Rheological and Structural Properties of Gels Cross-linked with Fungal Laccases

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität
Hannover

zur Erlangung des Grades
Doktorin der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation
von

Sanaz Khalighi, Karshenasi Arshad

[2020]

Referentin/Referent: [Prof. Dr. rer. nat., Dr. Ing. Ralf Günter Berger]

Korreferentin/Korreferent: [PD. Dr. rer. nat. Ulrich Krings]

Tag der Promotion: [19.06.2020]

Acknowledgments

This study was carried out in the Laboratory of Institute of Food Chemistry, Hannover, Germany.

I would like to express my sincere gratitude to Prof. Dr. Ralf Günter Berger, who provided me the opportunity to finish my PhD in the Laboratory of Institute of Food Chemistry and guided me in all the time of research. His immense knowledge, positive criticism, guidance and motivation have given me more power and spirit to excel in the research area.

I am especially grateful to Prof. Dr. Jakob Franke, PD. Dr. Ulrich Krings and PD. Dr. Sascha Beutel for their efforts in reviewing my thesis and shouldering the responsibilities as examiners and chairman.

I really thank all my colleagues and friends in the Institute of Food Chemistry. I would always remember your great supports and substantial help in every second of my work in the Institute.

Last but not the least, I owe a deep debt of gratitude to my parents and my husband, who are always incredibly supportive. Thank you for giving me the strength to chase my dreams.

Abstract

Arabinoxylans (AXs) and sugar beet fibre (Fibrex) are substances which have the potential of forming cross-linked hard gels. AXs, which are classified as hemicelluloses, are the major non-starch polysaccharides in wheat bran. Sugar beet pectin is the main soluble fibre in Fibrex. The cross-linking potential of AX and Fibrex refers to the ferulic acid units, which are ester linked to the molecules´ main chain. The more ferulic acid moieties are present on the polysaccharide backbone, the more covalent cross-links may be generated. A modified AX extraction method, in which the incubation time and concentration of alkaline extracts were optimised, has been applied to extract AX from wheat bran. In addition, a modified hydrothermal extraction method has been used to extract Fibrex.

The extractable AXs from wheat bran were cross-linked by the commercial laccase C (LccC) and self-produced laccases from *Funalia trogii* (LccFtr) and *Pleurotus pulmonarius* (LccPpu). Dynamic oscillation measurements on AX gels presented mechanical spectra, including storage modulus, loss modulus and loss factor of the gels. Gel samples demonstrated viscoelastic properties, which remained constant for four weeks in the gels formed with LccFtr and LccPpu. Arabinoxylan gel characteristics, including high water holding capacity, swelling ratio in saliva, and heat resistance indicated a covalently cross-linked network. Mediators (caffeic acid and ABTS) and citrus pectin did not enhance the elastic properties of the gels.

Fibrex was successfully cross-linked with LccFtr to an edible gel. Analysing rheological and structural characteristics of the Fibrex gels including high water holding capacity, swelling ratio in saliva, and heat resistance) showed the viscoelastic behaviour of the gels which was attributed to the covalently cross-linked network. However, vanillin, as a mediator, and citrus pectin did not enhance covalent cross-links and elastic properties of the Fibrex gels. Rheological properties and storage stability of sweetened Fibrex gels indicated a constant storage modulus during four weeks storage.

Rheological behaviour and structural properties of the Fibrex gels and AX gels were compared with gelatin gels as a reference. Using laccases as an oxidant provided gels with a solid and stable texture, comparable in firmness to traditional gelatin gels. Cross-linked gels may find uses in pharmaceutical and other industrial applications, which require a heat resistant gel that forms easily at room temperature. They also represent an ethical alternative for manufacturing vegan, halal, and kosher food.

Key words: Arabinoxylan gel; Fibrex gel; Laccase; Cross-linking; Rheology; Viscoelastic properties

Zusammenfassung

Arabinoxylane (AX) und Zuckerrübenfasern (Fibrex) haben das Potenzial, vernetzte harte Gele zu bilden. AXe, die als Hemicellulosen klassifiziert werden, sind die wichtigsten Nicht-Stärke-Polysaccharide in Weizenkleie. Zuckerrübenpektin ist der wichtigste lösliche Ballaststoff in Fibrex. Das Vernetzungspotential von AX und Fibrex beruht auf Ferulasäuresubstituenten, die mit der Hauptkette verestert sind. Je mehr Ferulasäureeinheiten auf dem Polysaccharid-Rückgrat vorhanden sind, desto mehr kovalente Vernetzungen werden erzeugt. Zur Extraktion von AX aus Weizenkleie wurde die AX-Extraktionsmethode modifiziert, indem Inkubationszeit und Konzentration der alkalischen Extrakte optimiert wurde. Für Fibrex wurde eine modifizierte hydrothermale Extraktionsmethode entwickelt.

