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THE POTENTIAL IMPACT OF MAILLARD REACTION PRODUCTS AGAINST LOW DENSITY LIPOPROTEIN OXIDATION *IN VITRO*

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El-Massry, K.F.[1]; R. Dittrich[2]; K. Kunz[2] and M. Pischetsreider[3]

ABSTRACT

Maillard reaction products (MRPs) are formed during processing by the reaction of reducing sugars with amino acids. Oxidation of low density lipoproteins (LDL) is a crucial step in the development of atherosclerotic lesions.The protective role of several components in plant foods, mostly polyphenolic compounds, against LDL-oxidation *in vitro* and *in vivo* is well established and increased consumption of plant food was therefore, related to a reduced risk of heart disease. During food processing, antioxidative activity of food is liable to changes, for example by the degradation of flavanoids. In this study, we have investigated the potential of MRPs to contribute to the overall antioxidative capacity of food to prevent LDL oxidation *in vitro*. Several amino acids were heated with glucose under different reaction conditions. The activity of the mixtures to prevent oxidation of human LDL was measured. Human LDL were incubated with different test compounds. LDL oxidation was initiated by the addition of copper ions and recorded by measuring UV absorbance at 230 nm. Antioxidative activity was recorded by the length of lag-time and compared to the antioxidative activity of L-ascorbic acid. These reaction mixtures of different amino acids with glucose showed, in the tested concentrations, antioxidative activity. Variation of the antioxidative activity depending on the amino acid moiety was observed. The control mixtures which contained only sugar or amino acid hardly showed any protective activity. Thus it can be concluded that during the Maillard reaction which takes place during heating or processing of certain food stuff, products are formed which can have a protective activity against LDL oxidation. The reaction mixtures are then analyzed by HPLC and five structurally defined Maillard products were tested for their antioxidative activity against LDL oxidation as described before. Some of the Maillard products, particularly those with aminoreductone structures showed similar activity as L-ascorbic acid, whereas other structures proved to be rather inactive. It can be concluded that some of the tested Mailllard products have beneficial effect on health. The concentration of the active Maillard compounds in different foods, their contribution to the total antioxidative activity of plant based foods and their availability *in vivo* are currently investigated.

1- Flavour and Aromatic Chemistry Department, National Research Center, Giza, Egypt.
2- Obstetrics and Gynecology Department, University of Erlangen, Germany.
3- Institute of Pharmacy and Food Chemistry, University of Erlangen, Germany.

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INTRODUCTION

Antioxidative compounds which are naturally occurring in many food stuffs are important for food technology, because they inhibit lipid oxidation and as a result, prolong shelf life of the product (**Henderson *et al* 1999**). Besides the antioxidative vitamins, phenolic compounds derived from spices, tea, fruits and berries or wine are shown to be very active (**Kondo *et al* 1999**). More recently, antioxidative food components also gained interest because it was suggested that their intake is beneficial for health. There is substantial evidence that antioxidative food components have a protective role against coronary heart diseases[CHD] by inhibition of low-density lipoprotein (LDL) oxidation *in vivo*. Oxidized LDL plays a major role during the development of atherosclerotic plaques and as a result of cardiovascular disease (**Steinberg *et al* 1989**). Furthermore, fruits with high content of antioxidative components show strong inhibition of tumour- cell proliferation (**Eberhardt *et al* 2000**). However, the inhibition of such diseases by isolated antioxidant(s) remains to be established by ongoing and future clinical trials.

The two most important series of reaction for the formation of volatile aroma compounds in cooked and thermally processed foods are the Maillard reaction (between amino compounds and reducing sugars) and lipid degradation (thermal and oxidative). This reaction affects the development of colour and flavourings and has become a significant focus of attention in the food industry. During the past 75 years, numerous studies have been published dealing with Maillard reactions such as chemistry, flavour development, nutrition, toxicology, antimutagenicity, and antioxidant properties (**Danely, 1986; Namiki, 1988; O'Brien and Morrissey, 1989; Bailey and Um, 1992; Eiserich *et al* 1995**).

