tocopherol (250-400 mg kg^{-1}) in virgin olive oil enhance its antioxidant properties and its beneficial health effects. (Tsimidou, 2010)

4.2.3 Hydroxytyrosol (3,4-dihydroxyphenethyl alcohol)

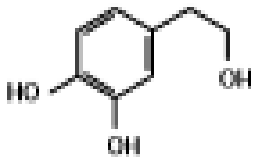


Figure 8: Structure of Hydroxytyrosol ((3,4-dihydroxyphenyl)ethanol) ***Invalid source specified.***

The polar fraction of virgin olive oil consistes of various polar phenolic compounds, that paly an important role in the beneficial effects on human health, the oxidative stability and the organoleptic characteristics of olive oil.. One of the phenolic compounds that is present in high amounts in olive oil is hydroxytyrosol ((3,4-dihydroxyphenyl) ethanol). The content of phenolic compounds in olive oil can be easily estimated, by examining the methanol-aqueous extract with colourimetric methods such as Folin-Ciocalteu assay.The result is expressed as total phenolic content. The phenolic content of virgin olive oils has a huge range from 50 to 1000 mg caffeic

acid kg^{-1} with a usual value of 100 to 300 mg caffeic acid kg^{-1}. More precise instrumental methods such as HPLC can separate and determine the concentration of phenolic components (Kalogeropoulos & Tsimidou, 2014). Phenolic compounds have also been examined for their inhibition properties on AGEs formation. Specifically, Hydroxytyrosol has been linked with protective activity against Methylglyoxal (MGO) that is produced during glycation , as well as against the formation of fructosamine. Due to the preventing effects of Hydroxytyrosol on early Maillard stages, it can also prevent the formation of advanced products, such as, N-ε-(carboxymethyl)lysine (CML), as an inhibitor of their precursors (Khan, et al., 2019). This property of Hydroxytyrosol is also examined in this study as a significant antioxidant component of virgin olive oil. Finally, the beneficial effects of phenolic components of virgin olive oil are investigated and reported by EFSA and a relevant health claim have supported their antiglycative properties and their protective capacity against oxidative damage. (NDA, 2011)

EXPERIMENTAL PART

3.1 Scope

The scope of this study is the observation of the Maillard reaction progression and AGEs formation, during thermal processing of glycation model systems, consisted of an essential, highly reactive amino acid (L-lysine) and a reducing sugar that exists in abundance in foods (D-glucose). Extra virgin olive oil, which is the most common component of Mediterranean diet was examined for its inhibitory activity as well as, some of its strongly apparent bioactive components. The heating treatment was held at different temperatures (80 and 100℃), in order to observe potential differences between the results of the measurements, based on different temperatures. Different molar ratios between D-Glucose and L-Lysine were also examined (10:1 and 1:1). For the examination of inhibitory capacity, squalene, a-tocopherol and hydroxytyrosol were chosen as the most common bioactive components of virgin olive oil. Finally, two samples of extra virgin olive oil according to their total phenolic content were chosen for the examination of their inhibitory effect on glycation model systems. The model systems were prepared, heated and tested through colour measurement, fluorescence spectroscopy and UV-Vis spectrophotometry, so as the colour development, fluorescent AGEs and Maillard products of different stages to be evaluated in different glycation model systems.

3.2 Experimental procedure

3.2.1. Reagents

- D-Glucose (Mr= 180,16, Sigma-Aldrich, CAS: 50-99-7)
- L-Lysine monohydrochloride (Mr= 182.65, Sigma-Aldrich, CAS: 657-27-2)
- K_2HPO_4, Dipotassium Phosphate (Mr= 174.18, Supelco, CAS: 7758-11-4)
- KH_2PO_4, Potassium Dihydrogen Phosphate (Mr= 136.09, Supelco, CAS: 7778-77-0)
- Tween 80, Polysorbate 80 (Sigma-Aldrich, CAS: 9005-65-6)
- Squalene, 2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene (Mr= 410,72, Supelco, CAS: 111-02-4)
- α-tocopherol, 2,5,7,8-Tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol (Mr= 430,71, Sigma-Aldrich, CAS: 10191-41-0)
- Hydroxytyrosol, 3,4-Dihydroxyphenethyl alcohol (Mr= 154,16, Sigma-Aldrich, CAS: 10597-60-1)
- Dichloromethane (Sigma-Aldrich, Mr= 84,93, CAS: 75-09-2)

- Two samples of Greek Virgin Olive Oil, EVOO_1 (TPC = 380 mg/kg) and EVOO_2 (TPC = 251 mg/kg) (Origin: Rethimnon, Crete, Variety: Koroneiki)

3.2.2 Materials

- Volumetric flasks (25, 50, 100, 500, 1000 mL)
- Pipettes (10 mL)
- Graduated measuring cylinders (25, 100 mL)
- Graduated beakers (50, 100, 250 mL)
- Pasteur pipettes
- Falcons (2, 15, 50 mL)
- Disposable plastic cuvettes
- Magnets

3.2.3 Instruments

- Automatic Transfer Pipette (1000 μL, Brand, Germany)
- Analytical Balance AE20 (METTLER TOLEDO, Ohaio, USA)
- Heating plate (VELP Scientifica, Usmate, Italy)
- Vortex (VELP Scientifica, Usmate, Italy)
- Thermomix HCM100-Pro (Sinosource, China)
- Centrifugal K-80 (Hellenic Labware, Greece)
- UV-Vis Spectrophotometer (JASCO V-530, Tokyo, Japan)
- Spectrocolorimeter LC100 SV100 Kit (Lovibond, UK)
- Fluorimeter 2030 VICTOR X2 (Perkin-Elmer, UK)

3.3Preparation of Working solutions

Phosphate Buffer solution (PB 0,1 M)

To prepare 0.1M of Phosphate Buffer solution (pH 7.6), 13,506 g ± 0,1 mg of Dipotassium Phosphate (K_2HPO_4) and 3,056 g ± 0,1 mg of Potassium dihydrogen Phosphate (KH_2PO_4) are weighed in a beaker and a quantity of deionized water is added. Homogenization is done using a magnetic stirrer. After the adjustment of the pH (using potassium hydroxide or phosphoric acid) the mixture is transferred into a volumetric flask of 1L. The flask is filled with deionized water and is mixed until a clear solution is observed.

Glucose solution in PB (0,15 M) (1)

To prepare 0.15M of Glucose in PB, 6,756 g ± 0,1 mg of Glucose are weighed into a volumetric flask of 250 mL containing a quantity of the buffer (prepared as described

above). The homogenization is done under magnetic stirring. The rest of the volume is filled up with PB.

Lysine in PB (0,1 M) (2)

To prepare 0.1M of Lysine in PB, 3,6547 g ± 0,1 mg of Lysine are weighed into a volumetric flask of 250 mL containing a quantity of the buffer (prepared as described above). The homogenization is done under magnetic stirring. The rest of the volume is filled up with PB.

3.3 Preparation of Glycation Model Systems

Glucose : Lysine mixture in 10:1 molar ratio – S1 (3)

To prepare 100 mL of Glucose:Lysine mixture in PB, with a molar ratio 10:1, 91 mL of the solution (1) and 9 mL of the solution (2) are transferred into a volumetric flask of 100 mL, containing 0,4 g ± 0,1 mg of TWEEN 80 (Polysorbate 80). The mixture is homogenized in an ultrasonic bath for 10 minutes until the quantity of TWEEN 80 is completely dissolved.

Glucose : Lysine mixture in 1:1 molar ratio – S2 (4)

To prepare 100 mL of Glucose:Lysine mixture in PB with a molar ratio 1:1, 50 mL of Glucose the solution (1) and 50 mL of the solution (2) are transferred into a volumetric flask of 100 mL containing 0,4 g ± 0,1 mg of TWEEN 80 (Polysorbate 80). The mixture is homogenized in an ultrasonic bath for 10 minutes until the quantity of TWEEN 80 is completely dissolved.

TWEEN-80 solution (0,4% w/v) (5)

In volumetric flask of 100 mL, containing 0,4 ± 0,1 mg of TWEEN-80, is filled with freshly prepared mixture (3) or (4). The relevant mixture is homogenized in an ultrasonic bath until the whole quantity of TWEEN-80 is dissolved. In that case, a 0,4% w/v solution in 10:1 or 1:1 glucose solution is prepared.

Glucose:Lysine:Squalene mixture in 10 : 1 : 0.2 & 1:1:0,2 molar ratio (6 & 7)

Into a beaker, 1 mL of squalene (0,82 g ± 0,1 mg) is weighed, and a quantity of the freshly prepared mixture (5) in molar ratio 10:1 is added, until the total weight of the mixture reaches 100 g. The mixture is mixed under magnetic stirring for 1 minute and

further homogenization is performed using an UltraTurrax appliance for 1 minute to achieve better dispersion of the lipophilic droplets into the aqueous phase. The same procedure is followed for the preparation of 1:1 model system containing squalene.

Glucose : Lysine : Tocopherol mixture in 10 : 1 : 0,2 & 1:1:0,2 molar ratio (8 & 9)

A mixture of 0,4% TWEEN 80 is prepared using solution (3) as described above [A]. In another volumetric flask of 100 ml, 1 g ± 0,1 mg of TWEEN 80 is weighed and the flask is filled with PB. Both mixtures are homogenized in an ultrasonic bath until the quantity of TWEEN 80 is completely dissolved. Into a beaker, 1 g ± 0,1 mg α-tocopherol is weighed and a quantity of 1% TWEEN 80 solution is added until the total weight of the mixture is 100 g [B]. The mixture [B] is homogenized under magnetic stirring for about 1 minute followed by further homogenization in an Ultra Turrax homogenizer for about 1 minute in order to achieve better dispersion and emulsification. Into another beaker 90 ml of solution [A] and 10 ml of solution [B] are mixed under magnetic stirring followed by further homogenization in an Ultra Turrax homogenizer. The same procedure is followed for the preparation of 1:1 model system containing tocopherol.

Glucose : Lysine : Hydroxytyrosol mixture in 10 : 1 : 0.1 & 1:1 :0,1 molar ratio (10 & 11)

0,4 g ± 0,1 mg of TWEEN 80 are added into a volumetric flask of 100 mL and filled with solution (3) [A]. The mixture is homogenized in an ultrasonic bath until the quantity of TWEEN 80 is completely dissolved. Into a beaker 25 mg ± 0,1 mg of Hydroxytyrosol (2,3-Dihydroxyphenylacetic acid) are weighed and solution [A] is added until the total weight is 100 g. The mixture is homogenized by magnetic stirring until the quantity of Hydroxytyrosol is completely dissolved and the homogenization is completed by stirring with Ultra Turrax homogenizer. The same procedure is followed for the preparation of 1:1 model system containing HT

Glucose : Lysine mixture in 10 : 1 & 1:1 molar ratio + 10 ml Olive Oil (12 & 13)

At first, in a volumetric flask of 100 ml capacity containing 0,4 g ± 0,1 mg of TWEEN 80 (0,4% w/v) is filled with mix (3) [A]. Another volumetric flask of 100 ml capacity containing 1 g ± 0,1 mg of TWEEN 80 (1% w/v) is filled with Phosphate Buffer 0,1 M [B]. The two mixtures are homogenized in ultrasonic bath until the quantity of TWEEN 80 is completely dissolved. Into a beaker are mixed 90 ml of solution [B] and 10 ml of Olive Oil are added gradually under magnetic stirring. Stirring by Turrax follows for 1 minute [C]. The final mixture is consisted of 90 ml of solution [A] and 10 ml of mixture [C], which is homogenized by magnetic stirring and stirring using an Ultra Turrax homogenizer (1 min). The same procedure is followed for the preparation of 1:1 model system containing extra virgin olive oil.

3.5 Thermal process

Thermal process of all the above mentioned solutions, is accomplished in a thermomix. 2 mL Eppendorf tubes were filled with 1,7 mL of each solution. The temperature was set at 80 and 100℃ and the rotational speed was fixed at 600 rpm (rounds per minute). The system is heated for a total of three hours (180 min) and two eppendorfs were withdrawn from the heating block at the time intervals of 20, 60, 120 and 180 minutes and transferred into an ice bath in order to stop the reaction. The content of the tubes is then transferred into a falcon of 15 ml and kept into ice. The extraction of the water-soluble phase is performed with the use of 1:1 volume ratio of Dichloromethane as described below. The procedure for each time interval is performed in triplicate. The control for all measurements was the sample at t = 0 min.