Die extrahierbaren AX aus Weizenkleie wurden mit der kommerziellen Laccase C (LccC) und eigenen Laccasen aus *Funalia trogii* (LccFtr) und *Pleurotus pulmonarius* (LccPpu) vernetzt. Dynamische Oszillationmessungen an AX-Gelen ergaben mechanische Spektren, darunter Speichermodul, Verlustmodul und Verlustfaktor der Gele. Die Proben zeigten viskoelastische Eigenschaften, die in den mit LccFtr und LccPpu gebildeten Gelen vier Wochen lang konstant blieben. Die Eigenschaften des Arabinoxylan-Gels wie die hohe Wasserbindekapazität, das Quellverhältnisses und die Hitzebeständigkeit deuteten auf ein kovalent vernetztes Netzwerk hin. Mediatoren (Kaffeesäure und ABTS) und Zitruspektin verbesserten die elastischen Eigenschaften der Gele nicht.

Fibrex wurde mit LccFtr zu einem essbaren Gel vernetzt. Die Analyse der rheologischen und strukturellen Eigenschaften der Fibrex-Gele (hohe Wasserbindekapazität, Quellverhältnis und Hitzebeständigkeit) zeigte erneut das viskoelastische Verhalten eines kovalenten Netzwerks. Auch hier wurde weder mit Vanillin noch mit Zitruspektin eine Verbesserung der elastischen Eigenschaften erzielt. Rheologie und Lagerstabilität der gesüßten Fibrex-Gele zeigten ein konstantes Speichermodul während einer vierwöchigen Lagerung.

Das rheologische Verhalten und die strukturellen Eigenschaften der neuen Gele wurden mit Gelatinegelen als Referenz verglichen. Laccasen als Oxidationsmittel ergaben Gele mit einer festen und stabilen Textur, die mit traditionellen Gelatinegelen vergleichbar waren. Diese können in pharmazeutischen und anderen industriellen Anwendungen eingesetzt werden, die ein hitzebeständiges und bei Raumtemperatur rasch gebildetes Gel benötigen. Sie stellen zudem eine ethische Alternative für die Herstellung veganer, halaler und koscherer Lebensmittel dar.

Schlüsselwörter: Arabinoxylan-Gel; Fibrex-Gel; Laccase; Vernetzung; Rheologie; Viskoelastische Eigenschaften

Abbreviations list

ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure)
AX	Arabinoxylan
BMM	Buffered Minimal Medium
CA	Caffeic acid
Da	Dalton
Di-FA	Diferulic acid
DNSA	3,5-Dinitrosalicylic acid
DSC	Differential Scanning Calorimetry
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
FA	Ferulic acid
FTIR	Fourier Transform Infrared
h	Hours
HBT	1-Hydroxybenzotriazole
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography

Hz	Hertz
IR	Infrared
KOH	Potassium Hydroxide
LccC	Laccase from *Trametes versicolor*
LccFtr	Laccase from *Funalia trogii*
LccPpu	Laccase from *Pleurotus pulmonarius*
LC-MS	Liquid Chromatography- Mass Spectrometry
LVE	Linear Viscoelastic Region
mbar	Millibar
min	Minutes
mM	Millimolar
m/z	Mass-to-charge ratio
NaOH	Sodium Hydroxide
Pa	Pascal
Pa.S	Pascal.Second
Pectin C	Citrus Pectin
Phe	L-Phenylalanin

rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
SNL	Standard nutrient media
TEMPO	2,2,6,6-Tetramethylpiperidine-N-oxyl
TGA	Thermogravimetric Analysis
Tri-FA	Triferulic acid
U	Unit ($\mu mol.min^{-1}$)
UV	Ultraviolet
V	Volt
WBAX	Wheat bran arabinoxylan
WHC	Water Holding Capacity
W.N.	Week Number

