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### Food Protein-Polysaccharide Conjugates obtained via the Maillard Reaction: A Review

Fabíola Cristina de Oliveira<sup>a,b</sup>, Jane Sélia dos Reis Coimbra<sup>b</sup>, Eduardo Basílio de Oliveira<sup>b</sup>, Abraham Damian Giraldo Zuñiga<sup>c</sup> & Edwin E. Garcia Rojas<sup>d</sup>

<sup>a</sup> Departamento de Ciéncia e Tecnologia de Alimentos, Instituto Federal de Educaçao Ciéncia e Tecnologia do Sudeste de Minas Gerais - Campus Rio Pomba, AV. Dr. José Sebastião da Paixão S/Nº - Bairro Lindo Vale, 36180-000 Rio Pomba-MG, Brasil.

<sup>b</sup> Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, Av. P. H. Rolfs, 36570-000 Viçosa - MG, Brasil.

<sup>c</sup> Universidade Federal do Tocantins - UFT, Av. NS 15, ALCNO 14, CEP 77123-360, Palmas - TO, Brasil.

<sup>d</sup> Departamento de Engenharia de Agronegócio, Universidade Federal Fluminense (UFF). Av. dos trabalhadres, 420, 27255-250 Volta Redonda-RJ, Brasil.

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# ACCEPTED MANUSCRIPT

## FOOD PROTEIN-POLYSACCHARIDE CONJUGATES OBTAINED VIA THE MAILLARD REACTION: A REVIEW

Fabíola Cristina de Oliveira<sup>1,2</sup>, Jane Sélia dos Reis Coimbra<sup>2\*</sup>, Eduardo Basílio de Oliveira<sup>2</sup>, Abraham Damian Giraldo Zuñiga<sup>3</sup>, Edwin E. Garcia Rojas<sup>4</sup>

<sup>1</sup>Departamento de Ciéncia e Tecnologia de Alimentos, Instituto Federal de Educação Ciéncia e Tecnologia do Sudeste de Minas Gerais – Campus Rio Pomba, AV. Dr. José Sebastião da Paixão S/Nº - Bairro Lindo Vale, 36180-000 Rio Pomba-MG, Brasil.

<sup>2</sup>Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa,  
Av. P. H. Rolfs, 36570–000 Viçosa - MG, Brasil.

<sup>3</sup>Universidade Federal do Tocantins – UFT, Av. NS 15, ALCNO 14, CEP 77123-360, Palmas - TO, Brasil.

<sup>4</sup>Departamento de Engenharia de Agronegócio, Universidade Federal Fluminense (UFF). Av. dos trabalhadres, 420, 27255-250 Volta Redonda-RJ, Brasil.

\* Jane Sélia dos Reis Coimbra

Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa,  
Av. P. H. Rolfs, 36570–000 Viçosa - MG, Brasil.

## Abstract

The products formed by glycosylation of food proteins with carbohydrates via the Maillard reaction, also known as conjugates, are agents capable of changing and improving techno-functional characteristics of proteins. The Maillard reaction uses the covalent bond between a group of a reducing carbohydrates and an amino group of a protein. This reaction does not require additional chemicals as it occurs naturally under controlled conditions of temperature, time, pH and moisture. Moreover, there is growing interest in modifying proteins for industrial food applications. This review analyses the current state of art of the Maillard reaction on food protein functionalities. It also discusses the influence of the Maillard reaction on the conditions and formulation of reagents that improve desirable techno-functional characteristics of food protein.

**KEYWORDS:** *Glycation, techno-functional properties, food industry.*

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## Abbreviations

AGE advanced glycation endproducts

MR Maillard Reaction

ARP Amadori-rearrangement product

ATR attenuated total reflectance

aw water activity

$\beta$ -lg beta-lactoglobulin

BCA bicinchoninic acid method

BSA bovine-serum albumin

DLS dynamic light scattering

DSC differential scanning calorimetry

DX dextran

ESI electrospray ionization

FTIR Fourier transform infrared

HMF hydroxymethylfurfural

MALDI matrix-assisted laser desorption

MALLS multi-angle laser light scatter

MRP Maillard reaction product(s)

MS mass spectrometry

NMR nuclear magnetic resonance

OPA o-phthaldialdehyde

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pI isoelectric point

RH relative humidity

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC size exclusion chromatography

SEM scanning electron microscopy

SWPI soy whey protein isolate

TNBS trinitrobenzenesulfonic acid

TOF time-of-flight

UV ultraviolet

WGP wheat germ protein

WPI whey protein isolate

## INTRODUCTION

Proteins are important in foods due to their rich composition of essential amino acids, which are not produced by the human body despite being necessary for cellular metabolism. Moreover, food proteins present physico-chemical and structural properties that enable them to act as food ingredients capable of altering the appearance, taste, texture and rheological behaviour of food products under different thermal and mechanical processing conditions.

However, the industrial application of food proteins is limited due to their instability under extreme flow conditions, high temperature and the presence of organic solvents and proteolytic agents. Food protein properties are affected by intrinsic factors such as molecular structure and composition, and extrinsic factors such as temperature, chemical composition of the medium, pH and shear stress. Thus, if proteins could be converted into more stable forms they could be used in more industrial applications (Oliver et al., 2006; Liu et al., 2012a).

The functional characteristics of macromolecules are defined by physico-chemical and structural properties that affect the behaviour of biomolecules during food processing, storage and consumption (Damodaran, 2007). Moreover, the functional properties of macromolecules influence both the quality of the final product and the selection of the processing steps. In technical terms, a functional property is any property of a biomolecule, in addition to its nutritional value, which affects its use as a food ingredient. Examples of techno-functional properties of biomolecules are solubility, emulsion forming ability, foam-forming ability, gel-forming ability and the ability to retain fat or water (Ahmedna et al., 1999; Liu et al., 2012a).

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Techno-functional characteristics of proteins can be intensified by means of physical, chemical or enzymatic treatments to obtain food ingredients for different applications (Kato, 2002; Flanagan and Sing, 2006; Du et al., 2012). However, the use of modified proteins as food ingredients when achieved by chemical methods is still limited due to the use of reagents that are harmful to human health (Hattori et al., 1996; Nagasawa et al., 1996; Spotti et al., 2013). One example is the use of cyanogen bromide to form the ovalbumin-dextran compound that exhibits improved emulsifying properties compared to matrixes containing pure ovalbumin (Kato et al., 1988). Thus, the search for naturally occurring chemical reactions remains necessary.

The Maillard reaction satisfies these conditions, since it does not require additional chemicals and occurs naturally under conditions of controlled time, temperature, pH and moisture (Kato, 2002; Jimenez-Castano et al., 2007; Liu et al., 2012a). In the Maillard reaction, a protein and polysaccharide are linked through a covalent bond between the amino group of proteins, especially the  $\epsilon$ -amino group of lysine residues and the terminal reducing group of the carbonyl of polysaccharide (Oliver et al., 2006; Liu et al., 2012a).

Studies indicate that heating protein mixtures and carbohydrates below the protein denaturation temperature and under controlled water activity generates complexes formed by covalent bonding, known as protein-polysaccharide conjugate (Haar et al., 2011; O'Regan and Mulvihill, 2009; Álvarez et al., 2012, Wong et al., 2011; Corzo-Martinez et al., 2008).

Polysaccharides are highly stable, safe, non-toxic, hydrophilic and biodegradable natural biopolymers. They are abundant in nature and highly diverse, and may have low processing cost. Polysaccharides comprise diverse and numerous reactive groups, exhibiting a wide range of molecular weights and different chemical compositions, contributing to their structural

variability (Liu et al., 2008). These advantages are associated with the use of polysaccharides in protein conjugate. Several factors, such as reaction temperature and time, ratio of amino group and reducing sugar, water activity ( $a_w$ ), pH, nature of the reactants influence the rate of the glycation and the types and amounts of Maillard Reaction products (MRP); and consequently the properties of the modified proteins (Oliver, 2011).

The food industry produces an increasing variety of formulated foods containing mixtures of proteins and polysaccharides. Interactions between these macromolecules affect the structural and functional properties of many foods (Cassiani et al., 2011).

The glycosylation of proteins with polysaccharides, via the Maillard reaction, is an effective way to improve functional properties of food proteins (Sanmartín et al., 2009; Corzo-Martinez et al., 2012; Álvarez et al., 2012, Spotti et al.; 2013; Kasran et al., 2013a,b); and the conjugates formed by glycosylation have potential for use in controlled release systems and nanoencapsulation (Deng et al., 2010; Markman and Livney, 2012). Hence, protein-polysaccharide conjugates obtained via the Maillard reaction have potential for use in the food industry.

Due to growing interest in converting food proteins into more stable forms for industrial applications, this review presents the current state of the Maillard reaction on the functionality of food proteins. Moreover, it discusses the influence of reaction conditions and reagents on improving techno-functional properties of food proteins.

## MAILLARD REACTION

The Maillard reaction was first described by French chemist Louis Maillard in 1912. The first coherent scheme of the Maillard reaction was presented by Hodge in 1953 (Figure 1).