3.6 Extraction

The sample is vortexed with a 1:1 volume ratio of Dichloromethane for 45 seconds and centrifuged for 10 min at 40000 rpm (Hellenic Labware, K-80). The upper layer, containing AGEs and MRPs is transferred into a clean 15 ml falcon. The samples are kept in the refrigerator at -20℃ until analysis. The same procedure is followed for the blank sample.

3.7 Analysis of the Reaction Products

Colour measurement of Maillard Reaction products (MRPs)

Considering that MRPs are an effective way to enhance the colour in food, the evolution of colour during the reaction was recorded. The colour was determined by a Lovibond Spectrocolorimeter LC100, readably calibrated using a white standard plate. The results were displayed as L*, a*, and b* values, indicating lightness, redness, and yellowness, respectively (Li et al. 2018). All samples were determined in triplicate. The following equation were used to calculate the **difference in colour (△E*):**

$$\Delta E^* = \sqrt{(L^* - L^0)^2 + (a^* - a^0)^2 + (b^* - b^0)^2}$$

where L^0, a^0, and b^0 are the colour of the reaction samples at **t=0.**

Measurements of AGEs fluorescence

AGE fluorescence was measured in all samples at an excitation wavelength 377nm and an emission wavelength 445nm. In a 96-well microplate 200 μl of each sample is transferred by an automatic pipette in each well. AGE fluorescence was measured as

arbitrary units (AU) in triplicate against the control, in a Fluorimeter 2030 VICTOR X2 (Perkin-Elmer, UK) plate reader

$$(A.U.) = Fluorescence\ Intensity_{sample} - Fluorescence\ Intensity_{control}$$

Absorbance measurement

In the first stage of the MR, reducing sugars react with amino acids, giving rise to non-colour compounds which do not absorb in the visible spectra (Delgado-Andrade et al. 2010). For this reason, the formation of the early low molecular weight compounds was monitored at 280 nm, and a pool of more advanced ones at 360 nm. The progress of the reaction involves the production of high molecular weight compounds, named melanoidins, with chromophore groups displaying maximum absorbance at 420 nm. In the present study the measurement of absorbance at 360 and 420 nm was performed in a double-beam UV-Vis Spectrophotometer as follows:

Preparation of samples for absorbance measurement

Samples were dissolved in PB, when necessary, in order to follow the linear relation between absorbance and concentration of Lambert-Beer's law. For samples heated at 80℃, dilution wasn't necessary and were transferred directly into the cuvette. The samples of 60, 120 and 180 minutes that were heated at 100℃, were submitted at 2-fold, 4-fold and 10-fold dilution respectively.

Sample dilution

The dilution of the samples takes place with PB as described below:

Dilution Factor = 2: 1500 μL of sample are transferred into the cuvette using an automatic pipette.1500 μL of PB are then added and the mixture is homogenized carefully.

Dilution Factor = 3: 1000 μL of sample are transferred into the cuvette using an automatic pipette. 2000 μL of PB are then added and the mixture is homogenized carefully.

Dilution Factor = 4: 750 μL of sample are transferred into the cuvette using an automatic pipette. 2500 μL of PB are then added and the mixture is homogenized carefully.

Dilution Factor = 10: 300 μL of sample are transferred into the cuvette using an automatic pipette. 2700 μL of PB are then added and the mixture is homogenized carefully.

3 ml of appropriately diluted sample, are transferred into a 1cm plastic cuvette using an automatic pipette. The spectrophotometer is set at fixed wavelength mode and it is set at 360 or 420 nm. PB is used as blank. The absorbance signal is recorded and the final results are expressed after multiplication of the dilution factor used for each sample as. Each time interval is measured in duplicate. Control measurements were recorded in t=0.

$$ABS_{final} = ABS_{measured} * (DF)$$

3.8 Statistical Analysis

Analyses were performed in duplicate **with the data expressed as mean ± standard** deviation, in the environment of Statgraphics Centurion 18 (Statgraphics Technologies, Inc., The Plains, Virginia). For the determination of statistical differences, analysis of variance and Multiple Range Tests were done. **Differences among means at P < 0.05 were considered to be significant**. Statistical differences were tested for inhibitory capacity into S1 & S2, for difference between molar ratios of Glucose-Lysine 10:1 & 1:1 respectively and between heating time points (20, 60, 120 & 180 min)

RESULTS

4.1 Effect of Inhibitory agents monitored by Fluorescence Spectra

The tables below, show the results of Fluorescence Intensity, expressed as Arbitrary Units (AU). Each table represents one heating temperature and one molar ratio system.

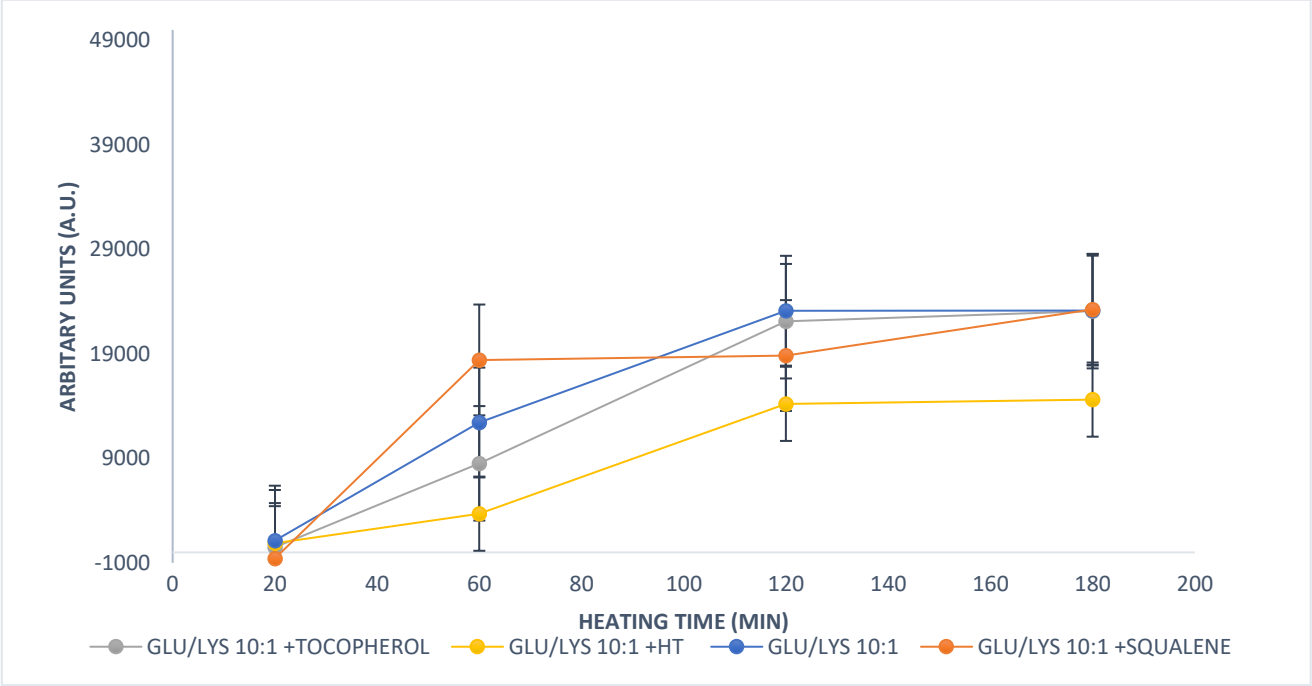


Figure 1: Diagram of Fluorescence Intensity expressed as Arbitary Units, of S1 (10:1) heated at 80°C and the impact of inhibitory agents, described by different colours of lines

Figure 1, shows the trend of Fluorescence Intensity of the Glucose-Lysine system in a molar ratio 10:1 with or without the incorporation of an inhibition agent (squalene, tocopherol and hydroxytyrosol). As it is obvious, at 80°C, the Glucose-Lysine, S1, shows an increase in Fluorescence Intensity during heating time. The presence of squalene enhances the Fluorescence Intensity at 20 and 60min heating in 80°C. In order to explain this finding, the interaction between squalene and lysine was examined and a production of Fluorescence signal was obvious. The interaction of squalene with lysine was tested for all temperatures and molar ratio combinations and squalene seems to produce Fluorescence signal after its interaction with lysine during the heating process, giving additional Fluorescence signal, due to its pro-oxidant activity and formation of mainly epoxides in temperatures higher than 62°C (Naziri, et al., 2014) which after reacting with nitrogen can create fluorescent products, that can be detected by Fluorescence spectroscopy (λexc=360±20 nm, λem=460±20 nm) (Badalassi, et al., 2004). This leads to the conclusion that addition of squalene to glycation model system for heating treatment can produce fluorescent non-AGEs compounds. For this reason, a mixture of Lysine and squalene in PB was heated for maximum 5h and the results of Fluorescence Intensity of the heated samples is shown on diagram which is part of the Annex. On the other hand, tocopherol seems to inhibits AGEs formation at least for the first 2h of heating, where a significant inhibition on Fluorescence Intensity was observed. The same pattern of inhibition is noticed by the addition of hydroxytyrosol to S1.

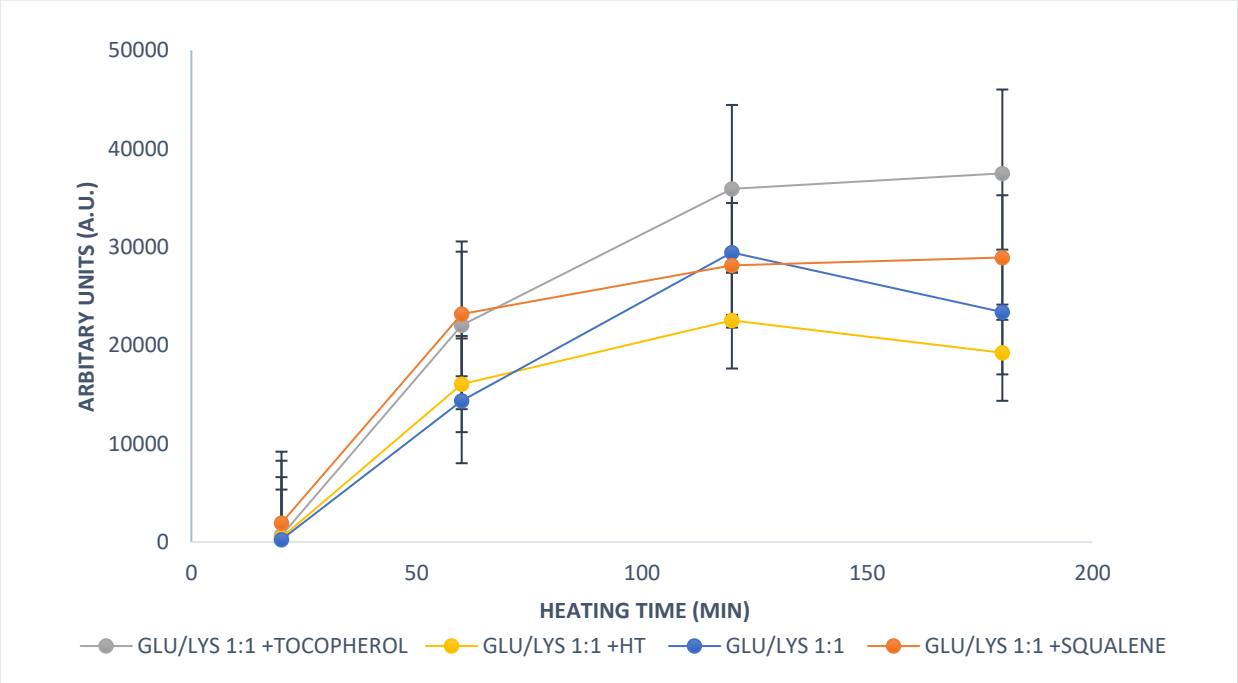


Figure 2: Diagram of Fluorescence Intensity expressed as Arbitary Units, of S2 (1:1) heated at 80°C and the impact of inhibitory agents, described by different colours of lines

Figure 2 shows mean values of Fluorescence Intensity expressed in Arbitary Units, accompanied by standard deviations, for S2 and the impact of inhibitory agents (squalene, tocopherol and hydroxytyrosol). Heating of the S2 (Glucose-Lysine in molar ratio 1:1) at 80°C results in higher Fluorescence Intensity levels with respect to the 10:1 molar ratio. Again, squalene produces higher Fluorescence signal with significant difference to the other two inhibitors tested. The addition of α-tocopherol resulted in significantly lower Fluorescence signal only after two hours of heating. The same pattern of inhibition is noticed by the addition of hydroxytyrosol into S2.