Table of Contents

1. Introduction

1.1. Gels

There are different explanations to define the term "gel", but there is still no generally adopted definition of this term. Gels come under the subject of colloidal science and has been described in the Polymer Dictionary as "polymers and their swollen materials with three-dimensional network structures that are insoluble in any solvent." **(Nazir *et al.*, 2017)**. Gel formation is the phenomenon involving the association or cross-linking of the polymer chains to form a three dimensional network that traps or immobilises the water within it to form a rigid structure that is resistant to flow. In other words, it is an intermediate between a solid and liquid possessing both elastic and viscous characteristics **(Saha and Bhattacharya, 2010)**.

Hydrocolloids are a heterogeneous group of long chain polymers (polysaccharides and proteins) characterised by their property of forming viscous dispersions and/or gels when dispersed in water **(Saha and Bhattacharya, 2010)**. There are various industrially important gelling hydrocolloids from diverse origins, including: starch, pectin, cellulose (plant source and applied as thickeners and gelling agents), agar and carrageenan (derived from red seaweeds and consider as gelling agent), Alginate (extracted from brown seaweeds and used as gelling agent), curdlan (microbial source and applied as thickener and gelling agent) and gelatin (derived from an animal protein and widely used as gelling agent and thickener) **(Burey *et al.*, 2008)**. Among commercial hydrocolloids used in the food industry, gelatin has been regarded as special and unique, performing multiple functions with a wide range of applications in various industries. Gelatin has been used as a food ingredient (gelling and foaming agent), in the preparation of soft and hard capsule in pharmaceutical products and in the biomedical field **(Karim and Bhat, 2008)**. The issue of gelatin replacement has existed for many years for the vegetarian, halal and kosher markets, but there is a growing interest in the last decade, particularly within Europe with the emergence of bovine spongiform encephalopathy in the 1980s **(Morrison *et al.*, 1999)**. Many polysaccharides were introduced to food industry as gelatin alternatives, such as agar, alginate, modified starch and pectin (the latter was suggested as a replacement for gelatin in marshmallows production) **(Moorehouse, 2004)**. In addition, wheat bran arabinoxylan and sugar beet fibre (Fibrex) are polysaccharides which can act as substitutes for gelatin.

1.2. Wheat bran

Wheat grain has three main parts: endosperm (80- 85% of the grain), germ (3% of grain) and bran (13- 17% of grain) **(Micard *et al.*, 2015)**. Wheat bran is a by-product of wheat milling

of wheat into white flour and generated worldwide in enormous quantities (14- 19% of the grains' weight) **(Maes and Delcour, 2002)**. Wheat bran is a source of cell-wall polysaccharides and its major constituents are non-starch polysaccharides (46%), starch (10- 20%), proteins (15-22%) and lignin (4- 8%) **(Wang *et al.*, 2014)**. The main non-starch polysaccharides are arabinoxylan (70%), cellulose (24%) and (1–3), (1–4)- β-glucan (6%) **(Maes and Delcour, 2002)**.

1.2.1. Wheat bran arabinoxylan

Arabinoxylans are the most abundant hemicellulose polymers in wheat bran. They mainly consist of a chain of D-xylopyranosyl with β-(1–4) links, substituted on position 2 and/or 3 with α-L-arabinofuranosyl residues **(Jacquemin *et al.*, 2015)**. Arabinoxylan can have several substituents, including ferulic acid, which is mostly esterified to O-5 arabinose that is linked via an O-3 linkage to the xylose backbone **(Skendi and Biliaderis, 2016)** and 3-methoxy and 4-hidrocinnamic acid substituents **(Bataillon *et al.*, 1998)**. Figure 1.1 presents a chemical structure of a ferulated arabinoxylan.