The Maillard reaction refers to a complex group of reactions, beginning with the covalent bond between the amine groups and carbonyl compounds. The Maillard reaction, for purposes of simplicity, can be divided into three stages: early, advanced and final (Figure 2). All these stages are interrelated and can occur simultaneously, and they are affected by reaction conditions (Silván et al., 2011). The early stage of the Maillard reaction is characterised by the initial glycosylation reaction. This is a condensation reaction between the carbonyl group of a reducing sugar with the available amine group, which is also deprotonated, from an amino acid (or protein) to form an N-glycosylamine with the release of one water molecule. N-glycosylamine undergoes an irreversible rearrangement generating the Amadori product (ARP), 1-amino-1-deoxy-ketose. The  $\epsilon$ -amino group of lysine residue is most likely to react, as it is the major source of primary amines in proteins. The amine-terminus groups of the amino acid may undergo a condensation reaction. Imidazole groups from histidine residue, indole from tryptophan residue, and guanidil from arginine residue also react to a lesser extent. With ketoses, such as fructose, a ketosylamine form, rearranges to form the product of Heyns (2-amino-2-desoxialdose). No colour changes are observed at this stage of the reaction (Damodaran, 2007; Martins et al., 2001; Echavarría et al., 2012). The nutritional value may be reduced during the early stages of the Maillard reaction because of the decrease in amino acid availability.

The advanced stage begins with the degradation of the Amadori product, or Heyns product, which may be altered by oxidation, fragmentation, enolization, dehydration, acid hydrolysis and free radical reactions, resulting in multiple poorly-characterised compounds. The degradation of the Amadori product depends on system conditions, such as pH, time and temperature. The Amadori product suffers mainly 1,2 enolization with the formation of furfural (when pentoses

are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved) when the pH is equal to or lower than 7. At pH values above 7, the degradation of the Amadori compound involves mainly 2,3 enolization, with reductones being formed, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one, and a variety of fission products, including acetal, pyruvaldehyde and diacetyl. These compounds are highly reactive and participate in new reactions (Martins et al., 2001; Echavarría et al., 2012).

Carbonyl groups can be condensed with free amino groups, resulting in nitrogen incorporated in the reaction products. Dicarbonyl compounds react with amino acids, resulting in aldehydes and aminoketones. This pathway is known as Strecker degradation (Martins et al., 2001).

Other reactions occur later in the advanced stage, including cyclisation, dehydration, retroaldolization, rearrangement, isomerization and condensation. A final phase leads to the formation of nitrogenous polymers and co-polymers of brown colouration, known as melanoidins (Friedman, 1996; Martins et al., 2001, Zhang and Zhang, 2007).

In contrast to the early stages, the advanced and final stages of the Maillard reaction contain a high degree of complexity. The chemistry of compounds formed in these stages are not well-known and their mechanism are not well understood, although the results are easily recognised in terms of heating reactions that cause changes in the colour and flavour (Oliver et al., 2006; Echavarría et al., 2012).

The Maillard reaction occurs naturally and may have beneficial or harmful effects on the physical, chemical, biological and organoleptic characteristics of food products in which it occurs. The harmful effects may be observed when the extent of the Maillard reaction is not controlled, e.g. during production or storage of powdered milk. However, under controlled

conditions, the identity of certain products undergoing heat treatment is favoured by the Maillard reaction. This improvement is in terms of acceptance by consumers associated with the development of flavour, aroma, texture and colour in the food product, which are essential for “dulce de leche”, meat products, breads and grains (soybeans, peanuts, coffee and barley).

Under controlled conditions, changes in the protein structure combined with carbohydrates lead to different functionalities, which are useful when the glycosylated protein is used as an ingredient to enhance functional properties such as thermal stability and solubility (Liu et al., 2012a). During its advanced stages, the Maillard reaction can also result in various diseases, such as diabetes and Alzheimer’s disease (Fenaille et al., 2004).

To use conjugates as a food ingredient, the Maillard reaction must be conducted properly to avoid the advanced stages, which generate harmful compounds (Kato, 2002; Oliver et al., 2006). The conjugates formed via the Maillard reaction consist of various glycoforms and are produced in combination with a large number of poorly-characterised products. The types and quantities of the products obtained from the Maillard reaction are influenced by reaction conditions such as pH, temperature, mass ratio of the amine group and the carbonyl group, relative humidity and intrinsic properties of reagents (Gu et al., 2009; Oliver et al., 2006; Liu et al., 2012a).

Studies focused on the functional properties of conjugates are numerous and relate both property intensification (Medrano et al., 2009, 2011; Zhu et al., 2010; Mu et al., 2011; Li et al., 2013; Spotti et al., 2013; Kasran et al., 2013b) as well as reducing biocompound functionality, especially related to solubility (Al-Hakkak and Al-Hakkak, 2010). Regardless of the results of protein glycosylation via the Maillard reaction, conjugates have the potential to manipulate

protein properties. However, many questions remain unanswered regarding the operating conditions for the industrial processing of the conjugates.

Understanding the influence of the reaction parameters on the glycosylation step and the functionality of the protein will aid the development of safe and well-defined industrial processes for producing new value-added food ingredients.

## Food safety of glycated proteins

The Maillard reaction products (MRPs) frequently result in the formation of antinutritional and toxic substances. Studies have revealed some harmful effects including mutagenic, carcinogenic and cytotoxic effects. The progress of the Maillard reaction causes the formation of advanced glycation end-products (AGEs) that may play an important role in the pathogenesis of chronic diseases such as diabetes, atherosclerosis, renal failure, premature aging, and Alzheimer's disease. (Echavarría et al., 2012; Corzo-Martinez et al., 2010).

Glycation under controlled conditions, in terms of incubation time, pH, aw, and temperature, may prevent the Maillard reaction from progressing to more advanced stages, and, thus, preventing the formation of harmful components (Sanmartín et al., 2009). Nevertheless, considering the complexity of the Maillard reaction, further insight into those parameters is needed to more thoroughly control this reaction (Corzo-Martinez et al., 2010).

Highly glycated proteins produced by such reactions have fewer safety issues than chemically modified food proteins (Pan & Melton, 2007), so the proteins glycated through the MR can be added to foods as functional ingredients to improve emulsion and gelation and alter flavour, appearance, and texture. Many studies have reported beneficial effects associated with advanced

Maillard reaction products including antioxidant, antimicrobial, antihypertensive, antigenicity properties (Rufian-Henares and Morales, 2007, 2008; Gu et al., 2010; Guan et al., 2010; Laparra et al., 2011; Li et al., 2011; Huang et al., 2012).

Any food ingredient created by the Maillard reaction is likely to be considered a “novel food” and, hence, it should be subjected to safety testing before being consumed by humans. Controversially, either beneficial or detrimental effects have been attributed to the Maillard reaction products and, unfortunately, no definitive results about the toxicological effects of individual compounds are available (Sanmartini et al., 2009).

Jing and Nakamura (2005) indicated that egg lysozyme–galactomannan and ovalbumin–galactomannan Maillard reaction conjugates had enhanced emulsifying capacity, antimicrobial, and antioxidative activities. Furthermore, both the mutagenesis and the rat toxicity tests demonstrated that these neoglycoconjugates were neither mutagenic nor toxic, pointing out their potential for safe and effective use as functional ingredients in food products.

## CURRENT STATE OF THE MAILLARD REACTION

Protein-polysaccharide conjugates, formed through the Maillard reaction, are carriers of active ingredients and act as agents that enhance techno-functional characteristics of food proteins. These include gelling properties, emulsifiers, stabilisers, antioxidants and antimicrobial activity, as well as reduced protein allergenicity. Table 1 lists several studies involving conjugates from proteins, such as milk, egg, soy, wheat, rice and peanuts, and carbohydrates such as glucose, lactose, dextran, chitosan, pectin and galactomannan.

These studies show that the functional properties of polysaccharide-protein conjugates obtained via the Maillard reaction depend on the conformation of the protein and particular characteristics of the polysaccharides, such as hydrophobicity and viscosity. In contrast with the high reactivity of monosaccharides and disaccharides, the low reactivity of polysaccharides and steric hindrance limit the extent of the Maillard reaction and decrease subsequent glycosylation reactions, preventing excessive colour changes and protein polymerization. However, the functional properties are dependent on the polysaccharide chain length and the number of bonds between the amine and carbonyl groups.

## CONJUGATES FOR FOOD APPLICATIONS

Conjugates intended for food applications must provide enhanced functionality with minimal changes to colour or flavour. Therefore, the Maillard reaction must be conducted under carefully controlled conditions to avoid further colour and flavour changes (Oliver et al., 2006).

Parameters controlled during the glycosylation of proteins to form conjugates with improved properties include temperature, water activity, pH and time. Temperature affects the rate and mechanism of the Maillard reaction. The activation energy varies between 10 and 160 kJ/mol. Thus, energy in the form of heat is necessary for the Maillard reaction to take place, and the temperature increase results in a higher reaction velocity. The water content has a major impact on the activation energy, with the reaction achieving maximum speed when water activity ranges from 0.3 to 0.7. With low water content, the Maillard reaction is slow. Increased water content results in increased velocity, but with high amounts of water the reactant concentration is

lowered, thus decreasing the reaction velocity. The reaction speed also increases with pH, reaching its maximum at slightly alkaline pH (Damodaran, 2007).

The nature of the reducing sugars and amino groups also affects the speed of the Maillard reaction. Different sugars react at different speeds; for example, reactions with glucose are faster than with fructose, which in turn are faster than those with polysaccharides (Damodaran, 2007).

The influence of experimental conditions, such as pH, temperature and reagent concentrations on polysaccharide-protein conjugate production, has been extensively studied (Table 1). The proteins and carbohydrates used interfere with selecting the best process conditions.