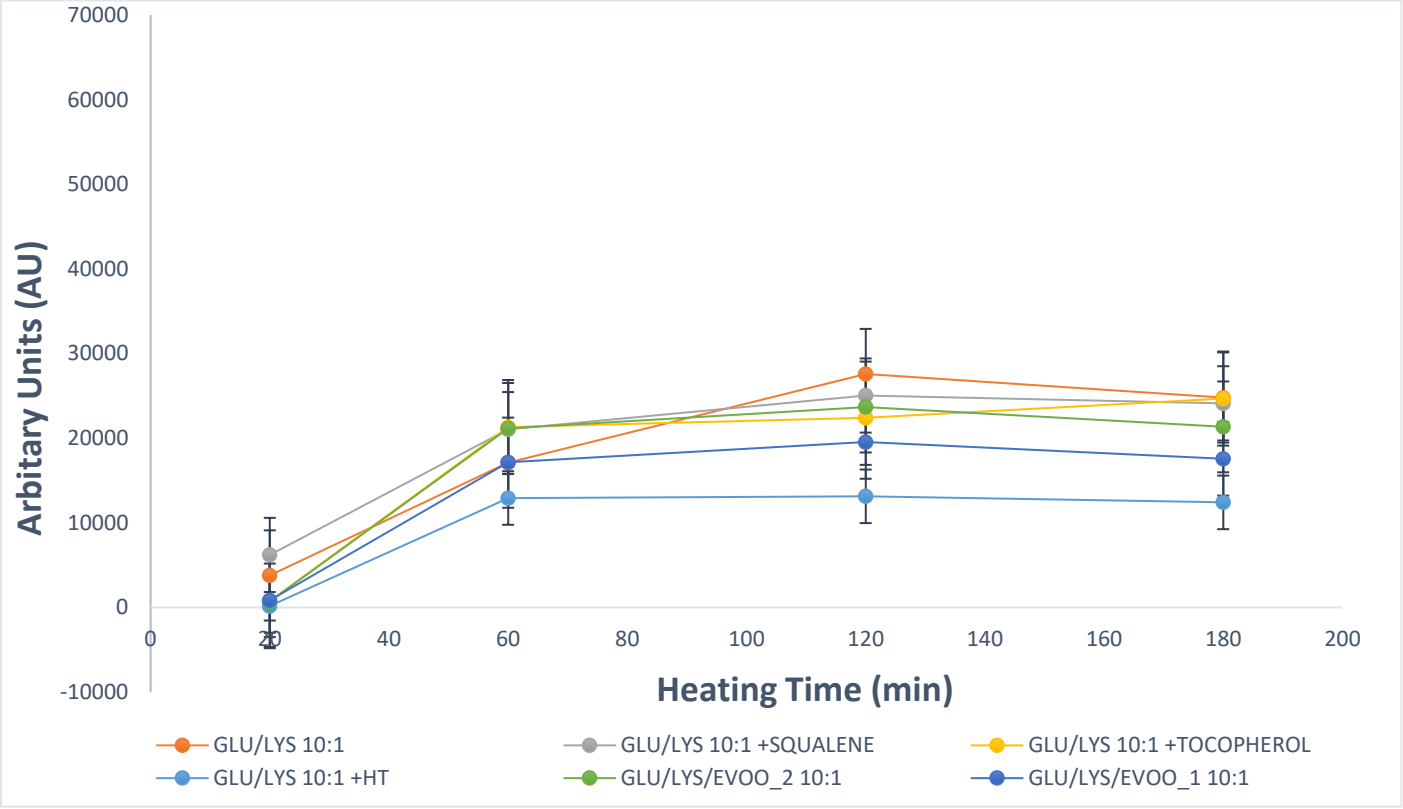


Figure 3: Diagram of Fluorescence Intensity expressed as Arbitary Units, of S1 (10:1) heated at 100°C and the impact of inhibitory agents, described by different colours of lines

Figure 3 shows the trend of Fluorescence Intensity values for the Glucose-Lysine system in a molar ratio 10:1 (S1) and the impact of bioactive compounds and intact extra virgin olive oil, during heating at 100°C. The Fluorescence signals of S1 in 100℃ became more intense in this temperature, with statistical difference, compared to 80°C ($p<0,05$). The addition of squalene resulted in significantly higher signals during the first hour of heating whereas significant inhibition seems to be accomplished after 2hrs of heating. The same inhibitory behaviour was observed in the case of α-tocopherol. The addition of both inhibitory factors, squalene and α- tocopherol, resulted in non-significant differences. HT showed the most effective inhibitory effect to the formation of AGEs in S1 during the heating process, resulting to significantly lower Fluorescence.

Comparing the two samples of extra virgin olive oil, EVOO_1 is the one with significant inhibitory effect to S1 ($p<0,05$)

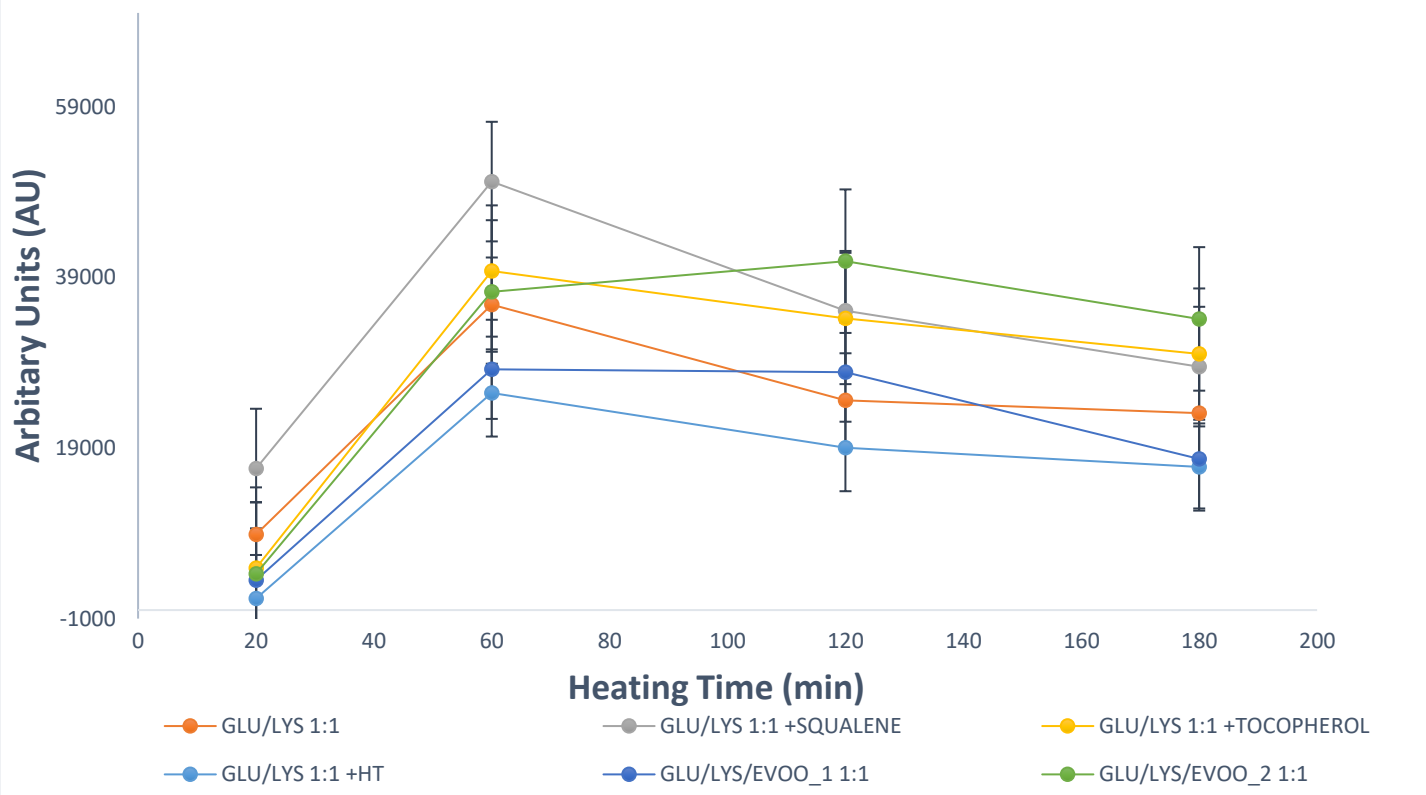


Figure 4: Diagram of Fluorescence Intensity expressed as Arbitary Units, of S2 (1:1) heated at 100°C and the impact of inhibitory agents, described by different colours of lines

Figure 4 shows the Fluorescence Intensity, expressed in Arbitary Units during heating of Glucose-Lysine in 1:1 molar ratio at 100°C. Squalene and α-tocopherol showed significantly higher Fluorescence Intensity when added to the system. The addition of HT significantly lowers the Fluorescence signal of the System confirming its inhibitory effect on AGEs formation. The olive oil sample with high total phenolic content (EVOO_1) showed inhibitory effect on the formation of Fluorescent components, except for the case of the 120min heating time, whereas the EVOO_2 enhances the formation of fluorescent compounds.

4.2 Effects of inhibitory agents monitored by Colour Development

The evolution of ΔE* with treatment time at different temperatures are shown below.

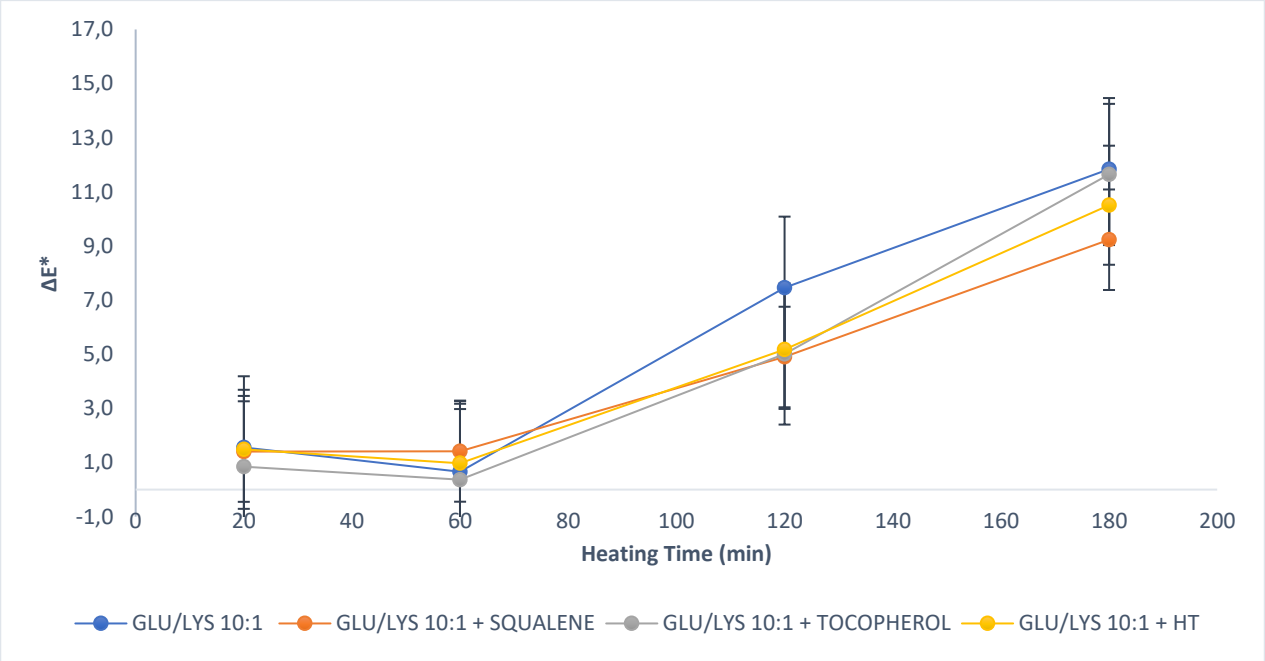


Figure 5: Diagram of Colour Development expressed as ΔE, of S1 (10:1) heated at 80°C. The impact of bioactive compounds is shown by different colours of points

Figure 5 monitors the colour development (ΔE) during heating at 80°C of the model system Glucose-Lysine in 10:1 molar ratio. The addition of squalene into the system resulted in significant lower colour development ($p<0,05$) at all time intervals tested. The inhibitory effects of α-tocopherol are mainly observed at 20min and 120min whereas at the end of the thermal processing no significant difference was present between the ΔE values of the S1 and the one with the addition of α-tocopherol. On the other hand, the inhibitory effect of hydroxytyrosol is mainly observed at the later time intervals of the heating process (120 and 180min), resulting in significantly lower ΔE values (5.15±0,18 and 10.5 ±0,15 respectively) with respect to the ΔE values of the System 1 (7.45±0,11 and 11.82±0,03, respectively).