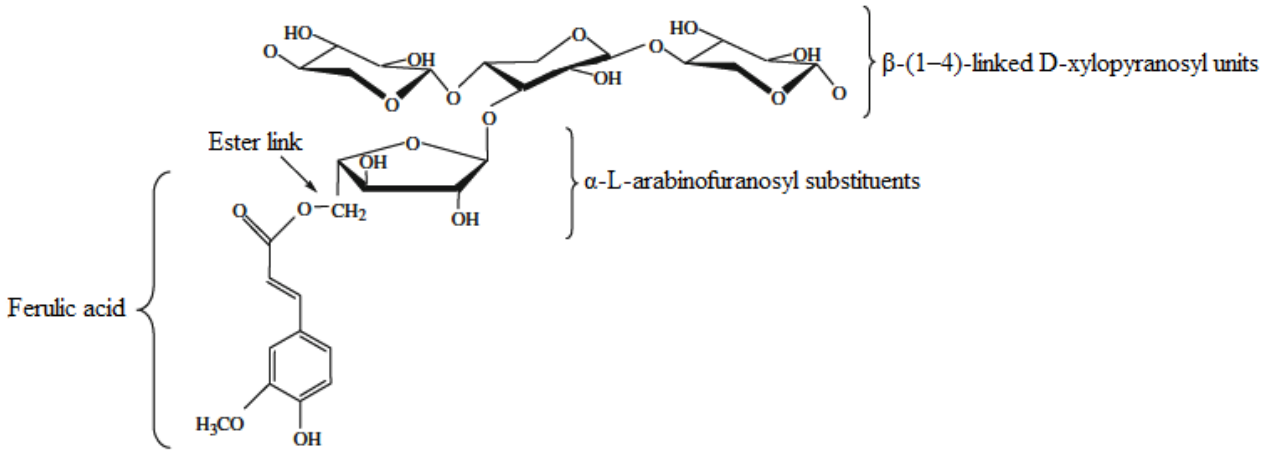


Figure 1.1. Chemical structure of a ferulated arabinoxylan **(Nino- Medina *et al.*, 2010)**

Arabinoxylans play a vital role in humans' health as well as food industry. Arabinoxylan is a dietary fibre, which resists digestion in the human small intestine and is fermented by the microbiota in the large intestine. Therefore, it is considered a prebiotic that assists in the production and growth of useful bacteria in the intestines. Health benefits of consuming arabinoxylan includes, cancer prevention, management and prevention of diabetes, assistance with weight management and lowering risk of heart disease **(Anderson and Simsek, 2018)**.

Arabinoxylans effectively contribute to various food industries to enhance food properties, such as textural characteristics, shelf life, water binding capacity, stability of foams and improvement baking qualities **(Hemdane *et al.*, 2016)**. In addition, arabinoxylans make highly viscous solutions which can form oxidative gels under the action of certain oxidizing agents **(Nino- Medina *et al.*, 2010)**.

1.2.2. Arabinoxylan extraction method

The most common method to isolate arabinoxylan from wheat bran is by water extraction. However, a limited amount of arabinoxylans can be isolated via water extraction method due to the fact that arabinoxylans are mostly enclosed in a complex matrix with other cell wall components or with themselves. Therefore, harsh methods such as chemical, enzymatic or mechanically assisted treatments have been applied **(Bender *et al.*, 2017a)**.

Alkaline solvents (including, NaOH, KOH and $Ca(OH)_2$) break the cell wall matrix by disrupting hydrogen and covalent bonds. Consequently, arabinoxylans are effectively solubilized from cell wall materials **(Bender *et al.*, 2017a)**. According to experiments on extraction rye arabinoxylan, increasing temperature (up to 70 °C) and higher pH (NaOH concentration) boost AX yield up to 3.3% **(Bender *et al.*, 2017b)**. On the other hand, the more severe extraction conditions, the less intact ferulic acids remains for cross-linking **(Berlanga-Reyes *et al.*, 2011)**. The ferulic acid (FA) content in wheat bran arabinoxylan (WBAX) is greatly affected by length of AX extraction time and pH of the system, and can be varied from 0.009 to 3 μg/mg WBAX **(Berlanga-Reyes *et al.*, 2011)**.

Arabinoxylans can also be solubilized by xylanases as an enzymatic extraction method which is not suggested for gel making purposes.