During food processing and storage, natural antioxidants are considerably degraded. On the other hand, chemical reactions among food components lead to the formation of secondary antioxidants (**Nicoli *et al* 1997**). One of the major candidates of secondary food antioxidants seem to be Maillard reaction products (MRPs). The first report concerning the antioxidative effect of Maillard products was given by **Franzke and Iwainsky (1954)**. Since then, antioxidant activities of MRPs have been extensively studied using different test systems (**Monti *et al* 1999**). In most cases, crude Maillard mixtures or uncharacterized melanoidines were tested, whereas in few cases antioxidative activity was related to structurally defined Maillard products (**Pischetsrieder *et al* 1998 and Ando *et al* 2000**).

The protective role of MRPs against oxidative damage gained particular interest, because it is assumed that it considerably prolongs shelf life of processed food (**Bressa *et al* 1996**). Furthermore, Maillard products could be useful as natural antioxidants to be added to food susceptible to oxidative damage (**Smith and Alfawaz 1995**).

However, hardly anything is known about a potential benificial role of Maillard products *in vivo* and it can be assumed that similarly to natural products, secondary food components (MRPs) are resorbed and might protect for example LDL from oxidative damage. So, in the present study, the ability of Maillard products to inhibit copper induced LDL oxidation *in vitro* was investigated. First, Maillard mixtures were compared using different amino acid components and reaction conditions and finally some structurally defined Maillard products were tested.

MATERIAL AND METHODS

All chemicals were of Analar grade wherever possible and were obtained from BDH, Aldrich and Sigma Chemicals.

Production of Model Mixtures

The reaction mixtures consisted of D-glucose (1M) and amino acids (1M) (arginine, phenylalanine, methionine, alanine, aspartic acid, lysine and glycine), individually dissolved with glucose in 100 mL distilled water, and adjusted pH to 7.1 with 0.1 N sodium hydroxide or 0.1 N phosphoric acid. For the preparation of glutathione - reaction mixture, an equal molar ratios of D-glucose (1 M) and glutathione amino acid content (glutamic acid, cysteine and glycine, as an equal molar ratio) were dissolved in distilled water (500 mL) and also adjusted pH to 7.1. All the reaction mixtures were heated over a boiling water bath (100 ^{0}C) for 1h under reflux with circulating cooling system. After reaction, the mixtures were rapidly cooled to bring the temperature down and terminate the reaction. The reaction masses were stored in a refrigerator till assay.

Some structurally characterized compounds which were shown to be major Maillard products were used in the LDL assay. These compounds were previously isolated from Maillard mixtures or synthesized and their structures were elucidated by spectral analysis (**Pischetsrieder and Severin, 1997**).

Isolation or Synthesis of Different Maillard Products

1– Deoxy – 1 – propylammonium– D-fructose oxalate (C4- morpholino-AR) (**Micheel and Hagemann, 1959**), piperidino hexose reductone (**Weygand *et al* 1961**), galactosylisomaltol (**Hodge and Nelson, 1961**), acetylformin (**Shawn *et al* 1967**), 3-hydroxy-4-(alkylamino)-3-buten-2-one (Propylaminoreductone)(C4-AR) were prepared and analyzed by HPLC as described by **Pischetsrieder *et al* (1998)**.

LDL Assay

Blood samples were collected after an overnight fasting of ≥ 12 h from healthy male volunteers. Plasma was separated by low speed centrifugation at 600 x g for 10 min at 4°C. After separation of fresh plasma, LDL samples were isolated immediately using single – step ultracentrifugation. Plasma (2ml) was adjusted to a density of 1.21 g /ml with KBr and layered under 10 ml saline (density 1.006 g/ml) containing 0.01% EDTA in 12 ml quick – seal tubes (Beckman Instruments, Munich,

Germany).The tubes were centrifuged at 65000 rpm for 6 h at 4°C using a Ti75 fixed angle rotor using Beckman L5-75 ultracentrifuge (Beckman Instruments, Munich, Germany). The yellow LDL-band was removed through the side of the tube with a needle and syringe. Immediately prior to the oxidation incubations, LDL was separated from EDTA by gel filtration using Econo-Pac 10 DG columns (BioRad GmbH, Munich, Germany).