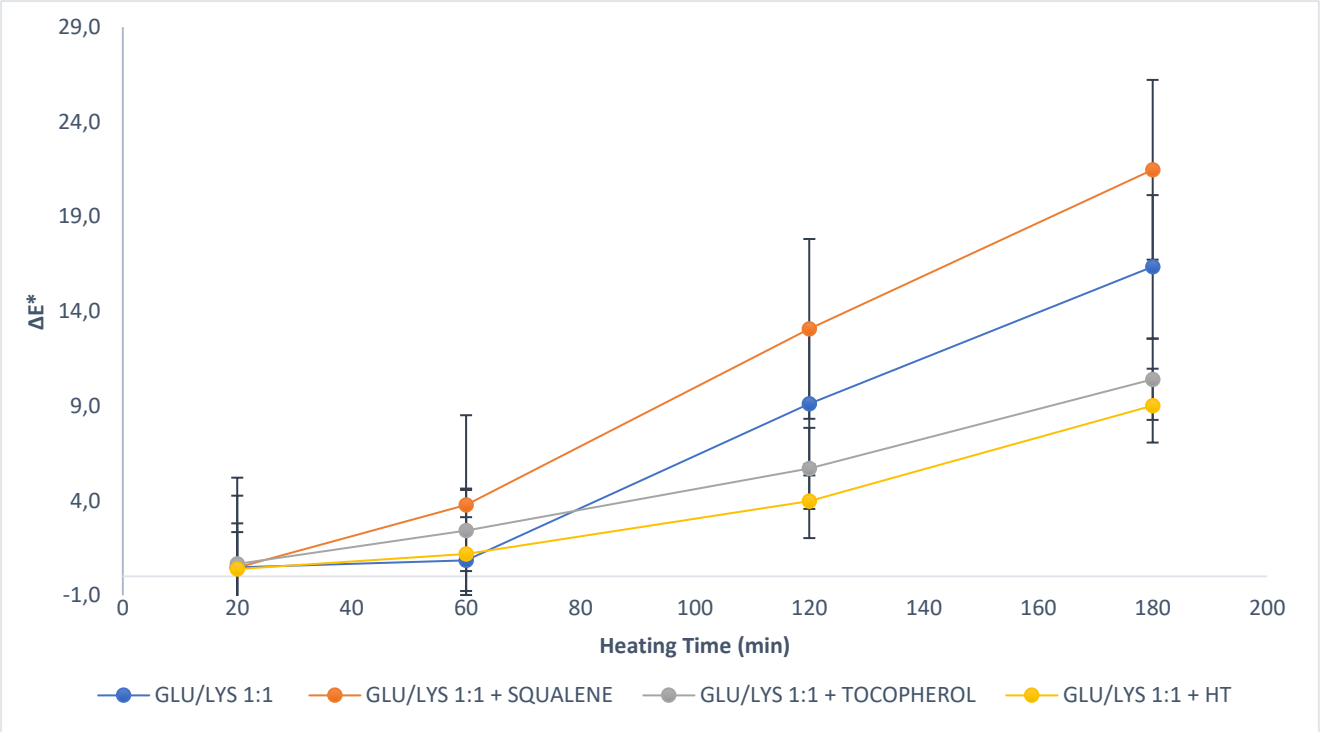


Figure 6: Diagram of Colour Development expressed as ΔE, of S2 (1:1) heated at 80°C. The impact of bioactive compounds is shown by different colours of lines

Again, in Figure 6, the trend of the Colour Development of the Glucose-Lysine model system in 1:1 molar ratio at 80℃ are presented. In the case of 1:1 molar ratio, the effect of squalene, led to statistically higher ΔE* values. The addition of α-tocopherol into the system showed inhibitory effect on colour development with statistically different results only at 120 and 180 mins of heating procedure. HT had the highest inhibitory effect on colour development compared to the initial model system, with statistical differences only at 120 and 180 min.

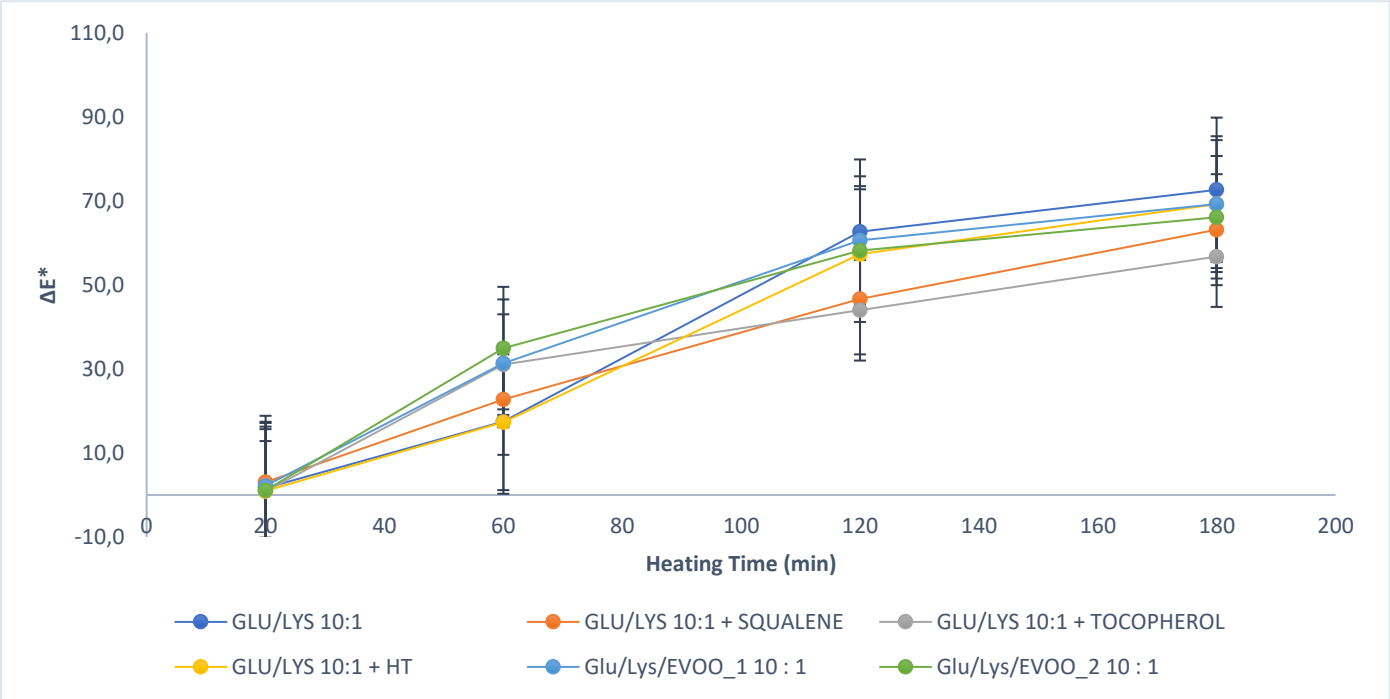


Figure 7: Diagram of Colour Development expressed as ΔE, of S1 (10:1) heated at 100°C. The impact of bioactive compounds is shown by different colours of lines

The colour development of the Glucose-Lysine model system in a molar ratio 10:1 is monitored on Figure 7 and the impact of bioactive compounds is also examined. Inhibitory effect of squalene is obvious only after 120 and 180 min of the heating process ($p<0,05$), which is also observed in the case of α-tocopherol. HT did not show significant differences in colour development. Virgin Olive Oil samples showed no significant inhibitory effect on colour development as well.

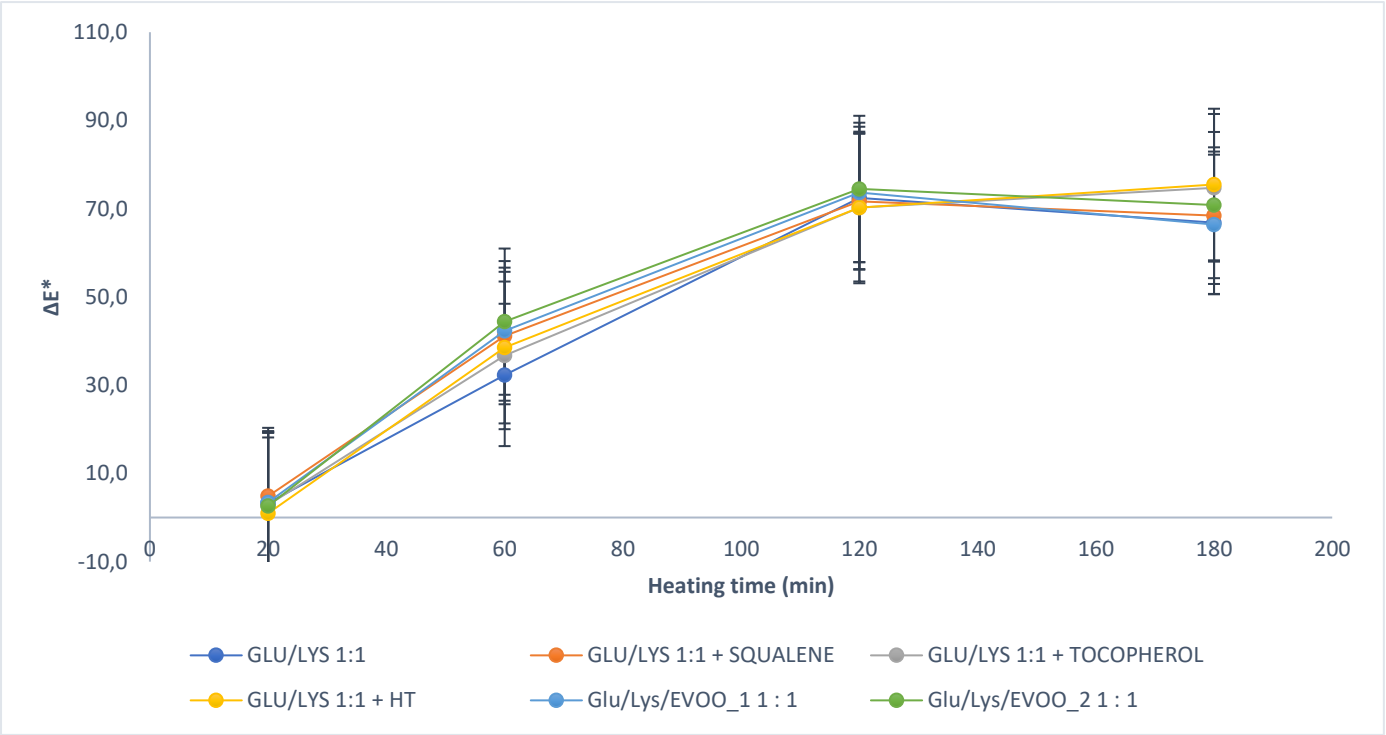
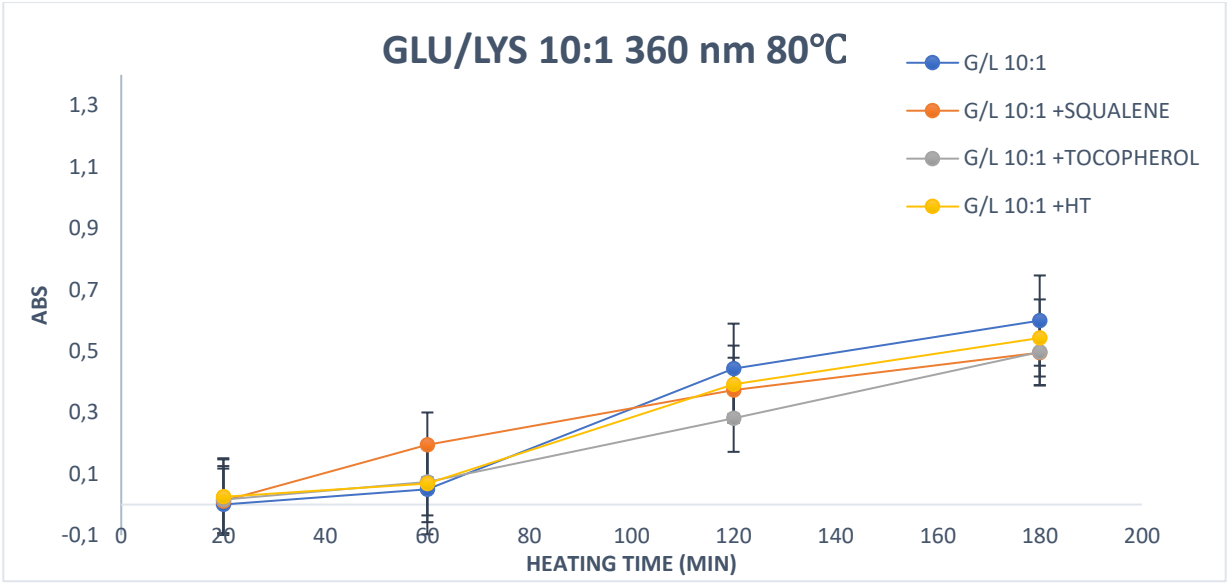