1.2.3. Arabinoxylan cross-linked gels

A unique property of arabinoxylans is their ability to form covalent gels by an oxidative coupling reaction which catalysed by different enzymatic systems, such as peroxidase/H_2O_2, manganese peroxidase, laccase/O_2 or chemical systems **(Nino- Medina *et al.*, 2010)**. The ability of arabinoxylan to gel depends on the concentration of polysaccharide, molecular weight and particularly the ferulic acid content **(Izydorczyk and Biliaderis, 2007)**. The oxidative gelation of arabinoxylan stems from the dimerization of ferulic acid residues of polysaccharide chains which leads to the formation of the three-dimensional network. The ferulic acid dimerization mechanism, which is shown in Figure 1.2, can be described as follow: first, an oxidizing agent attacks the H atom of the OH group at the ring position of ferulic acid and forms a phenoxy

radical. Then, this radical is stabilized by resonance and located at three different positions onto the whole molecule, two on the aromatic ring (C-4 and C-5) and one at the double bond (C-8) of its side chain. As the next step, the cross-linking between two phenoxy radicals is completed by coupling of unpaired electrons of two different radicals and forming a covalent linkage which connects two polysaccharide chains. Therefore, the structure of dimers depends on the radical position. Five diferulic acids have been detected in arabinoxylan gels, including: 8-5′, 8-O-4′, 5-5′, 8-5′ benzo, and 8-8′, in which 8-5′ and 8-O-4′ are the most abundant. In addition, the formation of superior ferulic acid oligomers, during gel formation, have been proposed by several authors **(Mendez- Encinas *et al.*, 2018)**.

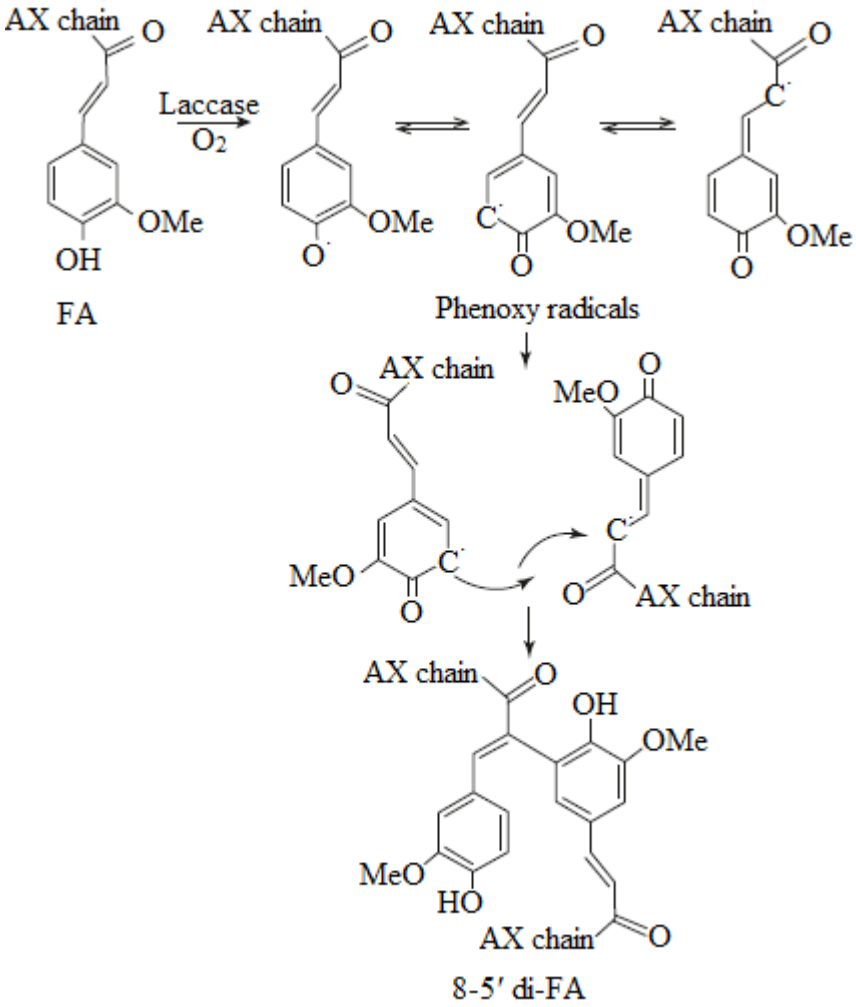


Figure 1.2. Schematic representation of FA dimerization **(Mendez- Encinas *et al.*, 2018)**