The most commonly used methods of conjugate synthesis include those using heat, either dry or in aqueous solution. The first method involves the heating of a dry dispersion of a protein and saccharide under controlled temperature and relative humidity. A disadvantage of this method is that the reaction can take several days or weeks. The reaction is limited by the uneven contact between reactants, and in the case of folded or rigid proteins, the resulting product is a mixture of protein-polysaccharide conjugates, free proteins and unreacted polysaccharides (Li et al., 2013; Zhuo et al., 2012). The second method involves a protein/saccharide (mainly monosaccharide and disaccharide) mixture in a buffer solution, which is heated at a specific temperature. This method has the advantage of better control and shorter reaction times. Its disadvantages include the removal of the water and buffer when recovering the products (Li et al., 2013; Zhuo et al., 2012). Figure 3 shows the major steps involved in conjugate formation by means of both dry-heating and heating in aqueous solution.

Xu et al. (2010) prepared conjugates from  $\beta$ -conglycinin and dextran under high pressure.  $\beta$ -conglycinin and dextran were dissolved in a phosphate buffer pH 7.2 at 1:1 (w/w) ratio and

placed in a high pressure system at controlled temperature. The solution was heated to 100 °C and subjected to two values of pressure, atmospheric pressure and 10 MPa. The experiments were performed for 6 hours with samples taken every hour. The results showed that an increased pressure accelerated the reaction rate of the free amine groups and decreased the rate of browning. The conjugates subjected to high pressures exhibited better emulsifying properties compared to conjugates obtained at atmospheric pressure. According to these results, conducting the reaction in pressurised systems may be a practical method for obtaining conjugates. The drawback of this method on an industrial level is the high cost associated with higher pressures. New processing technologies can be applied, using non-thermal treatment technologies to obtain conjugates via the Maillard reaction, one example being treatment with pulsed electric fields (Guan et al., 2010; Sun et al., 2011). Sun et al. (2011) used this methodology to synthesise conjugates from whey protein isolate (WPI, 1% w/v) and dextran. Mixtures of dextran and WPI in a 1:1 ratio (w/w) were dissolved in sodium phosphate buffer (pH 10) and subjected to a pulsed electric field with intensities of 15 and 30 kV/cm for 7.35 ms, with each pulse lasting 25 µs. Increased pulse intensity increased the browning. The conjugates subjected to electrical pulses showed better solubility and emulsifying properties compared to whey protein isolate.

### **Reaction in dry-heating conditions**

The first step in the dry-heating reaction is to prepare an aqueous solution of protein and carbohydrates and mix them in a desired weight (or molar) ratio, depending on the proteins and carbohydrates used. The mixture then is frozen and lyophilised. The conjugates are produced during storage of the freeze-dried powder at a certain temperature, usually ranging from (40 to

80 °C, with 60 °C being most common. Relative humidity (RH) is controlled during storage, with 65% or 79% RH most commonly being used. The RH is controlled in an oven or desiccator containing a saturated solution of potassium iodide or potassium bromide, to obtain values of 65% and 79% moisture, respectively. The reaction time for conjugate formation depends on the type and conformation of protein, as well as the type of reducing sugar. The reaction time can vary from hours to weeks. Afterward, the synthesised conjugates are immediately cooled to stop the reaction, then kept refrigerated for further characterization and use (Du et al., 2012; Liu et al., 2012b; Akhtar and Dickinson, 2003; Miralles et al., 2007; Aminlari et al., 2005).

To illustrate the effect of the protein structure on the reaction time, Kato et al. (1992) and Nakamura et al. (1992) described the duration of conjugates formation from  $\alpha$ -casein-galactomannan and lysozyme-galactomannan, respectively. The  $\alpha$ -casein, a protein with a flexible structure, easily formed a conjugate and four lysine residues in  $\alpha$ -casein reacted with the polysaccharide in 24 hours. In contrast, lysozyme, a globular protein, formed a conjugate slowly. Even after two weeks, only two lysine residues reacted with the polysaccharide without loss of lytic activity. This behaviour is probably due to the difference in reactivity of the exposed lysine residues among proteins with different structures, for example, highly structured proteins and proteins with lower structural levels (Kato, 2002).

Polysaccharides have only one reactive group, the carbonyl group at the reducing end, which binds to the amino group of the protein. The number of polysaccharides that react with the proteins depends on the protein conformation and is limited by the polysaccharide steric hindrance. Kato (2002) proposed a model for the formation of polysaccharide-protein complex

(Figure 4). According to this author, a network structure does not form because the steric hindrance limits the number of polysaccharides bonded to the protein.

In their native state, proteins can react with one or two polysaccharides, while denatured proteins may bind to several carbohydrates. For example, in casein four lysine residues react with polysaccharides in the denatured state, since in this condition lysine residues are more available for further reaction than protein in its native state.

### **Reaction with heating in aqueous solution (wet Maillard reaction)**

Most studies have used the dry-heating method for conjugate synthesis. However, this method is not feasible for large scale production, since it requires the material to be dried prior to the reaction and needs controlled humidity and temperature during the reaction. The reaction time can be on the order of weeks using this method (Zhu et al., 2008). Thus, from the industrial point of view, the dry-heating method is a costly process that prevents the marketing of conjugates as ingredients (Zhu et al., 2010).

Zhu et al. (2008, 2010) prepared conjugate of protein isolate (WPI) and dextran (DX) using a concentrated mixture of WPI and DX. This method was based on the macromolecular crowding effect.

When the Maillard reaction is conducted in aqueous solutions, a likely adverse reaction is protein denaturation and polymerization at elevated temperatures. In the presence of high concentrations of reactants the glycosylation yield could increase, but it could also result in greater protein denaturation and polymerization. When macromolecular reactions are performed in elevated concentrations of macromolecules, excluded volume theory predicts that the reaction will be

shifted in the direction of species with smaller excluded volume, limiting the excluded volume available for unfolding. This helps stabilise the native protein structure (Zhu et al., 2008, 2010; Zhuo et al., 2012).

Zhu et al. (2008, 2010) monitored the formation of the Schiff base, which is the initial product of the Maillard reaction, confirming that the Dextran was covalently linked to the WPI. This method simplifies the conjugation process, since there is no need for lyophilisation or to control the humidity of the reaction. Zhu et al. (2008) prepared WPI-DX conjugates from solution containing 10% w/v of WPI and 30% w/v DX, at pH = 6.5, incubated at 60 °C for 48 hours. The conjugates were purified using chromatographic methods and showed higher heat stability, solubility and emulsifying properties compared to WPI.

Other studies have used the heating in solution method for conjugate formation using diluted solutions of proteins and polysaccharides (Mu et al., 2011; Niu et al., 2011). Niu et al. (2011) obtained conjugates from a solution containing 1% (w/v) wheat germ protein and a solution containing 1% (w/v) of carbohydrate, such as xylose, glucose, lactose, dextran and maltodextrin. The pH of the solutions was adjusted to 11 and the solutions were heated to 90 °C for 50 min. The results showed that the degree of glycosylation increased with reaction time and as the carbohydrate decreased in size. The conjugates of wheat germ protein and carbohydrates prepared by the heating in solution method exhibited better functional properties compared to wheat germ protein without glycosylation. Using circular dichroism analysis to evaluate changes in the secondary structure of the protein, these authors showed that the Maillard reaction significantly affects protein structure in aqueous solutions. However, Chevalier et al. (2001)

observed no significant change in the protein structure when the reaction was performed in dry-heating systems.

Various techniques for conjugate separation and purification can be used in isolation, such as gel permeation chromatography, or combined with other techniques, such as ultrafiltration followed by gel chromatography or ion exchange chromatography followed by gel chromatography (Aminlari et al., 2005; Qi et al., 2009; Gu et al., 2010).

To characterise the conjugates, various techniques including electrophoresis, spectrophotometry, infrared, light scattering and mass spectrometry are used (Table 1).

## FOOD CONJUGATE CHARACTERIZATION

Electrophoresis is the most widely used technique for conjugate characterization (Shu et al., 1996; Wong et al., 2009; Xu et al., 2010; Ledesma-Osuna et al., 2010; Liu et al., 2012b).

Conjugate synthesis by covalent bond between the amine group from a protein and the carbonyl group from a polysaccharide may be confirmed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique uses the sodium dodecyl sulfate as a denaturing agent and the principle of electrophoretic mobility of the molecule related to the molar mass of the protein.

Results obtained by this technique are assessed by comparing the electrophoretic pattern of the pure protein, heated either in the absence or presence of polysaccharide. Conjugate synthesis results in a decreased intensity band compared to pure protein; instead, a band appears that corresponds to a compound of higher molar mass, indicating covalent binding between the

protein and carbohydrate. In the case of polysaccharides with high molar mass, this band appears at the border between the stacking gel and the resolving gel (Kato et al., 1995).

Figure 5 shows analysis of conjugates of soy protein and dextran using the SDS-PAGE technique (Xu and Yao, 2009). Xu and Yao (2009) used soy protein and dextran (weight ratio 1:3, 1:6, 1:9) in conjugate formation. Prior to SDS-PAGE, conjugates and protein samples were treated with buffer containing SDS and  $\beta$ -mercaptoethanol to destroy the crosslinks and disulfide-bridge, as well as hydrophobic aggregation in the conjugates and proteins.