The purity of the LDL-solution was confirmed by means of a lipid-electrophoresis using system LIPIDOPHOR ALL IN (Immuno GmbH, Heidelberg, Germany). LDL concentration was determined by measuring total cholesterol using CHOD-PAP-method since more than 98% purity of LDL-solution after LDL preparation could be obtained (**Siedel *et al* 1983**). The LDL-oxidation was initiated by the addition of a freshly prepared aqueous $CuSO_4$ solution. The LDL-solution was diluted with oxygen-saturated phosphate buffer solution (PBS) pH 7.4. In all experiments, the final conditions were : Temperature 30°C, 0.08 g/L LDL-cholesterol and 5 µmol/L $CuSO_4$, final test volume 1 ml. The LDL-solution was supplemented with 0.5 µmol/L of the test substances (MRPs) added to the LDL-solution prior to the initiation of the oxidation of LDL by copper ions. LDL-oxidation was spectro-photometrically monitored in triplicates, by measurement of the change in absorbance at 234 nm in 1ml quartz cuvettes in a Perkin Elmer Lambada 2 ultraviolet / vis spectrophotometer (Perkin-Elmer, Ueberlingen, Germany) with absorbance readings made every 5 min at 37°C until there was no further increase in the formation of conjugated dienes. LDL susceptibility to oxidation was measured by continuous monitoring of conjugated diene formation and is characterized by lag-time, which seems to be an oxidation resistance marker.The measurement of the lag-phase of LDL-oxidation offers the possibility to study the antioxidative effects of test substances (**Esterbauer *et al* 1989**). Antioxidative activity was expressed by the prolongation of lag-time and compared to L- ascorbic acid and reduced glutathione (0.5 µM each). The prolongation of lag-phase caused by the test substances (all model mixtures and characterized Maillard compounds) in relation to the duration of the initial lag-phases nevertheless shows low interassay variability.

RESULTS AND DISCUSSION

In this study, LDL oxidation was induced by Cu^{2+} and recorded by measuring UV- absorbance of conjugated dienes which are formed during oxidation. The antioxidant activity is based on the inhibition of conjugated diene formation (LDL oxidation products). This system is widely used *in vitro* model to evaluate a possible protective activity of food components against cardiovascular diseases (**Vinson *et al* 1995**), because there is strong evidence that oxidized LDL is a major factor in the pathogenesis of atherosclerosis (**Lusis, 2000**). Also, it has been documented that Cu^{2+} induced oxidized LDL exhibits biological and immunological properties similar to those *in vivo* (**Schwenke, 1998**).

To investigate the potential of MRPs to prevent copper induced LDL oxidation *in vitro*, several amino acids were heated

with glucose under different reaction conditions and their activities to prevent oxidation of human LDL were measured compared with L-ascorbic acid and reduced glutathione (positive controls).

The obtained results revealed that all the reaction mixtures of different amino acids with glucose increased considerably the duration of the lag-phases of LDL oxidation (Figure 1). However, it is observed that the structure of the amino acid residue seems to have a major influence on the extent and the differences in the antioxidative activity depending on the amino acid moiety. It is found that lysine, aspartic acid or arginine mixtures show a significant prolongation of lag – phases (580, 520, 460 min, respectively),whereas methionine, alanine, glycine and phenylalanine mixtures seem to be less active (381,343, 289 and 260 min, respectively). The control mixtures which contained only sugar or amino acid had only very low or no protective activity. This could be explained by their different reactivity towards glucose (for e.g. lysine can be modified at the α- and ξ-amino group), by a different product spectrum which can be formed (modification of the guanidinium group in arginine) or by their solubility.Also, Maillard products from basic amino acids such as arginine, histidine or lysine with reducing sugars have been shown to have very strong activities *in vitro* **(Lingnert and Eriksson, 1980).**

Glutathione - glucose reaction mixture exhibited the greatest inhibitory effect on Cu^{2+}-induced LDL-oxidation (691 min) and even more stronger than L-ascorbic acid and reduced glutathione (311 and 259 min, respectively), which has been shown to act as an antioxidant against LDL-oxidation (Figure 2).