Figure 8: Diagram of Colour Development expressed as ΔE, of S2 (1:1) heated at 100°C. The impact of bioactive compounds is shown by different colourss of lines

Finally, on Figure 8 the results of Colour Development of the Glucose-Lysine system in 1:1 molar ratio is presented and the impact of bioactive compounds is examined. As it can be seen from the presented results, squalene enhances colour formation during heating process, with higher ΔE values. The addition of α-tocopherol into the system had an enhancing or non-significant difference on colour formation, which is also observed in the case of the HT. Virgin Olive Oil samples, did not display any inhibition activity on colour development.

4.3 Effects of inhibitory agents monitored by UV-Vis Spectrophotometry

The measurement of absorbance at 360 and 420 nm, represents the formation of advanced products and coloured polymers respectively (Delgado-Andrade, et al., 2010). Statistical differences were calculated using One-Way ANOVA. For the measurements of heated samples at 80°C, no dilutions were needed. On the other hand, samples heated at 100°C, needed dilution for the absorbance measurement, and the dilution factor was taken into consideration in the final absorbance calculation.



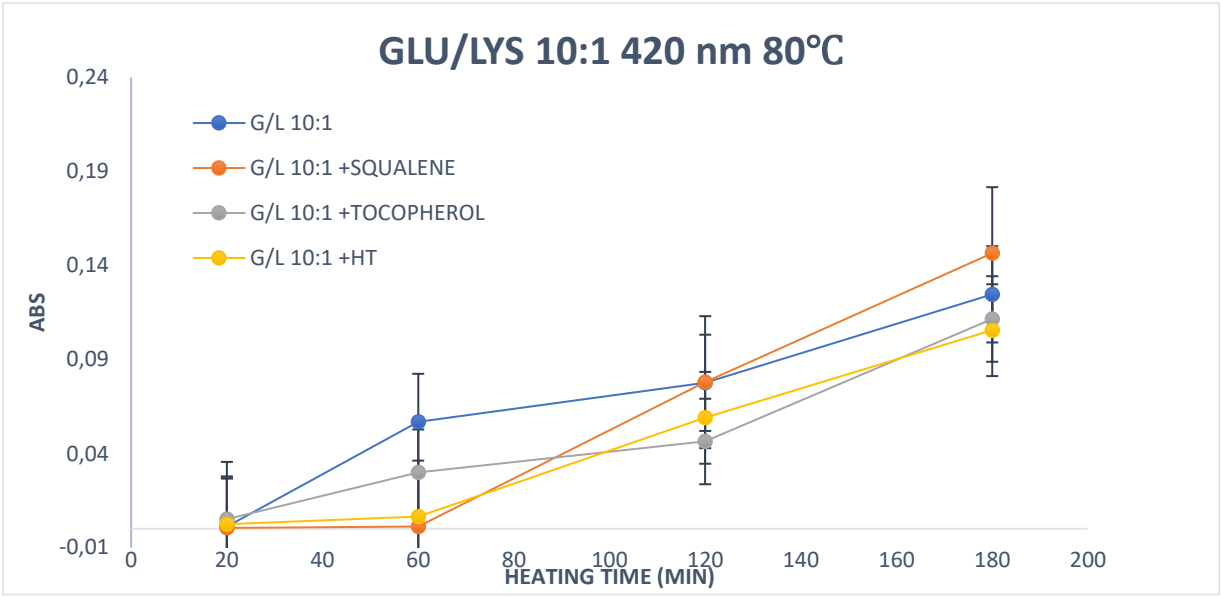


Figure 9: Absorbance levels of S1 (10:1) heated at 80°C, measured at 360 & 420 nm. The impact of bioactive compounds is shown by different colours of lines.

Figure 9, shows the absorbance trend at 360 and 420 nm of Glucose-Lysine systems of 10:1 molar ratio heated at 80℃, without or with bioactive compounds incorporated into the glycation model systems. For the initial time points of S1 and S2, very low Absorbance values were observed at 360 nm. When Squalene is added to the S1, higher absorbance levels are observed at 360 nm at the first heating time intervals, but furtherly during heating, significantly lower absorbance levels are present compared to the initial model system. On the other hand, squalene addition in S2, results to higher absorbance values with statistical significance ($p<0,05$). A maximum absorbance value of S1 containing squalene is reached with no significant changes until the end of the heating period ($p<0,05$). S2 containing squalene, shows an increase in absorbance levels

reaching the maximum in 180 minutes of heating, with statistically higher value ($p<0,05$).

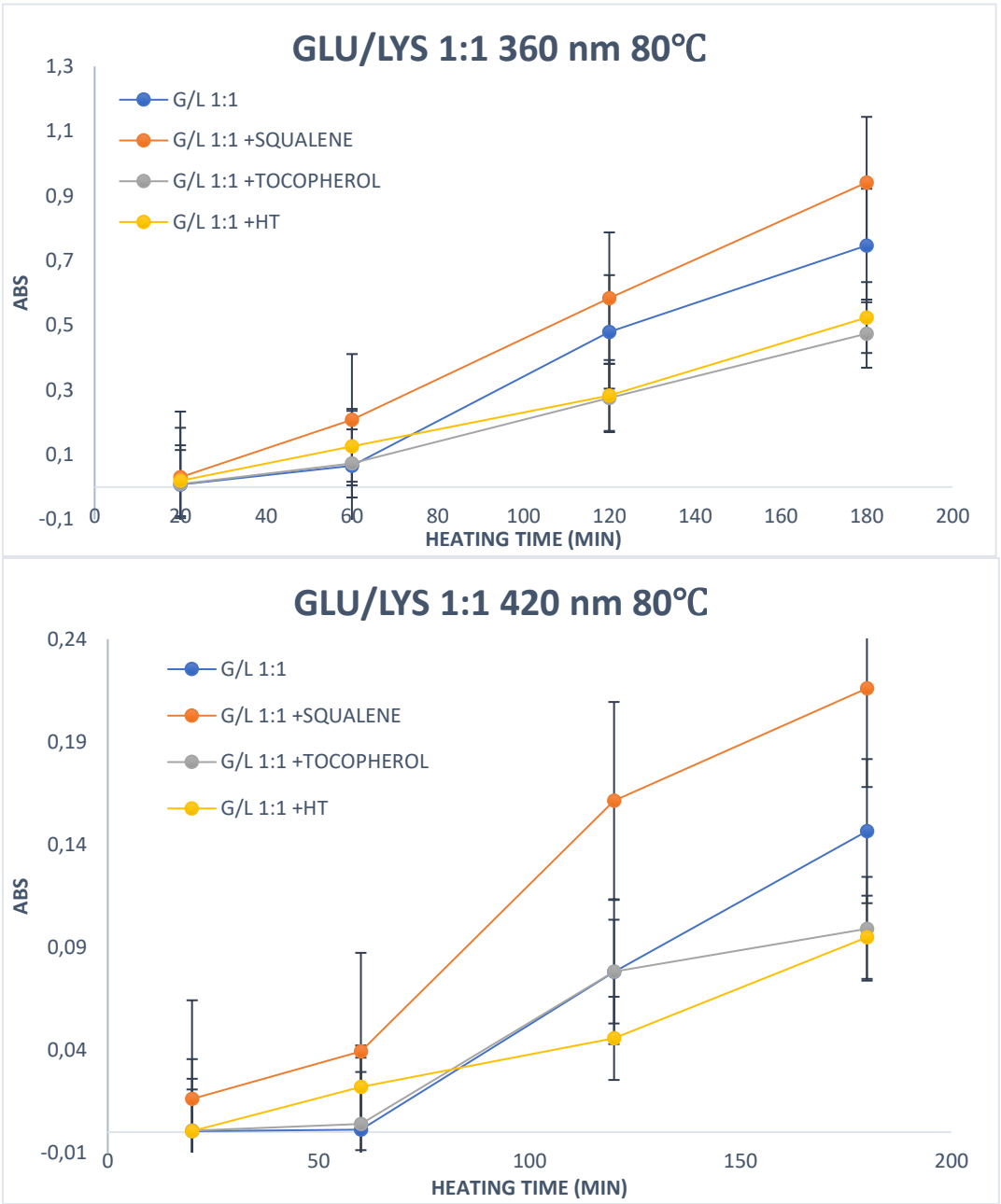


Figure 10: Absorbance levels of S2 (1:1) heated at 80°Cmeasured at 360 & 420 nm. The impact of bioactive compounds is shown by different colours of lines

The absorbance values of both S1 and S2 containing α-tocopherol at 360 nm in first time points, were very low, but during heat processing an increase in absorbance values is observed, but statistically lower overall, compared to initial systems S1 and S2. From this, it could be said that α-tocopherol, can show potential inhibition on intermediate products formation in the temperature of 80℃. Regarding the absorbance values of S1

and S2 with α-tocopherol, only in the last time intervals a worthy absorbance value is observed, but only in S2 with statistical difference. Generally, the formation of coloured polymers is low according to absorbance at 420 nm.

Finally, the addition of HT into S1 and S2, shows statistically lower absorbance values only after 120 and 180 min of heating at 360 nm, that can be translated as an inhibition of advanced products formation. Both glycation systems containing HT, showed very low absorbance values at 420 nm. As a general observation, in 420 nm the absorbance values are low in 80°C, showing low formation of browning rate in this temperature. The greatest inhibitory activity in 420 nm, is shown by HT after the first hour of heating.