The new band containing a much higher molecular weight than pure soy protein (line 2) indicates the formation of soy protein-dextran conjugates. Figure 5 indicates that the conjugates contain free proteins. The degree of conjugation was calculated by means of quantitative band analysis. Results showed that using 1:3, 1:6 and 1:9 mass ratios allowed the conjugation of approximately 9%, 32% and 34% of proteins with dextran, respectively. Moreover, using the same mass ratios resulted in 90%, 68% and 65% of free proteins after the Maillard reaction.

Browning may occur during the Maillard reaction, resulting in a brown colour. Many authors measure this browning by absorbance analysis, typically at 420 nm (Jiang et al., 2009; Xu et al., 2010; Li and Yao, 2009). Thus, the increasing absorbance values that result from browning can be used as an indicator of the browning degree caused by the Maillard reaction at more advanced stages (Sun et al., 2011).

Some techniques have been used to study the extent of the reaction and the degree of protein modification (Sun et al., 2011; Niu et al., 2011; Jimenez-Castano et al., 2007, 2005). The extent of the Maillard reaction is defined by the average number of lysines that react with the polysaccharide. One of these techniques is the *o*-phthalaldehyde (OPA) test, in which the extent

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of the Maillard reaction is measured indirectly using a colourimetric assay (340 nm) based on the reaction between the OPA and the free primary amine groups of proteins (Wooster and Augustin, 2006 ) (Figure 6).

The test with 2,4,6-Trinitrobenzenesulfonic acid (TNBS) is a sensitive and rapid chemical test used to quantify free amino groups. The reaction of primary amines with TNBS (Figure 7) generates a highly chromogenic product that can be quantified at 335 nm.

Another method to study the extent of the Maillard reaction is the furosine assay ( $\epsilon$ -N-furoyl-methyl-L-lysine), which consists of the quantitative determination of  $\epsilon$ -N-furoyl-methyl-L-lysine formed after acid hydrolysis of products obtained in the Maillard reaction.

Other techniques widely used in conjugate characterization include dynamic light scattering (DLS), Matrix Assisted Laser Desorption Ionization – Time of Flight – Mass Spectrometry (MALDI-TOF MS), infrared, fluorescence measurement, colourimetric measurements and circular dichroism (Álvarez et al., 2012; Ledesma-Osuna et al., 2010; Guan et al., 2010; Bund et al., 2012; Wang and Smail 2012).

## FOOD CONJUGATE APPLICATIONS

Proteins are widely used as ingredients in the food industry. Some protein applications include their use in meat products, dairy products and desserts, in which proteins act as emulsifying, gelling and foaming agents.

In order to have all these functions, proteins must be soluble. In addition, they must be thermally stable, since many food products are heat treated.

However, the use of proteins as food ingredients is hindered due to protein structural changes that occur during processing steps, which can result in limited protein functionality. Thus, studies are conducted with conjugates of protein and small carbohydrate/polysaccharides to compare their functional properties with the properties of pure proteins. In addition to research focused on functional properties of conjugates, conjugates have been compared with pure protein regarding their antioxidant activity (Jiang et al., 2009; Jiang and Brodkorb, 2012; Sun et al., 2006; Huang et al., 2012). Results have shown that casein-glucose conjugates have higher antioxidant activity than pure casein (Gu et al., 2010).

Nanostructures synthesised from conjugates have shown potential for use in controlled release systems (Li and Yao, 2009; Li et al., 2008; Deng et al., 2010) and nanoencapsulation, e.g., nanoencapsulation of hydrophobic nutraceuticals in clear beverages (Markman and Livney, 2012). Deng et al. (2010) used bovine serum albumin (BSA)-dextran conjugates to obtain nanoparticles for doxorubicin encapsulation. The use of nanoparticles in doxorubicin delivery systems is promising due to the increased antineoplastic efficacy and reduced side effects of doxorubicin. Doxorubicin is widely used in chemotherapy due to its effectiveness against many types of cancer, including breast and bladder cancer.

One hurdle related to the use of proteins as food ingredients is that most are allergens, such as  $\beta$ -lactoglobulin (Selo et al., 1999), a bovine whey protein, and  $\beta$ -conglycinin (Krishnan et al., 2009), a soy protein. However, studies have shown that the conjugation of proteins with polysaccharides decreased the level or degree of allergenicity of the protein, as reported by Li et al. (2011). These authors observed this effect in whey protein isolate-maltose conjugates, as did Usui et al. (2004) in soy protein-chitosan conjugates.

As shown in Table 1, several food proteins of animal and vegetable origin, as well as different types of polysaccharides and small carbohydrates, are used in conjugate formation to improve protein functional properties.

## **Functional properties**

Functional properties of proteins are important in determining the quality of the final product, and in facilitating processing (Singh et al., 2008). The most studied functional properties of conjugates include the emulsifying, gelling and foaming properties, as well as their solubility and thermal stability, as described below.

### ***Emulsifying properties***

Among the functional or technological properties, the emulsifying properties of conjugates are the most extensively studied (Sun et al., 2011; Mu et al., 2011; Alvarez et al., 2012; Li et al. 2013; Du et al. 2012, Medrano et al., 2012).

An emulsion is a thermodynamically unstable mixture of two immiscible liquids, comprised of an insoluble disperse phase in the dispersing phase. In foods, the immiscible phases are generally aqueous phases, such as aqueous solutions or dispersions of macro or micronutrients, and a lipid phase, such as oil or fat where liposoluble substances may be dissolved. Since water and oil are immiscible, mechanical agitation is needed to form a homogeneous dispersion of the two liquids. However, this system is considered thermodynamically unstable due to the high interfacial area created and followed by high interfacial tension, resulting in an increase of free energy in the system (Hill, 1996).

The emulsifying properties play an important role in food systems as they directly contribute to texture and sensory properties of food. The emulsifying properties of proteins essentially depend on two effects: (a) a substantial decrease of the surface tension due to the adsorption of the protein in the water-oil interface and (b) formation of a structural, mechanical and electrostatic barrier able to avoid the destabilization of the emulsified droplets (Wagner and Guéguen, 1999).

Proteins and polysaccharides play an important role in the stabilization of oil in water emulsions. Proteins are adsorbed in the water-oil interface during emulsification to form a viscoelastic layer, while polysaccharides confer colloidal stability through the thickening and gelling behaviour of the aqueous phase (Ru et al., 2009).

Thus, it is expected that protein-polysaccharide conjugates exhibit good emulsifying properties (Kato, 2002). Protein-polysaccharide conjugates showed better emulsifying activity and emulsion stability than the control mixtures of proteins with polysaccharides (Shu et al., 1996; Akhtar and Dickinson, 2003; Qi et al., 2009; Zhu et al., 2010; Liu et al., 2012b; Li et al., 2013; Kasran et al., 2013a,b).

Due to the binding layer, the conjugate surface is much more active than the surface of the polysaccharide or protein. Hence, conjugates are able to saturate the surface layer at a much lower concentration. At the same time, due to the covalent bond with the polysaccharide, a layer of adsorbed protein is protected against destabilization in unfavourable environmental conditions, such as heat, low pH, high electrolyte concentrations, etc. Both the large size and hydrophilicity of the polysaccharide molecule generate long range steric repulsion between the surfaces of the emulsion droplets (Dickinson, 2009).

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Factors affecting the emulsifying properties of conjugates include the polysaccharide molar mass, the polysaccharide load, polysaccharide structure, reaction time and the amount of lysine (Kato, 2002; Oliver et al., 2006).

Shu et al. (1996) studied the effect of polysaccharide chain lengths on the functional properties of conjugates using lysozyme as the model protein. Galactomannan of various sizes (from 3.5 to 24 kDa) was used as a polysaccharide (1:4 lysozyme: galactomannan molar ratio, 60 °C and 79% RH) and xyloglucan (1.4 kDa) as an oligosaccharide (1:8 lysozyme: galactomannan molar ratio, 60 °C and 79 % RH). The authors observed that the emulsifying properties of conjugates increased with increasing polysaccharide chain length, indicating that a higher molar mass resulted in improved emulsifying properties. However, the conjugation with the oligosaccharide and galactomannan (3.5 to 6 kDa) did not improve conjugate emulsifying properties. Thus, galactomannan with a molar mass greater than 6 kDa was essential for improving conjugate functional properties.

The balance between the hydrophobic and hydrophilic characters of protein-polysaccharide conjugates is critical for increasing the emulsifying properties of conjugates. During emulsification, the hydrophobic residues of the protein molecules are anchored in the oil droplets, while the saccharides attract water molecules around the oil droplet. This accelerates the formation of a thick layer around the stabilised emulsion by inhibiting oil droplet coalescence (Oliver et al., 2006).

Niu et al. (2011) evaluated the emulsifying properties of conjugates prepared with wheat germ proteins and various carbohydrates (glucose, xylose, lactose, dextran and maltodextrin).

Conjugates obtained with wheat germ protein and dextran (2% w/v solution), pH 11, heated to 90 °C for 20 min showed better emulsifying properties than the other carbohydrates used.

The initial protein:polysaccharide ratio used in conjugate preparation can affect their emulsifying properties. For example, using an excess of oligosaccharides causes a greater number of them to bind to the protein, leading to steric stabilization, enhancing emulsifying properties (Nakamura and Kato, 2000). Shu et al. (1996) showed that lysozyme conjugated with two moles of galactomannan had better emulsifying properties than lysozyme conjugated with one mole of this polysaccharide.