The above results showed the efficacy of all the tested Maillard products against copper catalyzed LDL oxidation and could be explained on the basis that when glucose was heated with different amino acids individually or with glutathione amino acid content (glutamic acid, cysteine and glycine), a variety of volatile heterocyclic compounds as thiophenes, furans, pyridines, pyrazines, thiazoles, thiazolidines, pyrroles and oxazoles were formed. However, it was reported that volatile heterocyclic compounds as thiazoles, oxazoles and furanones formed in L-cysteine/ D- glucose Maillard model system possessed antioxidative activity **(Eiserich and Shibamoto, 1994 and Eiserich *et al* 1995).**

The antioxidative mechanism for the aromatic thiol compounds may involve several models. The nucleophilic thiol group can behave as a one-electron-reducing agent and scavenge peroxyl and alkoxyl radicals. It can also act to decompose hydroperoxides by two-electrons reduction and subsequent disulphide formation. The five-membered heterocyclic aromatic ring may be able to scavenge reaction radicals. Direct attachement of the thiol groups to the aromatic ring appeared to be necessary for strong antioxidative activity.

The presence of these sulfur containing heterocyclic compounds in various foods may in part, explain and account for the increased oxidative stability of various cooked foods. The potential use of these compounds to inhibit the oxidation of lipid-rich foods is of interest to the food industry because the safety of synthetic antioxidants as BHA and BHT has been questioned **(Eiserich & Shibamoto, 1994 and**

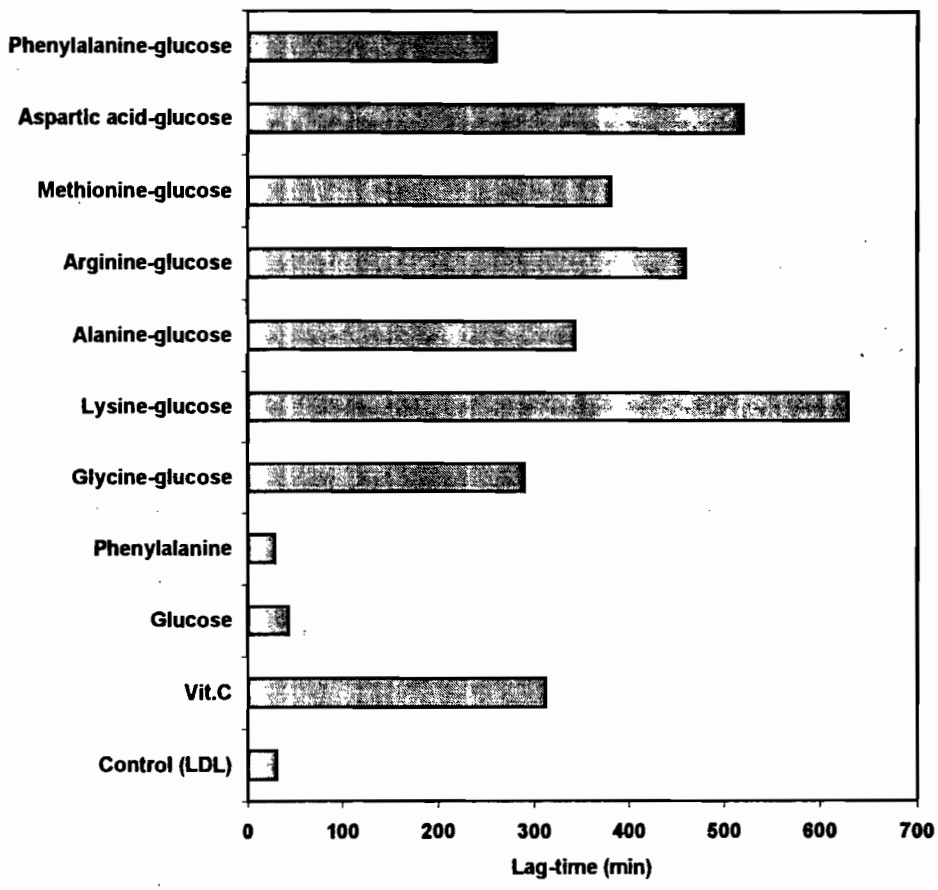

Fig. (1) : Prolongation of initial lag-phases of copper catalyzed LDL-oxidation (min) by different Maillard reaction mixtures (0.5 umol/L)