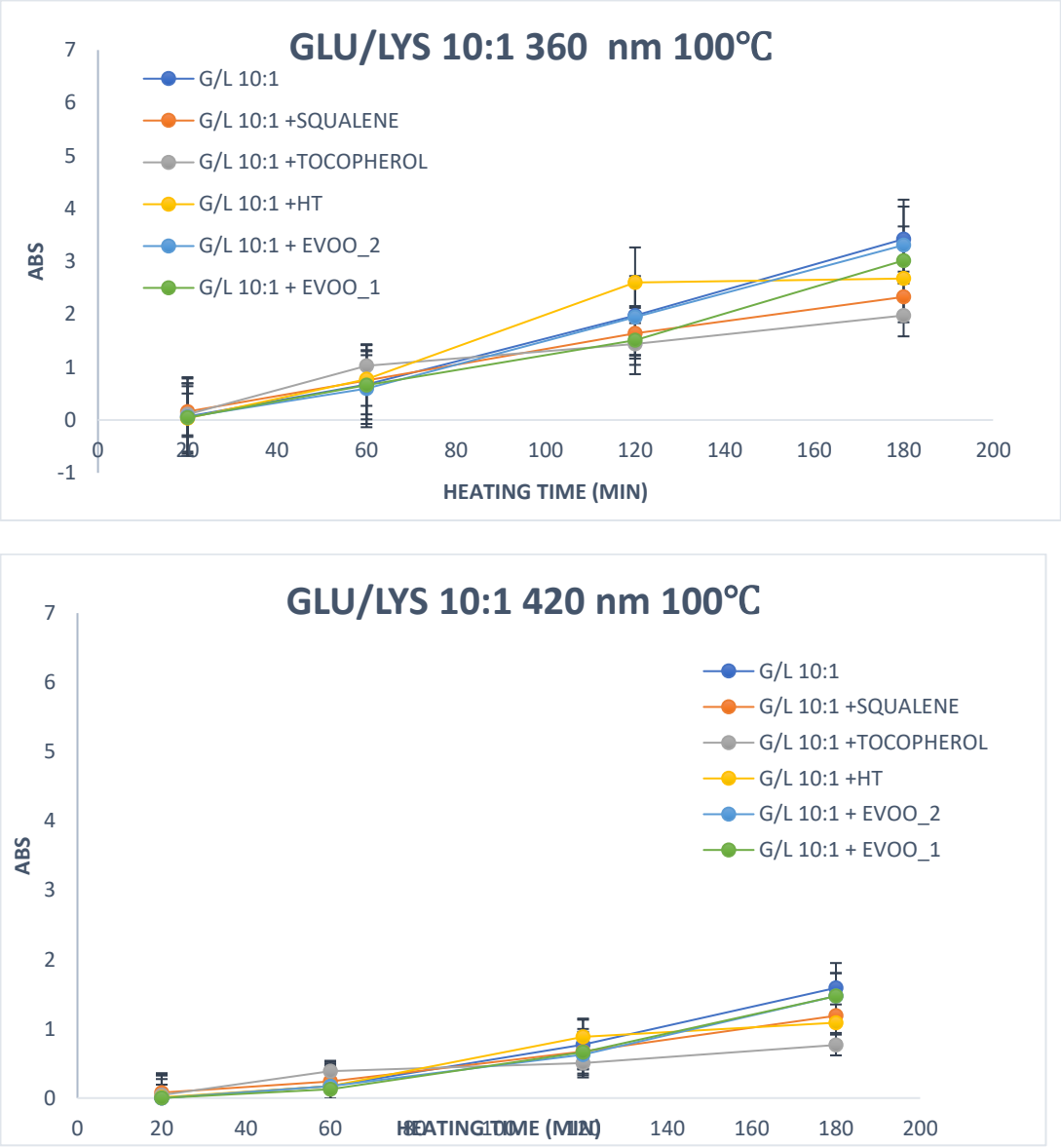
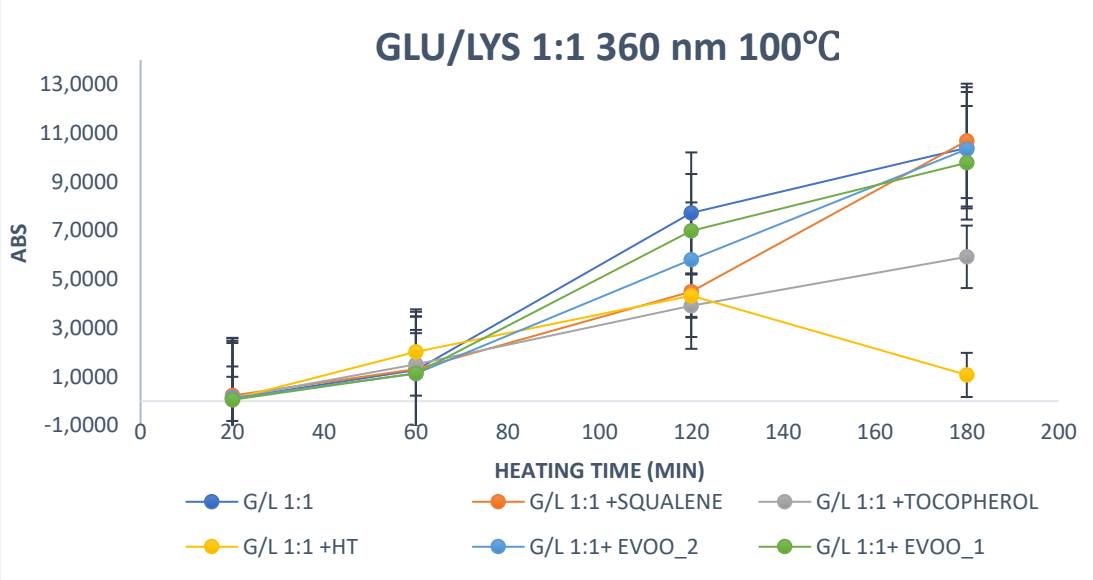


Figure 11: Absorbance levels of S1 (10:1) heated at 100°Cmeasured at 360 & 420 nm. The impact of bioactive compounds is shown by different colours of lines.

Figure 11, the results of the absorbance measurements of S1 are shown, with or without inhibitory. Higher temperature leads to higher absorbance values as it can be concluded and there is a noticeable increase of absorbance during heat processing until the end of the reaction.

Comparing the effect of inhibitory factors to the examined system, squalene, at 360 and 420, gives statistically higher absorbance levels at time intervals of 20 and 60 minutes, but during further heating a decrease in absorbance value is observed. The same pattern seems to be observed by the addition of α-tocopherol into the S1. HT addition gives higher absorbance values during the first 60 minutes of heat processing both at 360 and 420 nm. After the first hour of heating, HT shows inhibitory effect. The interaction between S1 and EVOO_2, showed no significant differences from the initial system. On the contrary, the addition of EVOO_1, with high total phenolic content, regarding the absorbance values at 360 nm, gives statistically significant lower values ($p<0,05$), especially during the last time points. A similar pattern seems to be observed at 420 nm, apart from the last time point, where the difference is not statistically significant, compared to S1.



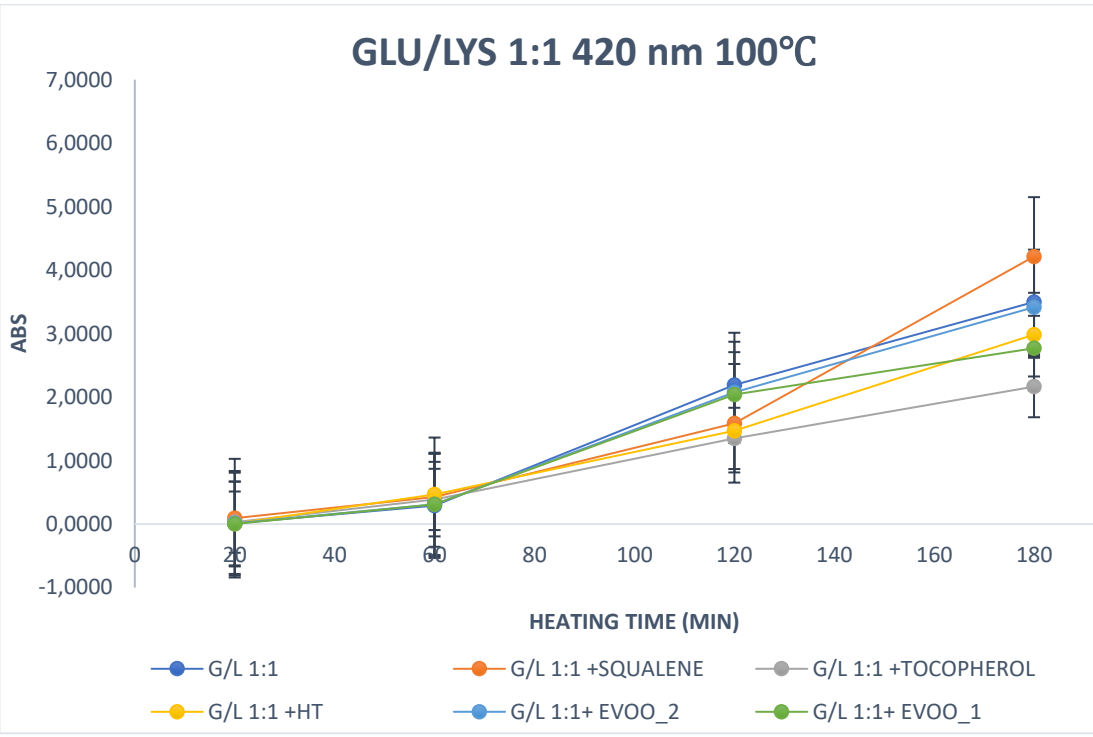


Figure 12: Absorbance levels of S2 (1:1) heated at 100°Cmeasured at 360 & 420 nm. The impact of bioactive compounds is shown by different colours of lines

Figure 12 shows the absorbance values at 360 nm and 420 nm, expressed as means, for glycation S2 at 100°C. This system compared to S1, gives statistically higher absorbance values ($p<0,05$), in both 360 and 420 nm. This might be explained by the presence of higher amounts of free amino acid residues, leading to higher amounts of glycation products. As a general observation on squalene's impact to the system, not significant inhibitory activity is observed with an exception at 120 minutes of heating. For α-tocopherol, it can be seen that at the initial time intervals the absorbance levels are enhanced compared to the initial S2 for both 360 and 420 nm. However, during further heating the absorbance shows significant reduction in both 360 and 420 nm. Regarding HT, there seems to be a similar tendency with that of α-tocopherol, as the inhibition impact is observed during two and three hours of heating. The addition of olive oil did not show significant inhibitory effect, with higher inhibitory activity for S2 showed by EVOO_1 ($p<0,05$).

DISCUSSION

Maillard reaction is definitely the most common reaction that takes place in processed foods. Between the various subsequent chemical reactions that take place in the three basic stages of protein glycosylation, one important category of produced compounds is Advanced Glycation End Products. These compounds which are derived from intermediate products of different stages, are connected to many chronic diseases such as, diabetes, neurodegenerative diseases, cardiovascular diseases, due to the oxidative stress which is enforced by dietary AGEs. During this study, the progress and the inhibition of this reaction by antioxidant compounds was examined. Many parameters play an important role to the reaction's evolution such as, temperature, concentration of the reactants, pH and water activity. General observations for each factor examined on this study, are referred below.

Heating temperature: As it can be concluded by the results of this study, temperature has a great impact on the Maillard reaction evolution. This can be easily observed by colour development, during heating treatment of model food systems. Higher production of colour polymers is displayed under intense heating in higher temperatures. Higher heating temperature accelerates the reaction's rate, which could be verified by studying kinetic model more specifically. (Nie, et al., 2013) in their study on glucose-glycine model systems, measuring furan production of heated samples, observed no significant differences between 80 and 100° C. Higher production was observed in higher temperatures. (Ajandouz, et al., 2008) on the other hand, observed faster browning and higher absorbance values in UV-Vis measurements at 420 nm, in the temperature of 100° C than in 80°C.

Molar ratio: The concentration between the reactants plays an important role to reaction's rate. Under the same heating temperature, a difference between the results is observed regarding the two molar ratios that were tested. An high molar content

between reducing sugars and amino acid content (10:1), can lead to lower AGEs formation, compared to an equal molar content of reducing sugars to amino acids (1:1) where the sugar molecules can react with equal number of free amino acids, ending to higher observed results. (Brands & van Boekel, 2001) after examining the model system of glucose-casein system in molar ratio of 10:1 in a heating temperature of 120°C and a 40-minute heating period, showed higher absorbance levels. In addition to this observation, (Liang, et al., 2016) mentioned a higher production of 3-DG in higher molar ratios of glucose and on the other hand, in 1:1 molar ratio observed a decreased concentration of 3-DG formation as higher amounts of Maillard reaction products are formed. As a conclusion, higher concentrations of glucose lead to higher amounts of 3-DG and in 1:1 molar ratio and above (higher amount of amino acid) higher amounts of advanced products are formed.