The polysaccharide structure also affects the properties of the conjugates. Studies show that ramified polysaccharides, such as galactomannan and dextran, improve functional properties of conjugates. Thus, ramified polysaccharides provide greater steric hindrance, preventing oil droplet coalescence.

Reaction time also influences the emulsifying properties of conjugates. Miralles et al. (2007) studied the formation of  $\beta$ -lactoglobulin ( $\beta$ -lg)-chitosan conjugates (1:4 mass ratio), 40 °C, 79 % RH at different reaction times, for up to 15 days. The emulsifying capacity increased with the incubation time for up to two days, then decreased. The increased time may lead to a degradation of Maillard reaction products, resulting in decreased emulsifying properties.

### ***Gelling properties***

A gel is defined as a continuous three-dimensional network of particles or molecules linked through covalent and non-covalent bonds. This network can trap a large amount of solvents and

other small molecules, thus retaining a large volume of a liquid continuous phase. In food systems, the solvent is water. The gel is an intermediate phase between the solid and liquid (Damodaran, 2007; Baier and McClements, 2005).

Heating solutions of globular proteins (lysozyme, BSA, soy protein) in the presence of reducing sugars of low molar mass (such as lactose, ribose or xylose) result in gels with higher breaking strength than samples heated in the absence of reducing sugars. In the presence of reducing sugars, gelling occurs at much lower protein concentrations in less time (Oliver et al., 2006).

Glycosylation of proteins via the Maillard reaction improves gelling properties (Dickinson and Izgi, 1996; Álvarez et al., 2012; Sun et al., 2011, Spotti et al., 2013). Matsudomi et al. (2002) studied the formation of conjugates from egg white protein with galactomannan by dry-heating (3 days, 60 °C, 65 % RH, 1:4 egg white protein:galactomannan ratio). The authors showed that gelling properties were improved after heating a conjugate solution (10% w/v of protein) at 90 °C for 30 min. The gel strength and water retention capacity of the gels formed using the conjugate were higher than those obtained from pure albumin heated without galactomannan. The appearance of gels from conjugates became transparent with increased heating time, while gels from pure albumin were turbid.

Spotti et al. (2013) prepared conjugates of WPI and dextran by dry-heating (2, 5 and 9 days, 60 °C and 63% RH). Reaction time greatly affected gelation properties. WPI/DX mixed gels fractured under uniaxial compression while conjugate gels did not fracture under the test conditions, subjected to 80% deformation in uniaxial compression test. In addition, Young's modulus values and stress-relaxation measurement allowed conjugate gels to be characterised and compared with mixed gels. Conjugate gels were less firm and hard.

### ***Foaming properties***

Foams are dispersed systems composed of two separate phases, where the liquid phase surrounds the dispersed phase, which is comprised of air bubbles. Proteins can act as stabilisers of these systems and accumulate in the air-water interface, changing surface properties (Damodaran, 2007). Foaming properties are important attributes in determining the quality of many food products, such as milk, meat, mayonnaise, ice cream, frozen desserts, cakes, breads, toppings, etc. The structure of many of these food products depends on the foam formation and its stability, which facilitates their mixing, confers product structure and contributes to product sensory quality. These dispersions are thermodynamically unstable and their relative kinetic stability depends on the properties of the surfactant components in the system (Corzo-Martínez et al., 2012; Sharma et al., 2012).

Conjugates obtained via the Maillard reaction present improved foaming properties compared to pure proteins (Medrano et al., 2009; Corzo-Martinez, 2010). Similar to emulsifying properties, the effect of glycosylation on foaming properties depends on the size and type of carbohydrate or polysaccharide, the reaction time and the protein-polysaccharide ratio.

Medrano et al. (2009) investigated the foaming property improvements of conjugates formed by  $\beta$ -lactoglobulin with lactose and glucose via the Maillard reaction (heating temperature of 50 °C and 65 % RH). All modified samples showed an increase in drainage stability compared with the sample that was not heat treated. Additionally, foam formed by lactose conjugates was more stable than that formed by glucose conjugates. An increase in foam stability with the reaction time was also detected, mainly in glycosylated samples.

The addition of lactose to  $\beta$ -lactoglobulin improved foaming properties due to increased adsorption of modified proteins in the air-water interface, which resulted in foams with greater stability to gravitational drainage. These changes can be attributed to structural differences in  $\beta$ -lactoglobulin induced by the bond with lactose. This bond leads to a molecular unfolding with increased hydrophobic residue exposure, resulting in greater protein stabilization as a monomer, with the simultaneous formation of a high number of tetramers and octamers. The binding of  $\beta$ -lactoglobulin with glucose may cause a greater aggregation of proteins, forming a foam more resistant to drainage than the one obtained from non-glycosylated proteins. However, they have lower stability compared to proteins glycosylated only with lactose.

Dickinson and Izgi (1996) studied foaming properties of polysaccharide-protein conjugates by prolonged dry-heating. Conjugates from protein mixtures (BSA, lysozyme or casein) and polysaccharide (dextran) were formed at 60 °C, with controlled relative humidity (40% or 79% RH) and a reaction time of 3 weeks. Foams were produced from diluted aqueous solutions, at pH 7, of conjugates of pure protein and polysaccharide-proteins using nitrogen gas, at constant temperature (15 and 30 °C). Conjugation of lysozyme with dextran intensified foaming properties, while conjugation of BSA with dextran resulted in a small improvements in foaming properties. Conjugation of casein with dextran had a negative effect. The effects of conjugation were intensified with increasing polysaccharide molar mass. According to the authors, there is an ideal reaction time in the dry-heating technique for improving protein functionality by covalent complexation, which depends on the nature of the evaluated protein. A reduction of the functional properties is observed in a given reaction time, probably due to a greater reaction extent, indicating that advanced stages of the Maillard reaction have been reached.

Corzo-Martinez et al. (2012) evaluated foaming properties of  $\beta$ -lactoglobulin conjugated with galactose. The glycosylation, at pH 7, did not significantly alter the interfacial characteristics and foaming properties compared to the control (protein heated in the absence of galactose). Moreover, the conjugates showed better dynamic adsorption in the air-water interface at pH 5, resulting in a better foaming ability compared to the control. According to the authors, the conjugation galactose with  $\beta$ -lg through the Maillard reaction is an alternative to consider when using this protein as a foaming agent. In this case, the applicability of  $\beta$ -lg can be used as a foaming agent in acid foods, such as carbonated beverages enriched with protein (fruit juices, sports drink beverage and varieties of these beverages with long shelf lives).

### ***Solubility and thermal stability***

Solubility directly affects other techno-functional properties of foods, such as emulsifying, foaming and gelling properties, as well as viscosity. Therefore, solubility is a key functional property for developing new formulations and food products.

Researchers have shown protein solubility increases after glycosylation (Qi et al., 2009; Mu et al., 2011; Li and Yao, 2009). Mu et al. (2011) evaluated the solubility of soy protein isolate-acacia gum conjugates. The solubility of conjugates was significantly higher than the mixture of soy protein isolate and acacia gum, without heat treatment and protein isolates at the same pH value. Jimenez-Castano et al. (2007) studied the glycosylation of whey proteins,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin and BSA with dextran, with molar mass (10 and 20 kDa) at 60 °C and a water activity of 0.44. Conjugates exhibited an increase in solubility at low pH, probably due to covalent bonding with polysaccharides, reducing the protein isoelectric point. Kasran et al.

(2013b) prepared conjugate of soy whey protein isolate (SWPI) with hydrolysed and unhydrolysed purified fenugreek gum. The protein solubility of SWPI-fenugreek gum conjugates improved compared to SWPI and SWPI-fenugreek gum mixture especially at the isoelectric protein point, when assessed in the pH range 3-8 at 22 °C.

However, some authors report decreased protein solubility as a result of the Maillard reaction. Al-Hakkak and Al-Hakkak (2010) observed a reduction in egg white protein-pectin conjugate solubility with increased reaction time. Alvarez et al. (2012) observed a decrease in the solubility of conjugates from porcine blood protein isolate and dextran.

This discrepancy may be attributed to the biochemical complexity of proteins as well as the reacting polysaccharide. It has been suggested that the increased solubility results from the limited degree of glycosylation, since the restricted attack of hydrophilic sugar residues to the protein should reduce the trend of protein self-association. However, the occurrence of disulfide bridges, as well as cross-linking among peptides, must be also considered as factors capable of causing the reduction of protein (Oliver et al., 2006).

Thermal stability analysis is widely used in the food industry to determine protein resistance to heat treatment. Heat treatment may damage the protein, resulting in loss of protein structure and precipitation, as well as causing a decrease in protein nutritional value. Studies show that the thermal stability of the glycosylated protein increases when compared to the native protein (Jimenez-Castano et al., 2007; Shu et al., 1996; Alvarez et al., 2012).

According to Alvarez et al. (2012), conjugates from isolated swine blood protein with dextran have better resistance to subsequent aggregation and precipitation after heat treatment. Dextran

prevents protein aggregation, avoiding protein-protein interactions and resulting in the protection of the protein native structure.

## FINAL CONSIDERATIONS

The Maillard reaction, performed under controlled conditions, is one of the few methods to achieve covalent bonds between food components without any risk to consumer health, since this reaction is accomplished without the addition of any toxic compounds. Additionally, the glycosylation of proteins via the Maillard reaction can improve techno-functional properties of proteins and has potential for use in matrixes for the controlled release of bioactive agents. However, additional studies to determine the feasibility of industrial applications of conjugates obtained via the Maillard reaction remain necessary. These studies must: i) develop and adapt methods for preparing conjugates with excellent functionality in different food systems, ii) provide fundamentals for better understanding the relationship between the conjugate structure and functional properties.