1.3. Sugar beet fibre

Cultivation of sugar beet is a major industry in Europe, USA and Russia. World production of sugar beet was 250 million tons per year in 2013. Sugar beet fibre is an agricultural by-product of beet processing, and its production yield is approximately 200 kg of beet pulp per ton of sugar beet root **(Sato *et al.,* 2013)**. Currently, the main utilization of sugar beet pulp is for cattle feed, but pectin can be extracted for a limited number of applications (stabilisation of flavoured oil emulsions in juice concentrates, lowering of cholesterol absorption from food, water-soluble pectin fibres and stabilisation of acidified drinking yoghurt) **(Holck *et al.,* 2011)**. The main fibre types in sugar beet fibre are insoluble hemicellulose and soluble pectin, and also cellulose and lignin **(Ağar *et al.,* 2016)**. Sugar beet pectin contains arabinan (α-(1, 5)-linked l-arabinofuranosyl residues), and/or galactan (β-(1, 4)-linked d-galactose residues) as side chains of the rhamnogalacturonan main chain **(Saulnier and Thibault, 1999)**. These side chains can be feruloyl substituted either on O-2 in the main backbone of rhamnogalacturonan -(1, 5)-linked arabinan, on O-5 in the terminal arabinose, or on O-6 in the main backbone of galactan. The content of ferulic acid can be up to 8.3 mg/g pectin **(Holck *et al.,* 2011)**.

1.3.1. Sugar beet fibre extraction

A large number of studies have addressed the extraction and properties of pectin from sugar beet pulp **(Pacheco *et al.,* 2019)**. Hydrothermal treatment has been introduced as an effective technology for Fibrex extraction which recovered most of the ferulic acid ester-linked to arabinan and/or galactan **(Sato *et al.,* 2013**). Autoclaving at 122 °C for 1 h increased the solubility, mainly of pectins and arabinan, and caused a growth in swelling (**Guillon *et al.,* 1992)**. Besides, the individual effects of pH and temperature were the most influential on the pectin yield. Indeed, at constant pH and temperature, the yields of pectin obtained for 1 h of extraction were lower than those for 4 h. The highest extraction yield was achieved when the dried beet pulp was treated at acidic pH, for 4 h at 90 °C. On the other hand, ferulic acid content in sugar beet pectin isolated at higher pH were slightly greater than acidic conditions **(Yapo *et al.,* 2007)**. Therefore, an autoclave treatment accompanying with 5 h heating in water at 95 °C, has been introduced as a non-chemical hydrothermal method, in which 45% of ferulic acid can be extracted **(Khalighi *et al.,* 2020a)**.

1.3.2. Sugar beet fibre cross-linked gels

A distinctive feature of sugar beet fibre is that contains feruloyl groups ester-linked to the sugar side chains. These substituents can be involved in cross-linking reactions with different enzymes, such as peroxidase/H_2O_2, manganese peroxidase, laccase/O_2 or chemical systems, leading to gel formation **(Levigne *et al.,* 2004)**. Previous experiments revealed that treatment of a sugar beet fibre with ammonium persulfate results in a gradual increase in viscosity, whereas the combination of hydrogen peroxide/peroxidase leads to an instantaneous increase in viscosity **(Oosterveld *et al.,* 2000)**. The amount of ferulic acid in oxidative cross-linked gels lowered by 78%, while an increase in ferulate dehydrodimers was observed. The most abundant dehydrodimers were 8-5′ and 8-O-4′ diferulic acids, also smaller amounts of the 5-5′ and 8-8′ were detected. A decrease in the total concentration of ferulic acids, indicated that a part of ferulates were converted to undefined oxidation products. It could be concluded that ferulic acid in beet pulp fibre is coupled into a variety of dehydrodimers via oxidative cross-linking reactions **(Oosterveld *et al.,* 1997)**. Figure 1.3 presents different enzymatically cross-linked ferulic acid dehydrodimers in sugar beet fibre.