Bioactive compounds: The inhibitory activity of bioactive compounds of extra virgin olive oil was also examined. Observations on the impact of each compound on the examined system were made.

Different bioactive compounds are already examined for their antiglycative properties, such as herbal extracts (Ho, et al., 2013)and phenolic components (Khan, et al., 2019). The most common bioactive components of virgin olive oil were tested for their inhibitory effect on glycation model systems. Extra virgin olive oil was also examined.

Squalene: The addition of squalene into the glycation model systems S1 and S2 seems to have no significant inhibition activity on the formation of fluorescent products of Maillard reaction. On the contrary, enhanced fluorescent results were obtained. It was noticed that when squalene is heated in the presence of lysine additional Fluorescence Signal was produced. Based on that, it was concluded that squalene behavior on AGEs formation couldn't be assessed by Fluorescence Spectrometry. From the absorbance results, especially at 360 nm which is highly connected to AGEs formation, squalene seemed to be an effective inhibitor of AGEs formation after 120 and 180 mins of heating at both temperatures and at both molar ratios. When color formation was investigated in S1 there is an inhibitory effect with lower colour difference between the initial system and the squalene treated one. S2 with the presence of squalene seems to have an enhanced colour formation meaning higher levels of coloured polymers. After what is known about the pro-oxidant activity of squalene in temperatures of 60°C (Naziri, et al., 2014) and above and the formation of fluorescent epoxides (Badalassi, et al., 2004), the inhibitory capacity of squalene on non-enzymatic glycation is controversial and further investigation is required.

α-tocopherol: The addition of this bioactive compound into the examined model systems, significantly inhibits the formation of fluorescent components in both heating temperatures, something that is also observed by the absorbance values at 360 nm (120 and 180 mins), leading to the hypothesis that α-tocopherol acts protectively against glycating procedures during heating. Considering the colour development, the same inhibitory activity is observed with an exception at 100℃, where S2 containing

tocopherol did not show significant reduction of colour development, which is also obvious by absorbance values at 420 nm. As a conclusion, α-tocopherol shows inhibitory activity in AGEs formation, but further study is needed.

Hydroxytyrosol: As far as HT is concerned, it seems to have the most effective inhibition capacity which is apparent in both examined temperatures and molar ratios, apart from the case of colour development where the addition of HT into S2 did not show significant differences. (Navarro & Morales, 2017) examining the formation of dietary AGEs/ALEs in a biscuit model system, observed that treatment with 0,25-0,75% wt. of hydroxytyrosol, downregulated the formation of free fluorescent AGEs and pentosidine.

Virgin Olive Oil: As the bioactivity of virgin olive oil is already known, its effectiveness on inhibition of glycation end products formation was tested at 100°C, due to the fact that temperatures equal and higher of 100°C are typically used for food processing. The samples of Extra Virgin Olive Oil used in this study, differed in their total phenolic content. Describing the results of colour formation, olive oil did not show significant inhibition. However, comparing Fluorescence results, EVOO_1, with the higher phenolic content, shows higher inhibitory impact on S1 and S2, which is not the case for EVOO_2. The same pattern is observed when the results of UV-Vis spectrophotometry are assessed at 360 nm, where EVOO_1 shows higher inhibitory capacity. As a conclusion, virgin olive oil with high phenolic content could be effective at the inhibition of AGEs formation through heating treatment of foods, but further study is needed in higher temperatures and various compositions of bioactive components.

CONCLUSIONS

Non-enzymatic browning reaction, is one of the most common process during heating treatment for food preparation. Apart from the good taste and appearance that gives to final product, this reaction is responsible for the formation of compounds, known as Advanced Glycation End Products. These compounds are strictly connected to oxidative stress and chronic diseases. The purpose of this study apart from the study of the formation of these products through heating treatment of glycation model systems, was the study of inhibition effects by bioactive components.

Results from different temperatures showed statistically higher results in higher temperatures ($p<0,05$) which shows a temperature dependent reaction. The different molar ratio, also shows an impact, as an equal molar ratio between sugar and amino group can give higher results, due to stoichiometric ratio of the reaction. This means that every amino acid molecule can react with each sugar molecule, leading to dietary

AGEs as the heating process continues. On the other hand, in 10:1 molar ratio with excess sugar content, only one analogue of sugar can react to AGEs formation and the rest will continue to different Maillard products.

The antiglycative properties of separate bioactive compounds of virgin olive oil were obvious by Fluorescence and UV-Vis spectrophotometry at 360 nm, with Hydroxytyrosol showing the most effective properties. Squalene showed an additive Fluorescence signal which does not correspond to Fluorescent AGEs. This can lead to the conclusion that Fluorescence determination of AGEs needs a preliminary step of the study of the interaction between reactants and bioactive compounds which may lead to fluorescent products formation. Virgin Olive Oil samples with high phenolic content showed antiglycative properties during heating procedure, however, further study is needed in order to be conducted a final conclusion.

As a future work, further examination of the Maillard reaction evolution is needed in higher heating temperatures and a more inclusive investigation of antiglycative properties of virgin olive oil is recommended. Specifically, more selective methods, such as HPLC, for CML determination in heated samples, could give the kinetic model of the reaction and more information about the antiglycative impact of bioactive compounds into glycation environment

ANNEX

In this section of this study all of the diagrams that have been designed according to results from each method.

Fluorescence Spectrometry

Table 1: Average values of Fluorescence Intensity (A.U. ± SD) of model systems heated at 80℃. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line

Time (min)	*System 1 Glucose/Lysine (10:1)*	*S1+Squalene*	*S1+ α-tocopherol*	*S1+ HT*
20	89,0[a] (± 8,49)	1172,0[c] (±142,84)	497,0[b] (±247,98)	81,5[a] (±54,45)
60	2466,0[b] (± 190,92)	3377,0[c] (±263,04)	364,0[a] (±101,82)	239,5[a] (±177,48)
120	11451,0[c] (± 12,73)	8112,0[b] (±323,85)	6180,0[a] (±14,14)	5913,0[a] (±340,83)
180	15265,5[b] (±1082,58)	12567,5[a,b] (±1962,22)	12546,0[a,b] (±1411,39)	9495,5[a] (±1718,98)

Table 2: Average values of Fluorescence Intensity (A.U. ± SD) of model systems heated at 80℃. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line

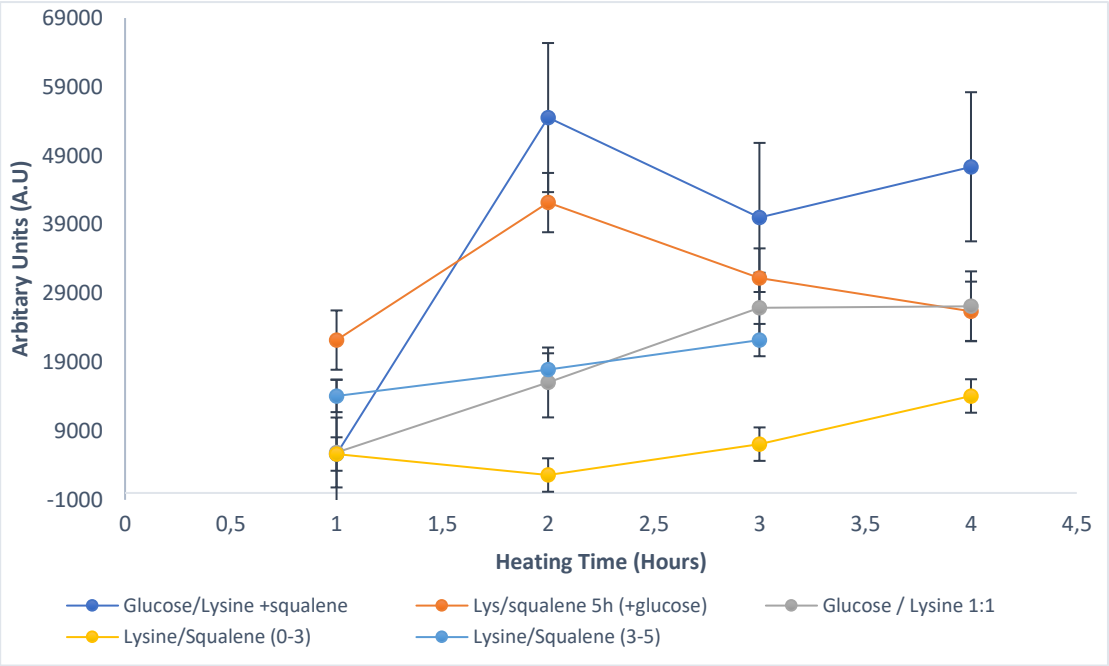
Time (min)	*S2 Glucose/Lysine (1:1)*	*S2+Squalene*	*S2+α-tocopherol*	*S2+HT*
20	551,5[a] (±41.72)	561,5[a] (±330,22)	528,5[a] (±94,05)	347,0[a] (±845,7)
60	2863,0[a] (±69,30)	9031,0[c] (±8,49)	2929,5[a] (±344,36)	3987,0[b] (±357,8)
120	16999,5[b] (±225,57)	21169,5[c] (±120,92)	10237,0[a] (±21,21)	8820,0[a] (±1496,2)
180	25541,5[a,b] (±2045,66)	28391,5[b] (±2161,63)	205711,5[a] (±2438,81)	21517,0[a] (±469,5)

Table 3: Average values of Fluorescence Intensity (A.U. ± SD) of model systems heated at 100°C. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line

Time (min)	*S1*	*S1+Squalene*	*S1+α-tocopherol*	*S1+HT*	*S1+EVOO_1*	*S1+EVOO_2*
20	3792,^{b} (±1652,51)	6213^{c} (±69,30)	750,0^{a} (±118,79)	188,5^{a} (±77,07)	848,5^{a} (±10,61)	712,0^{a} (±407,29)
60	17110,5^{b} (±348,60)	21073,0^{c} (±1549,98)	21323,0^{c} (±19,80)	12940,5^{a} (±55.86)	17163,0^{b} (±552,96)	21163,5^{c} (±1522,40)
120	27592,8^{e} (±1353,70)	25048,5^{d} (±174,66)	22412,5^{c} (±241,12)	13140,0^{a} (±121,62)	19556,0^{b} (±65,05)	23686,5^{c,d} (±553,66)
180	24813,5^{c} (±4541,75)	24125,0^{c} (±442,65)	24688,0^{c} (±15,56)	12421,0^{a} (±528,92)	17568,0^{b} (±340,83)	21510,0^{b,c} (±269,41)

Table 4: Average values of Fluorescence Intensity (A.U. ± SD) of model systems heated at 100°C. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line.

Time (min)	*S2*	*S2+ Squalene*	*S2+ α-tocopherol*	*S2+ HT*	*S2+ EVOO_1*	*S2+ EVOO_2*
20	8856,5^{c} (±1191,47)	16589,0^{d} (±1221,88)	4920,5^{b} (±37,48)	1351,0^{a} (±19,80)	3509,0 ^{a,b} (±989,95)	4248,0^{b} (±972,98)
60	35803,0^{b} (±323,85)	50227,5^{d} (±413,66)	39747,0^{c} (±373,35)	25449,5^{a} (±238,29)	28220,5^{a} (.±1595,94)	37290,0^{b,c} (±2374,46)
120	24577,0^{b} (±25,46)	35086,5^{d} (±185,97)	34179,0^{d} (±359,21)	19041,0^{a} (±873,98)	27890,0^{c} (±26,87)	41654,5^{e} (±1491,29)
180	23086,5^{b} (±113,84)	28540,5^{c} (±238,29)	30007,5^{d} (±20,51)	16770,0^{a} (±704,28)	17719,5^{a} (±137,89)	33189,5^{e} (±1958,69)



Colour Development

Table 5: Mean values of Colour Differences (ΔE ± SD) model S1 heated at 80 °C. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line

Time (min)	*S1*	*S1+ Squalene*	*S1+ α-tocopherol*	*S1+ HT*
20	$1,55^{c}$ (±0,06)	$1,4^{b}$ (±0,02)	$0,85^{a}$ (±0,09)	$1,5^{b,c}$ (±0,01)
60	$0,65^{a, b}$ (±0,02)	$1,4^{c}$ (±0,03)	$0,35^{a}$ (±0,17)	$0,95^{b}$ (±0,10)
120	$7,45^{b}$ (±0,11)	$4,9^{a}$ (±0,04)	$5,0^{a}$ (±0,09)	$5,15^{a}$ (±0,18)
180	$11,82^{c}$ (±0,03)	$9,25^{a}$ (±0,08)	$11,6^{c}$ (±0,14)	$10,5^{b}$ (±0,15)

Table 6: Mean values of Colour Differences (ΔE ± SD) of S2 heated at 80 °C. Different letters a, b, c indicate statistically significant difference (p≤ 0,05) at the same line.