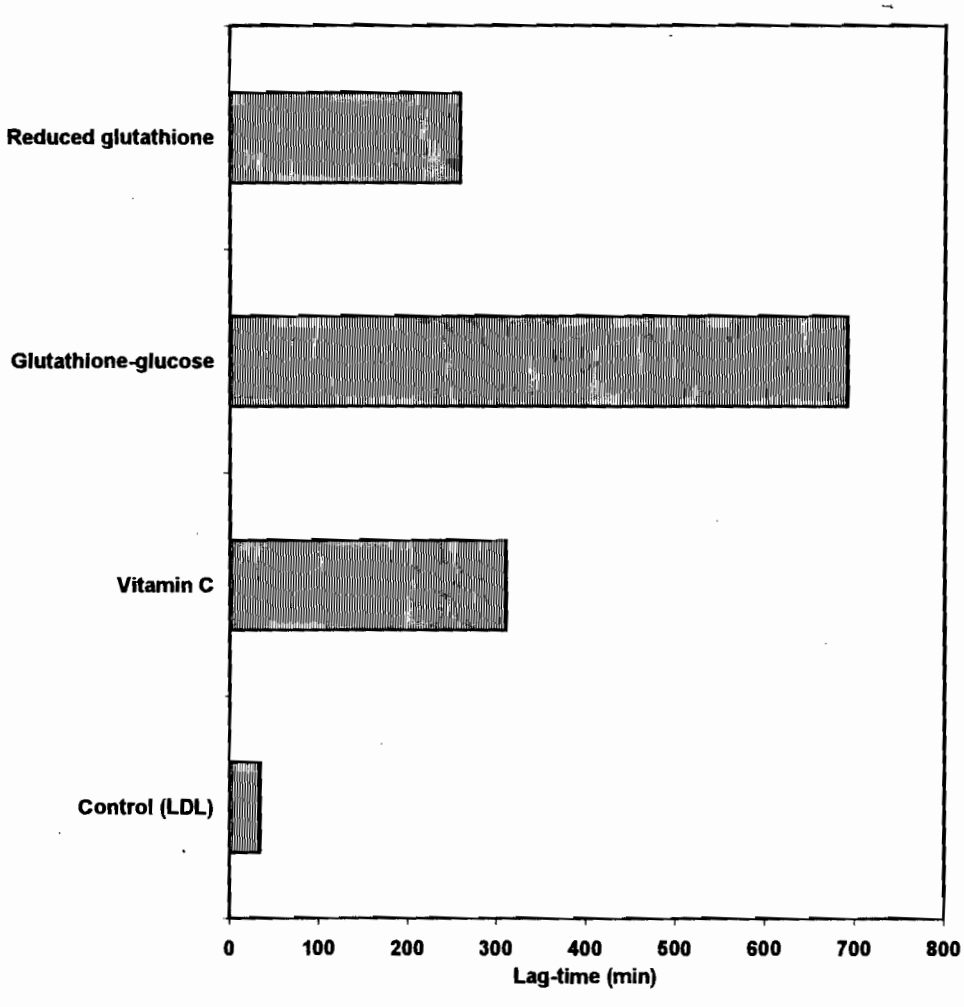

Fig. (2) : Prolongation of initial lag-phases of copper catalyzed LDL-oxidation (min) by glutathione -glucose system (0.5 umol/L)

Eiserich *et al* 1995).These data suggests that MRPs of glucose with different amino acids can protect LDL from oxidation *in vitro*.