Moreover, food protein glycosylation via the Maillard reaction is an area of research with potential use in the food industry. A better understanding of the synthesis and characteristics of these conjugates will allow their use in the food industry as ingredients to improve functional properties of food products.

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**Table 1.** Formation, characterization and functionality of food conjugates obtained via Maillard reaction

System	Reaction condition	Characterization technique	Application	Reference
Lysozyme and dextran	Dry-heating method. Mixture of protein:dextran, 1:5 mass ratio (w/w), (7.0 pH) was lyophilised. The product was incubated at 60 °C, 79% RH for 3 weeks.	Determination of free amino groups (TNBS method).	Improved emulsifying and antimicrobial properties of conjugates compared to pure protein.	Nakamura et al., 1991
Casein and dextran or galactomannan	Dry-heating method. Mixture of protein:polysaccharide, 1:3 mass ratio (w/w) was lyophilised. The obtained products were incubated at 60 °C, 79% RH for 24 h.	SDS-PAGE electrophoresis and determination of free amino groups by TNBS method.	Improved emulsifying properties of conjugates compared to pure protein.	Kato et al., 1992
Lysozyme and galactomannan or xyloglucan	Dry-heating method. Mixture of protein:galactomannan, 1:4 molar ratio and protein:xyloglucan 1:8 molar ratio, were lyophilised. The obtained product was incubated at 60 °C, 79% RH for 2 weeks.	SDS-PAGE electrophoresis and determination of free amino groups by TNBS method.	Emulsifying properties of conjugates were increased proportionately to the size of the polysaccharide, while conjugate thermal stability was increased regardless of the saccharide molar mass.	Shu et al., 1996
Soy protein and chitosan	Dry-heating method. Mixture of protein:dextran, 1:1 mass ratio (w/w), was lyophilised and the obtained product was incubated at 60 °C, 65% RH for 14 days.	SDS-PAGE electrophoresis	Allergens were reduced and antimicrobial and emulsifying properties increased compared to pure protein.	Usui et al., 2004

System	Reaction condition	Characterization technique	Application	Reference
Whey protein and dextran	Dry-heating method. Mixture of protein:dextran, 1:2 and 1:6 mass ratio, were lyophilised. The obtained product was incubated at 60 °C with 0.44 Aw and 55 °C with 0.65 Aw for 14 days.	Determination of free amino groups (TNBS method). Measurement of absorbance (420 nm)	Improved emulsifying properties of conjugates. Improved solubility and thermal stability at acid pH.	Jimenez-Castano et al., 2007
Albumin protein and fructose or inulin	Dry-heating method. Mixture of protein:sugar, 2:1 mass ratio, at pH 7.0 was lyophilised with the obtained product incubated at 60 °C, 79% RH for 3 days.	Fluorescence absorbance (420 nm) and colourimetric measurements.	Improved emulsifying and antioxidant activity of conjugates compared to pure protein.	Jiang et al., 2009
Soy protein and dextran	Dry-heating method. Mixture of protein:sugar, 1:1 mass ratio (w/w) was lyophilised. The obtained product was incubated at 60 °C, 79% RH for 1 to 7 days.	Absorbance measurement (470 nm) and SDS-PAGE electrophoresis.	Improved thermal stability and emulsifying properties of conjugates compared to pure protein.	Qi et al., 2009
Deaminated wheat protein and glucose or maltodextrin of various molar masses	Dry-heating method. Mixture of protein: carbohydrate in varying proportions according to the type of carbohydrate, with 2:1 molar ratio of -NH <sub>2</sub> available and glucose or reducing sugar of various maltodextrins at pH 6.5. The mixture was lyophilised and the lyophilised powder was incubated at 60 °C, 75% RH, for 24 h.	SDS-PAGE electrophoresis, amino acid composition analysis. Size-exclusion chromatography. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and circular dichroism.	The study evaluated the impact of glycosylation on protein secondary structure.	Wong et al., 2009

System	Reaction condition	Characterization technique	Application	Reference
Bovine serum albumin (BSA) and dextran	Dry-heating method. Mixture of BSA and dextran, 1:1 molar ratio, at pH 8.0 was lyophilised. The lyophilised powder was heated to 60 °C, 79% RH for 24 h.	SDS-PAGE electrophoresis.	Conjugates were used to produce nanoparticles to entrap and control the release of ibuprofen, a model compound.	LI and Yao, 2009
β-lactoglobulin and lactose or glucose	Dry-heating method. Mixture of protein and sugar, (1:10 and 1:100) molar ratio, at pH 7.0, was lyophilised. The lyophilised powder was stored at 50 °C, 65% RH for 96 h.	MALDI-TOF mass spectrometry, fluorescence measurement, SDS-PAGE electrophoresis and colourimetry.	Improved conjugate properties compared to pure protein. Foaming properties were more stable in the protein glycosylated with lactose than the protein glycosylated with glucose.	Medrano et al., 2009
β-conglycinin and dextran	High pressure method. Mixture of protein and dextran at pH 7.2, 1:1 mass ratio (w/w). The reaction was done in a solution with a 10 MPa thermal control at 100 °C for (1 to 6) h.	Absorbance measurement (420 nm), free amino acid group determination by OPA test, SDS-PAGE electrophoresis and circular dichroism.	Improved conjugate emulsifying properties compared to pure protein.	Xu et al., 2010
Milk protein and glucose, lactose, pectin and dextran	Dry-heating method. Mixture of protein:carbohydrates, 1:2 mass ratio (w/w), was lyophilised and the obtained product was incubated at 70 °C and 79% RH for varying times.	SDS-PAGE electrophoresis, free amino acid group determination by OPA test, amino acid analysis.	Improved functionality depending on the system type and reaction conditions.	Hiller and Lorenzen, 2010

System	Reaction condition	Characterization technique	Application	Reference
Casein and glucose	Heating in solution method. Mixture casein:glucose, 1:2 mass ratio (w/w), dissolved in water at a final concentration of 20% (w/v) and pH 12. The solution was heated to 100 °C for 130 min.	Size-exclusion chromatography. SDS-PAGE electrophoresis, Reversed-phase chromatography and infrared.	Conjugates with improved antioxidant activity compared to pure protein.	Gu et al., 2010
Egg white protein and pectin	Dry-heating method. Mixture of protein:sugar, 1:1 mass ratio (w/w) was lyophilised and the product incubated at 60 °C and 79% RH for (6 to 18) h.	SDS-PAGE electrophoresis and free amino acid group determination by TNBS method.	Improved rheological and emulsifying properties of conjugates, depending on reaction time. The solubility of the conjugate diminished with reaction time.	Al-Hakkak and Al-Hakkak, 2010
BSA oligosaccharides	Dry-heating method. Mixture of protein:carbohydrate, 1:2 mass ratio (w/w) lyophilised with the obtained product incubated at 60 °C and 43% RH for (6 to 12) h.	SDS-PAGE electrophoresis, fluorescence measurement and MALDI-TOF mass spectrometry.	Prophylactic agent of bacterial infections.	Ledesma-Osuna et al., 2010
BSA and dextran	Dry-heating method. Mixture of BSA:dextran, 2:1 molar ratio pH 8.0 lyophilised with the product heated to 60 °C and 79% RH for (18 to 48) h.	SDS-PAGE electrophoresis.	Developed conjugates were used in nanoparticle elaboration with high encapsulation capacity of doxorubicin. Conjugates showed potential use in controlled release systems.	Deng et al., 2010

System	Reaction condition	Characterization technique	Application	Reference
Whey protein isolated (WPI) and dextran	Heating in solution method. Aqueous solution with 10% (w/w) of WPI and 30% of dextran at pH 6.5 heated to 60 °C for 48 h.	SDS-PAGE electrophoresis, Size Exclusion Chromatography Multi-Angle Laser Light Scatter (SEC MALLS).  Protein analysis by BCA method (bicinchoninic acid).	Conjugates showed improved solubility, thermal stability and emulsifying properties compared to WPI.	Zhu et al., 2010
BSA and dextran	Pulsed electric field method. Mixture of equal proportions BSA and dextran at pH 10. The mixture was subjected to an electric field.	Free amino acid group determination by OPA test, Absorbance measurement (420 nm), SDS-PAGE electrophoresis and circular dichroism.	Improved antioxidant activity; influenced by reaction time.	Guan et al., 2010
Whey protein isolated (WPI) and dextran	Pulsed electric field method. Mixture of WPI:dextran, 1:1 mass ratio (w/w). WPI concentration was adjusted to 1%. The mixture was stirred for 2 h, then subjected to pulsed electric fields.	Absorbance measurement (420 nm), SDS-PAGE electrophoresis, free amino acid group determination by OPA test and circular dichroism.	Improved solubility and emulsifying properties of conjugates compared to WPI.	Sun et al., 2011
Soy protein isolate (SPI) and acacia gum	Heating in solution method. Mixture of protein:polysaccharide, 1:1 mass ratio (w/w), was heated at 80 °C for 48 h.	SDS-PAGE electrophoresis, free amino acid group determination by OPA test.	Improved solubility and emulsifying properties of conjugates compared to SPI and protein-gum mixture.	Mu et al., 2011