Time (min)	*S2*	*S2+ Squalene*	*S2+ α-tocopherol*	*S2+ HT*
20	$0,5^{a,b}$ (±0,03)	$0,45^{a}$ (±0,12)	$0,65^{b}$ (±0,12)	$0,35^{a}$ (±0,08)
60	$0,85^{a}$ (±0,03)	$3,75^{c}$ (±0,13)	$2,45^{b}$ (±0,19)	$1,15^{a}$ (±0,06)
120	$9,15^{c}$ (±0,09)	$13,05^{d}$ (±0,15)	$5,7^{b}$ (±0,08)	$3,95^{a}$ (±0,06)
180	$16,35^{c}$ (±0,05)	$21,45^{d}$ (±0,30)	$10,4^{b}$ (±0,28)	$9,05^{a}$ (±0,05)

Table 7: Mean values of Colour Differences (ΔE ± SD) of S2 heated at 100 °C. Different letters a, b, c indicate statistically significant difference (p≤ 0,05) at the same line.

Time (min)	*S2*	*S2+ Squalene*	*S2+ α-tocopherol*	*S2+ HT*	*S2+ EVOO_1*	*S2+ EVOO_2*
20	$3,3^{c}$ (±0,08)	$4,8^{d}$ (±0,15)	$2,85^{b}$ (±0,02)	$0,95^{a}$ (±0,06)	$3,45^{c}$ (±0,26)	$2,6^{b}$ (±0,01)
60	$32,35^{a}$ (±0,09)	$41,15^{d}$ (±0,15)	$36,75^{b}$ (±0,74)	$38,5^{c}$ (±0,14)	$42,3^{d}$ (±0,77)	$44,4^{e}$ (±0,87)
120	$72,45^{c}$ (±0,23)	$71,65^{b,c}$ (±0,08)	$70,25^{a}$ (±0,12)	$70,25^{a}$ (±0,05)	$73,65^{d}$ (±0,80)	$70,85^{a,b}$ (±0,53)
180	$66,8^{a}$ (±0,02)	$68,4^{b}$ (±0,01)	$74,75^{d}$ (±0,08)	$75,5^{e}$ (±0,12)	$66,4^{a}$ (±0,40)	$70,85^{c}$ (±0,22)

Table 8: Mean values of Colour Differences (ΔE ± SD) of S1 heated at 100 °C. Different letters a, b, c indicate statistically significant difference (p≤ 0,05) at the same line.

Time (min)	*S1*	*S1+ Squalene*	*S1+ α-tocopherol*	*S1+ HT*	*S1+ EVOO_1*	*S1+ EVOO_2*
20	$1,65^{b}$ (±0,17)	$3,1^{d}$ (±0,15)	$0,9^{a}$ (±0,01)	$1,0^{a}$ (±0,05)	$1,2^{a}$ (±0,06)	$1,1^{a}$ (±0,09)
60	$17,5^{a}$ (±0,01)	$22,75^{b}$ (±0,08)	$31,05^{c}$ (±0,05)	$17,35^{a}$ (±0,09)	$31,3^{c,d}$ (±3,14)	$34,95^{d}$ (±2,30)
120	$62,7^{b}$ (±0,13)	$46,65^{a}$ (±0,05)	$43,95^{a}$ (±0,10)	$57,35^{b}$ (±0,04)	$60,65^{b}$ (±5,74)	$58,2^{b}$ (±0,82)
180	$72,6^{d}$ (±0,03)	$63,15^{b}$ (±0,09)	$56,75^{a}$ (±0,18)	$69,2^{c,d}$ (±0,12)	$69,2^{c,d}$ (±1,85)	$66,1^{b,c}$ (±3,73)

UV-Vis Spectrophotometry

Table 9: Mean values of Absorbance ABS (± SD) at 360 and 420 nm of model systems heated at 80 °C. Different letters a, b, c indicate statistically significant difference ($p \leq 0,05$) at the same line.

Time (min)	*nm*	*S1*	*S1 + Squalene*	*S1 + α-tocopherol*	*S1 + HT*	*S2*	*S2 + Squalene*	*S2 + α-tocopherol*	*S2 + HT*
20	360	0,0001^{a} (±0,0001)	0,0116^{b} (±0,0014)	0,0159^{c} (±0,0006)	0,0252^{d} (±0,0015)	0,0765^{a} (±0,0005)	0,3030^{d} (±0,1933)	0,0095^{b} (±0,0002)	0,0194^{c} (±0,0003)
60		0,0500^{a} (±0,0003)	0,1944^{d} (±0,0016)	0,0738^{c} (±0,0005)	0,0682^{b} (0,0002±)	0,0665^{a} (±0,0040)	0,2081^{d} (±0,0023)	0,0729^{b} (±0,0004)	0,1259^{c} (±0,0007)
120		0,4424^{c} (±0,0205)	0,3728^{b} (±0,0010)	0,281^{a} (±0,0065)	0,3922^{b} (±0,0005)	0,4795^{b} (±0,0023)	0,5842^{c} (±0,0069)	0,2754^{a} (±0,0003)	0,2831^{a} (±0,0116)
180		0,5994^{c} (±0,0016)	0,4949^{a} (±0,0010)	0,4965^{a} (±0,0009)	0,5472^{b} (±0,0083)	0,7466^{c} (±0,0026)	0,9414^{d} (±0,0211)	0,4741^{a} (±0,0069)	0,5239^{b} (±0,0008)
20	420	0,011^{a} (±0,0001)	0,0042^{a,b} (±0,0023)	0,0051^{b} (±0,0004)	0,0024^{a,b} (±0,0002)	0,0004^{a} (±0,0001)	0,0161^{b} (±0,0001)	0,0007^{a} (±0,0001)	0,0005^{a} (±0,0001)
60		0,0568^{c} (±0,0030)	0,1037^{d} (±0,0117)	0,0300^{b} (±0,0029)	0,0064^{a} (±0,0004)	0,0011^{a} (±0,0001)	0,0392^{d} (±0,0008)	0,0040^{b} (±0,0013)	0,0219^{c} (±0,0006)
120		0,0776^{c} (±0,0018)	0,1117^{d} (±0,0032)	0,0464^{a} (±0,0006)	0,0590^{b} (±0,0011)	0,0779^{a,b} (±0,0093)	0,1614^{b} (±0,0590)	0,0781^{a,b} (±0,0257)	0,0456^{a} (±0,0008)
180		0,1246^{a} (±0,0296)	0,1008^{a} (±0,0019)	0,1115^{a} (±0,0023)	0,1056^{a} (±0,0066)	0,1465^{b} (±0,0018)	0,2160^{c} (±0,0086)	0,0990^{a} (±0,0037)	0,0948^{a} (±0,0008)

Table 10: Mean values of Absorbance ABS (± SD) at 360 and 420 nm of S2 heated at 100°C. Different letters a, b, c indicate statistically significant difference (p≤ 0,05) at the same line.

Time (min)	*nm*	*S2*	*S2+ Squalene*	*S2+ α-tocopherol*	*S2+ HT*	*S2+ EVOO_2*	*S2+ EVOO_1*
20	360	0,1140^{b,c} (±0,0085)	0,2340^{e} (±0,0006)	0,1442^{d} (±0,0148)	0,0949^{b} (±0,0002)	0,1275^{c,d} (±0,0205)	0,0579^{a} (±0,0014)
60		1,2811^{b} (±0,0211)	1,3201^{b} (±0,0007)	1,5105^{c} (±0,0273)	2,0205^{d} (±0,0011)	1,1322^{a} (±0,0602)	1,1395^{a} (±0,0233)
120		7,7206^{c} (±0,0246)	4,4958^{a} (±0,0031)	3,9152^{a} (±0,0079)	4,3276^{a} (±0,0198)	5,8082^{b} (±1,0926)	6,9910^{c} (±0,0056)
180		10,394^{d} (±0,0833)	10,697^{d} (±0,1690)	5,923^{b} (±0,1075)	1,0774^{a} (±0,0184)	10,336^{d} (±0,1223)	9,780^{c} (±0,3083)
20	420	0,0119^{c} (±0,0005)	0,0928^{e} (±0,0002)	0,0301^{d} (±0,0005)	0,0080^{b} (±0,0008)	0,0116^{c} (±0,0001)	0,0015^{a} (±0,0005)
60		0,2908^{a} (±0,0088)	0,4282^{c} (±0,0017)	0,3877^{b,c} (±0,0035)	0,4647^{c} (±0,0058)	0,3091^{a,b} (±0,0878)	0,3110^{a,b} (±0,0090)
120		2,1894^{d} (±0,0846)	1,5874^{b} (±0,0093)	1,3494^{a} (±0,1100)	1,4722^{a,b} (±0,0025)	2,0748^{c,d} (±0,0345)	2,0390^{c} (±0,0405)
180		3,4990^{c} (±0,0919)	4,215^{d} (±0,0580)	2,1650^{a} (±0,0721)	2,984 ^{b} (±0,0764)	3,4165^{c} (±0,2157)	2,7700^{b} (±0,2968)

Table 11: Mean values of Absorbance ABS (± SD) in 360 and 420 nm of Glucose-Lysine-Bioactive compound model systems in 10:1 (S1) and 1:1 (S2) molar ratio, in 80℃. Different letters a, b and c indicate statistically significant differences between the lines

Time (min)	*nm*	*S1*	*S1+ Squalene*	*S1+ α-tocopherol*	*S1+ HT*	*S1+ EVOO_2*	*S1+ EVOO_1*
20	360	$0,0649^{d}$ (±0,0002)	$0,1595^{f}$ (±0,0049)	$0,1060^{e}$ (±0,0001)	$0,0311^{a}$ (±0,0004)	$0,0562^{c}$ (±0,0022)	$0,0457^{b}$ (±0,0002)
60		$0,6709^{b}$ (±0,0370)	$0,7475^{c}$ (±0,0445)	$1,0285^{d}$ (±0,0165)	$0,7698^{c}$ (±0,0011)	$0,5932^{a}$ (±0,0059)	$0,6571^{b}$ (±0,0083)
120		$1,9585^{b}$ (±0,0098)	$1,6420^{a}$ (±0,0113)	$1,4382^{a}$ (±0,0048)	$2,6020^{c}$ (±),0198	$1,948^{b}$ (±0,0028)	$1,5116^{a}$ (±0,2138)
180		$3,4235^{e}$ (±0,0540)	$2,3285^{b}$ (±0,3330)	$1,9775^{a}$ (±0,0332)	$2,676^{b,c}$ (±0,0184)	$3,3095^{d,e}$ (±0,0785)	$3,018^{c,d}$ (±0,0042)
20	420	$0,0004^{a}$ (±0,0003)	$0,0795^{d}$ (±0,0008)	$0,0438^{c}$ (±0,0018)	$0,0109^{b}$ (±0,0008)	$0,0003^{a}$ (±0,0001)	$0,0013^{a}$ (±0,0002)
60		$0,1716^{b}$ (±0,0100)	$0,2417^{c}$ (±0,0072)	$0,3887^{d}$ (±0,0052)	$0,1731^{b}$ (±0,0058)	$0,1731^{b}$ (±0,0027)	$0,1275^{a}$ (±0,0041)
120		$0,7719^{c}$ (±0,0090)	$0,672^{b}$ (±0,0028)	$0,504^{a}$ (±0,0170)	$0,8842^{d}$ (±0,0025)	$0,6268^{b}$ (±0,0011)	$0,6636^{b}$ (±0,0548)
180		$1,5900^{c}$ (±0,0010)	$1,188^{b}$ (±0,0806)	$0,766^{a}$ (±0,0693)	$1,088^{b}$ (±0,0764)	$1,4725^{c}$ (±0,0233)	$1,4715^{c}$ (±0,0092)

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