Also, five structurally defined Maillard products with aminoreductone structures were tested for their antioxidative activity against LDL oxidation. Most of them showed similar or higher activity as L-ascorbic acid, whereas other structures proved to be rather inactive (Figure 3). However, the composition, structures and concentrations of the active compounds are difficult to determine in a complex mixture. Therefore, the activity of some structural defined Maillard compounds have been tested in the assay system. Particular MRP with reductone or amino reductone structure were suggested to possess strong reducing properties (**Pischetsrieder and Severin, 1997**). 4-Alkylamino-3-hydroxy-3-buten-2-one, a C4- aminoreductone (C4-AR) which is a major Maillard product of glucose showed similar antioxidative properties (310 min) as L- ascorbic acid in test system. On the other hand, C4-morpholino aminoreductone showed in the same assay system lower activity (267 min). The predominant role of the latter in the antioxidative activity of a Maillard mixture of lactose confirmed using a different test system (**Shimamura *et al* 2000**). Aminohexose reductone possesses a similar aminoreductone structure as C4-AR, whereas the Amadori product can be converted into an enaminol after enolization (**Pischetsrieder *et al* 1998**). Finally, antioxidative effect could be assumed for acetylformin due to its reductone ether moiety. Adverse activities of food reductones have been reported by **Mi *et al* (2001)**, who found that 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) and hydroxy hydroquinone (HHQ) prevent FeIII-induced lipid peroxidation of red blood cell membranes and LDL *in vitro*. Therefore, we have tested how these defined MRPs may contribute to the capability of the Maillrd mixtures to protect LDL oxidation.

To explain the observed variability in inhibition of LDL oxidation which is a free radical mediated process between the different tested compounds, differencies in their structural features can be considered. The differences in antioxidant activities towards LDL oxidation observed here could also be ascribed to other factors, including differences in solubilities and partitioning behaviour between the aqueous and lipid phases in the LDL system. Thus, the physicochemical properties of antioxidants are known to affect their antioxidant efficacy in complex, multiphase systems (**Frankel *et al* 1994**). Furthermore, the copper-mediated oxidation of tryptophan residues in the LDL-apolipoprotein B was shown to play an important role in initiating lipid oxidation in LDL particles and our compounds may affect it (**Giessauf *et al* 1995**).

The role of oxidized LDL in atherosclerosis remains controversial (**Jialal and Devaraj, 1996**). The polyunsaturated fatty acids in LDL are probably most susceptible to oxidation. The present study has demonstrated clearly that the polyunsaturated fatty acids in LDL could be protected from oxidation by the different Maillard reaction products.

Therefore, it can be concluded that secondary food components, such as Maillard products with aminoreductone structure, contribute to the overall anti-