System	Reaction condition	Characterization technique	Application	Reference
Wheat germ protein and xylose, glucose or galactose	Heating in solution method. Mixture of protein:sugar, 1:1 mass ratio (w/w) made with deionised water at pH 11.0 and heated to 90 °C for 50 min.	Absorbance measurement (420 nm), determination of substitution degree by TNBS method, fluorescence measurement, scanning electron microscopy (SEM), circular dichroism and amino acid analysis.	Improved functional properties of conjugates compared to pure protein. Results indicated carbohydrate size is important for conjugate functional properties.	Niu et al., 2011
WPI and maltose	Dry-heating method. Mixtures with different proportions of WPI:maltose were lyophilised. Resultant powder was incubated at different temperatures and 79% RH for different times.	Absorbance measurement (420 nm), free amino acid group determination by TNBS method and SDS-PAGE electrophoresis.	Diminished antigenicity of $\alpha$ -lactalbumin and $\beta$ -lactoglobulin.	Li et al., 2011
$\beta$ -lactoglobulin and sodium caseinate and galactose or lactose	Dry-heating method. Mixtures of protein:carbohydrate at varied proportions and conditions at pH 7.0 were lyophilised. The product obtained was maintained at 50 and 60 °C with (0.44 and 0.64) Aw.	MALDI-TOF mass spectrometry, Liquid chromatography electrospray ionisation tandem mass spectrometry (LC/ESI-MS) and fluorescence measurement.	Under controlled conditions, conjugates avoid adhering to pathogens such as <i>E. coli</i> . This is related to mucous colonization.	Laparra et al., 2011
Milk protein and corn fiber gum (CFG)	Dry-heating method. Mixture of protein:CFG, 3:1 mass ratio (w/w) was lyophilised. The lyophilised powder was heated to 75 °C and 79% RH for 2 days.	Size Exclusion Chromatography, Scanning electron microscopy (SEM) and nuclear magnetic resonance (NMR).	Improved stability of emulsion formed by conjugates compared to those formed by protein and CFG.	Yadav et al., 2012

System	Reaction condition	Characterization technique	Application	Reference
$\beta$ -lactoglobulin and galactose	Dry-heating method. Mixture of protein:galactose, 1:1 mass ratio (w/w), at pH 7.0 was lyophilised and the resultant powder was maintained at 40 and 50 °C, 0.44 Aw, for (24 or 48) h.	MALDI-TOF mass spectrometry.	Solubility variation of conjugates related to pure protein was pH dependent. Foaming properties were improved by protein glycosylation.	Corzo-Martínez et al., 2012
Protein isolated from porcine blood and dextran	Dry-heating method. Mixture of protein:dextran, 1:3 mass ratio (w/w), was lyophilised. Resultant powder was maintained at (70, 75 and 80) °C.	SDS-PAGE electrophoresis and free amino acid group determination by TNBS method.	Although conjugates had diminished solubility compared to pure protein, they presented improved thermal stability, emulsifying capacity and gelling properties.	Álvarez et al., 2012
Lysozyme and galactose, galacto-oligosaccharide and potato galactan	Dry-heating method. Mixture of protein:sugar, 1:7 mass ratio (w/w), at pH 7.0 was lyophilised. The obtained product was incubated at 60 °C, under differing Aw conditions: 0.79, 0.65 and 0.45 for (1 to 11) days.	Determination of free amino group by TNBS test, furosine analysis for glycosylation degree estimating and Mass spectrometry (ESI-MS).	Conjugates with prebiotic activity.	Seo et al., 2012
Rice dregs glutelin and $\kappa$ -carrageenan	Dry-heating method. Mixture of protein:polysaccharide, 1:2 mass ratio (w/w), was lyophilised. The resultant powder was heated to 60 °C, 79% RH for varying time periods.	Free amino acid group determination by OPA test, SDS-PAGE electrophoresis, infrared (FT-IR) and circular dichroism.	Higher solubility and emulsifying properties of conjugates compared to pure protein.	Du et al., 2012

System	Reaction condition	Characterization technique	Application	Reference
Peanut protein isolate and dextran	Dry-heating method. Mixture of protein:polysaccharide, 1:1 mass ratio (w/w), was lyophilised. The obtained powder was heated at 60 °C, 79% RH for 7 days.	SDS-PAGE electrophoresis, differential scanning calorimetry (DSC) and fluorescence emission spectroscopy.	Increased thermal stability, solubility, emulsifying and foaming properties of conjugates compared to pure protein isolate.	Liu et al., 2012b
Casein and maltodextrin	Dry-heating method. Freeze-dried solutions of caseinate and maltodextrin at different molar ratios were heated to 60 °C, 79% RH for (4, 6 and 8) h.	Free amino acid group determination by OPA test, SDS-PAGE electrophoresis.	Developed conjugates were used in nanoencapsulation of hydrophobic nutraceuticals to enrich clear beverages.	Markman and Livney, 2012
Rice protein hydrolyzate and glucose, lactose, maltodextrins and dextrins of different molar masses	Heating in solution method. Solutions prepared by adding saccharides (1% w/w) and protein (1% w/w). Solutions were incubated at 100 °C for 40 min.	Free amino acid group determination by TNBS method, absorbance measurement (420 nm), fluorescence measurement, hydrophobicity measurement and amino acid analysis.	Emulsifying properties and solubility of conjugates increased with increasing degree of substitution up to a certain value.	Li et al., 2013
Soy whey protein and fenugreek gum (hydrolysed and unhydrolysed)	Dry-heating method. Mixture of protein:gum, at different mass ratios (w/w), was lyophilised. The obtained powder was heated to 60 °C, 79% RH for 3 days.	SDS-PAGE electrophoresis, high performance size exclusion chromatography.	Improved functionality depending on the system type and mass ratio.	Kasran et al., 2013a

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System	Reaction condition	Characterization technique	Application	Reference
WPI and dextran (15-25kDa)	Dry-heating method. Mixed solutions of WPI (12% w/w) and DX (3.6 to 10.8% w/w), pH 7.0, were lyophilised. The powders obtained were heated to 60 °C, 63% RH for (2, 5 or 9) days.	Colour measurement, Free amino acid group determination by OPA test.	Maillard reaction significantly modified the mechanical properties of conjugate gels, preventing fracture under test conditions.	Spotti et al., 2013

## Figure Captions

Figure 1. Scheme of the Maillard reaction adapted from Hodge (1953).

Figure 2. Stages of the Maillard reaction and sub-products characteristics.

Figure 3. Major steps involved in the synthesis of conjugates where the Maillard. Reaction occurs under dry-heating (A) and heating in aqueous solution (B).

Figure 4. Schematic representation of protein-polysaccharide bond during Maillard reaction.

Figure 5. SDS-PAGE analysis that evidence the formation of conjugates obtained from dextran and soy protein reaction: molecular marker (line 1), soy protein (line 2), and soy protein-dextran conjugate of 1:3 (line 3), 1:6 (line 4) and 1:9 (line 5) of mass ratio, prepared after 12, 48 and 72 hours, respectively, at 60 °C and 79% RH. Reprinted (adapted) with permission from Xu and Yao, 2009. Copyright (2012) American Chemical Society.

Figure 6. Orthophtaldehyde (OPA) with primary amino groups reaction. OPA reacts with primary amino groups and with a compound which has SH group (such as  $\beta$ -mercaptoethanol) to produce a compound that will absorb light at 340 nm.

Figure 7. Reaction of TNBS with amino groups.