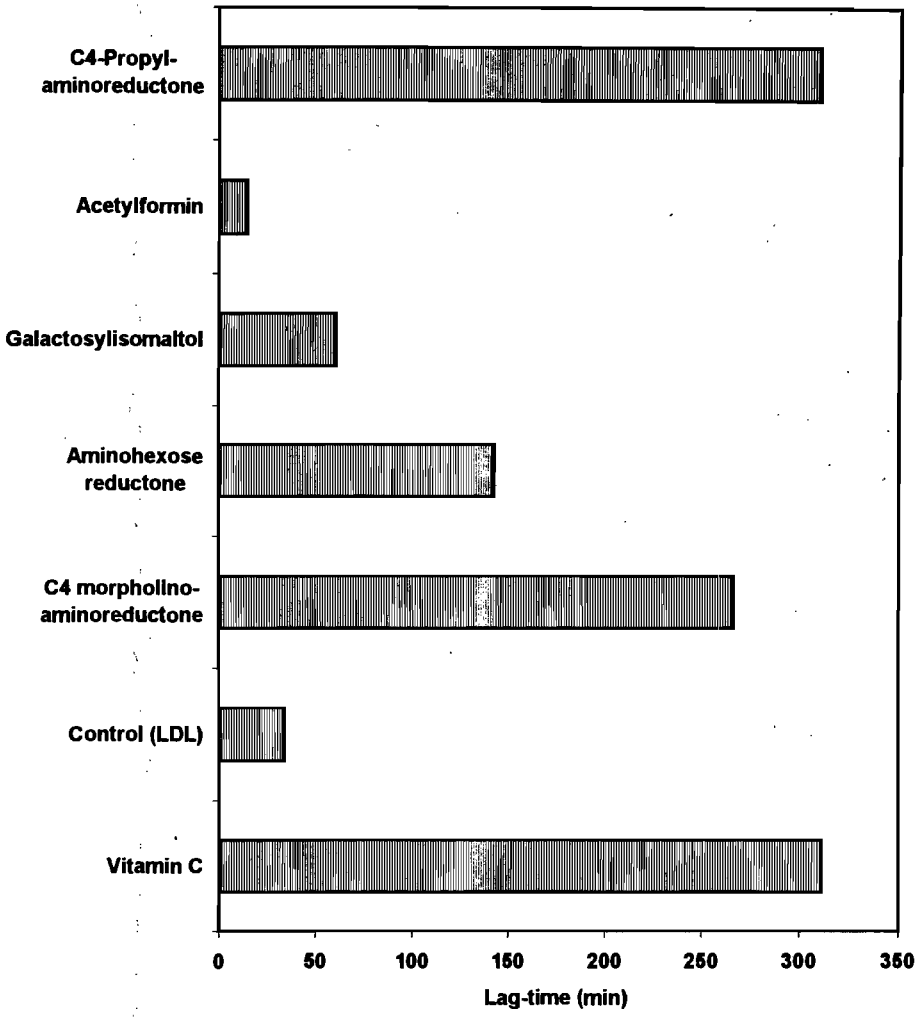

Fig. (3) : Prolongation of initial lag-phases of copper catalyzed LDL-oxidation (min) by structurally defined aminoreductone compounds

oxidative capacity of food to prevent LDL oxidation. However, the concentrations of the active Maillard compounds in different foods, their contribution to the total antioxidative activity of plant based foods and their biological availability still remain unclear. So, further investigations about the absorption rate and metabolism are required to establish their influence or physiological reactions.

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التأثير المحتمل لنواتج تحلل تفاعل ميلارد لمنع اكسدة دهون الدم قليلة الكثافة معمليا

[٢٤]

خالد فاروق المصري[١] – رالف ديترش[٢] – كارين كوندز[٢] – مونيكا بيشستريدر[٣]

١- قسم كيمياء مكسبات الطعم والرائحة – المركز القومي للبحوث- الدقي- القاهرة – مصر

٢- قسم النساء والتوليد – جامعة إيرلانجن – ألمانيا

٣- معهد الصيدلة وعلوم الأغذية – جامعة إيرلانجن – ألمانيا

تتكون نواتج تحلل تفاعل ميلارد ذات النكهات المختلفة أثناء عمليات التصنيع من تفاعل السكريات مع الأحماض الامينية. وتعتبر أكسدة دهون الدم قليلة الكثافة خطوة حاسمة فى حدوث أمراض تصلب الشرايين. ومن المعروف الدور الوقائي لبعض مكونات الغذاء النباتية ذات الطبيعة الفينولية بعد حدوث عمليات أكسدة لدهون الدم قليلة الكثافة ولكن هذه المركبات تتعرض للفقد أثناء التصنيع.

لذا تهتم هذه الدراسة لأول مرة بالتحقق من إمكانية التأثير المحتمل لنواتج تفاعل ميلارد كمواد مضادة للأكسدة وحامية من تأكسد دهون الدم قليلة الكثافة معمليا.

ولدراسة نشاط هذه النواتج تم تحضيرها من تفاعل سكر الجلوكوز مع الأحماض الأمينية المختلفة تحت ظروف التفاعل وتقدير كفاءتها لمنع أكسدة دهون الدم قليلة الكثافة المنشطة بأيونات النحاس معمليا مقارنة بفيتامين ج بقياس الوقت الفاصل لحدوث الأكسدة (lag- time) ،وقد وجد أن جميع النواتج تحت الدراسة لها قوة نشاط عالية كمضاد للأكسدة وحامية لدهون الدم

قليلة الكثافة من الأكسدة وعلى العكس من تأثير السكر او الحمض الامينى كل على حده. كما تم تقييم كفاءة خمسة مكونات أساسية لنواتج تفاعل ميلارد ذات الطبيعة المختزلةaminoreductones بعد تحضيرها معمليا وتحليلها بجهاز HPLC كمواد مضادة لأكسدة دهون الدم قليلة الكثافة. وقد وجد أن لبعضها تأثيرا واقيا.

ومن هذه النتائج نستخلص أهمية نواتج تفاعل ميلارد كمواد مضادة للأكسدة وحامية من أمراض تصلب الشرايين.

تحكيم: ا.د حمدى فرج حجاج
ا.د فؤاد عثمان محمد