Figure 1

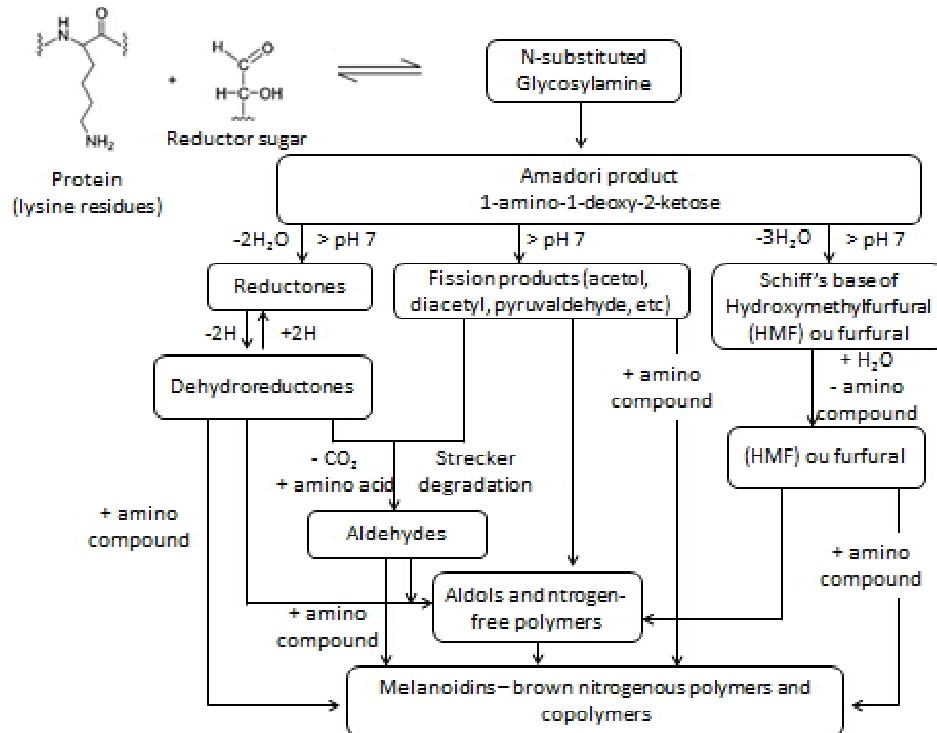


Figure 2

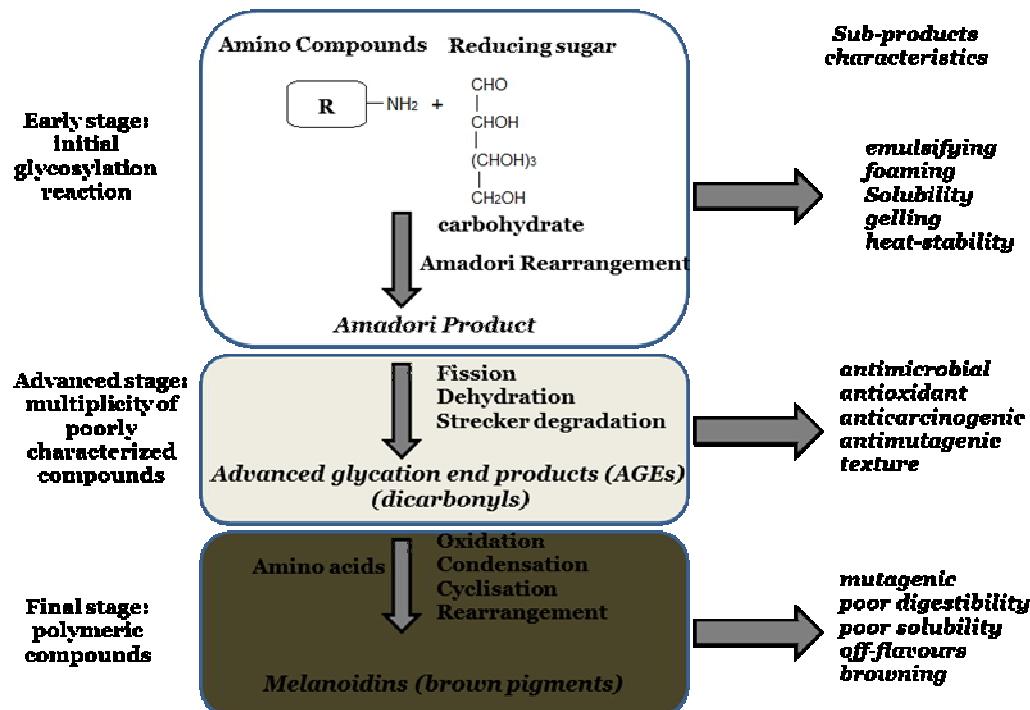


Figure 3

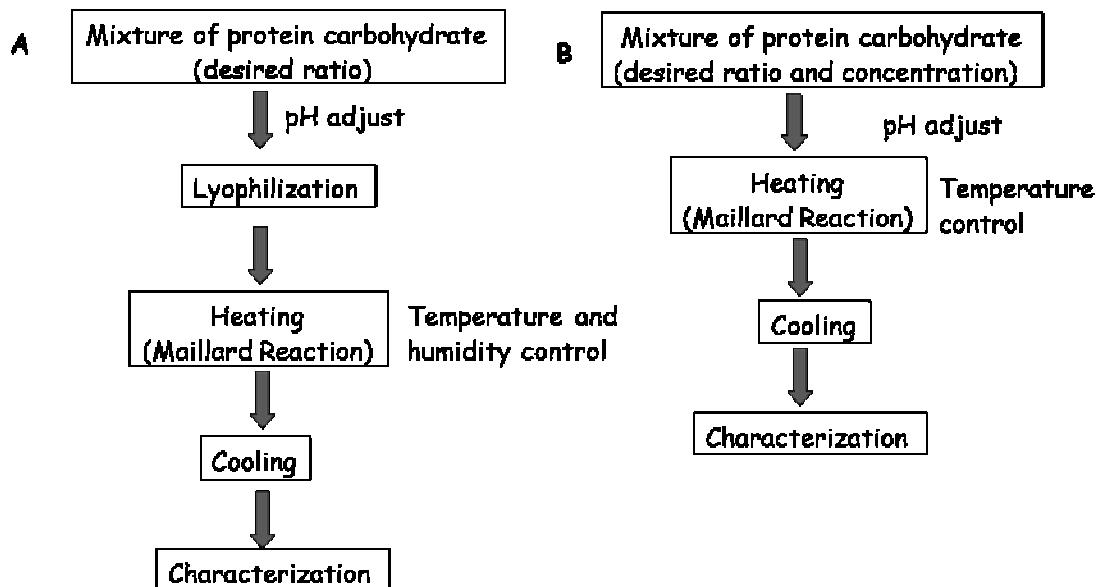


Figure 4

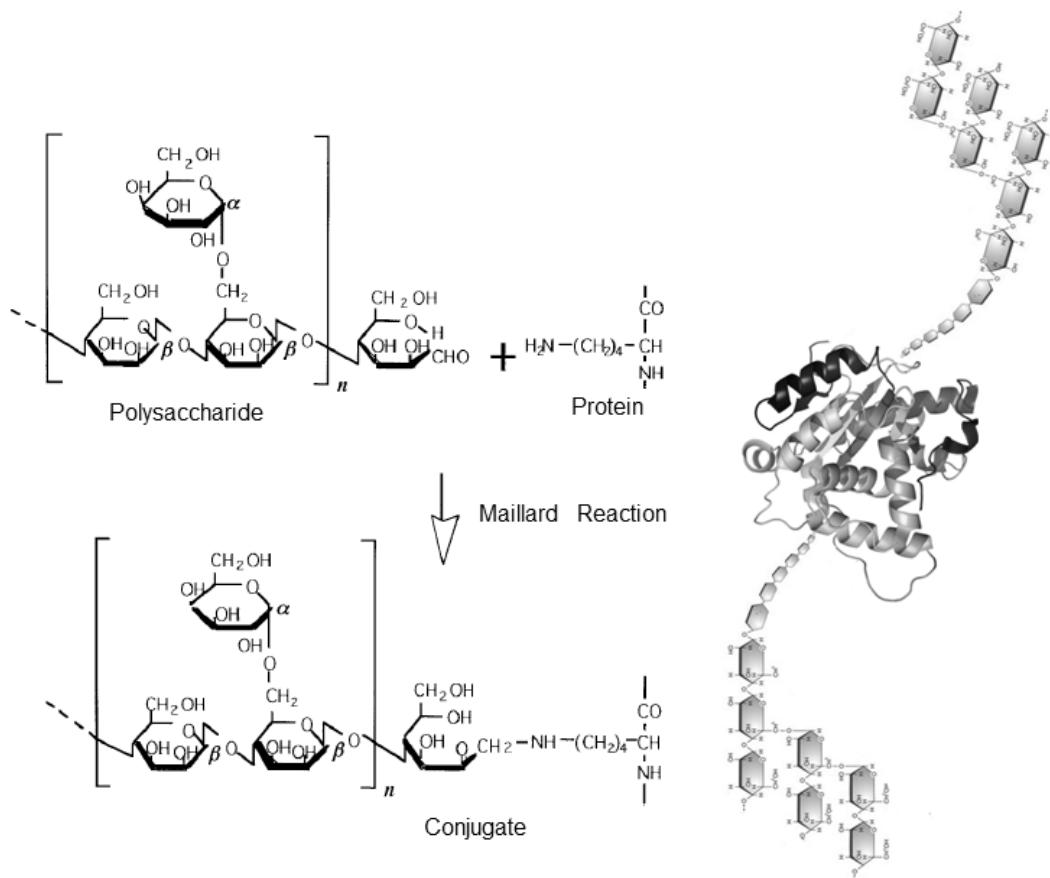


Figure 5

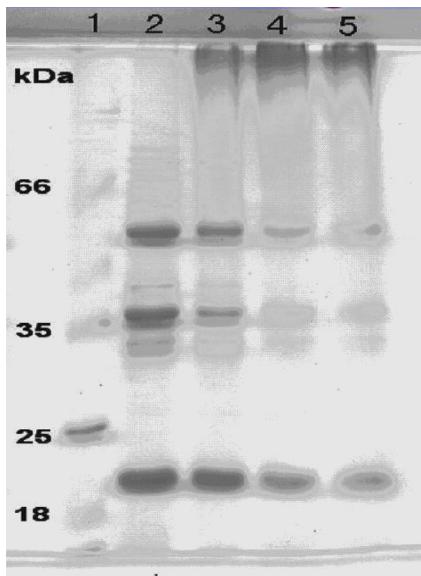


Figure 6

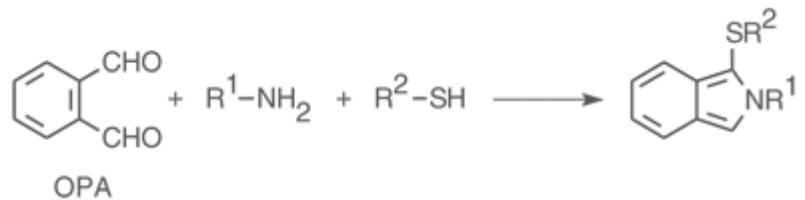


Figure 7