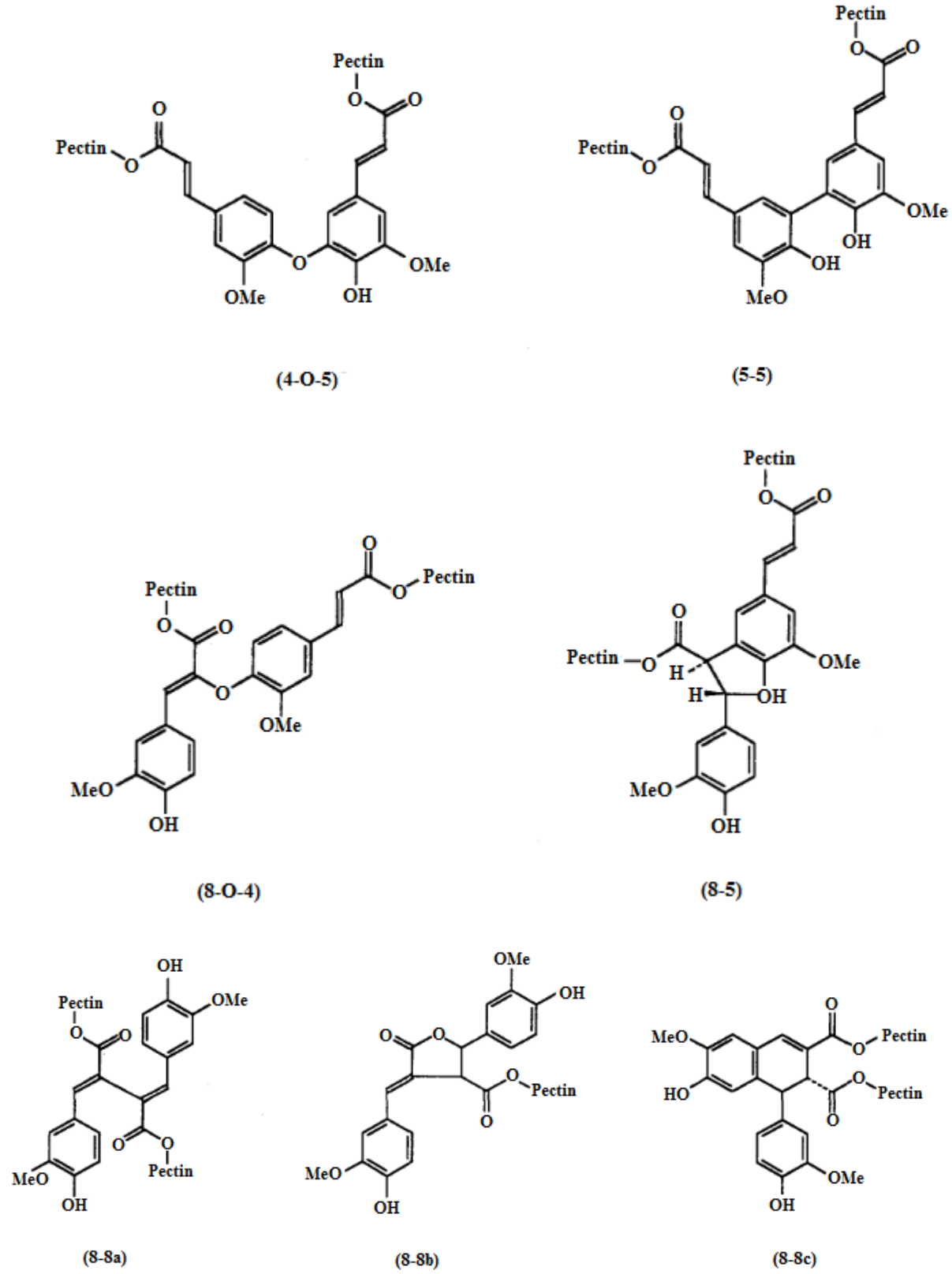


Figure 1.3. Structural representation of FA dehydrodimers in sugar beet fibre gel **(Oosterveld *et al.*, 1997*)***

1.4. Cross-linking agents

Cross-linking agent is an additive which links two polymer chains by the covalent or ionic bond **(Hanson and Wypych, 2019)**. Cross-linked polymers present unique physical properties, depending to degree of cross-linking and presence and absence of crystallinity. Cross-linking result in: elasticity, decrease in the viscosity, insolubility of the polymer, increase strength and toughness **(Maitra and Shukla, 2014)**.

Various oxidative cross-linking agents can provoke arabinoxylan and Fibrex gelation, including: ammonium persulphate, ferric chloride, hydrogen peroxide/ persoxidase, potassium periodate and laccase **(Figueroa-Espinoza and Rouau, 1998).**

Laccase and peroxidase are enzymatically cross-linking agents. However, they follow different oxidative mechanism within cross-linking reaction. Laccase oxidizes the substrate via direct interaction of the oxygen with the copper cluster in the enzyme, while peroxidase requires hydrogen peroxide as an oxidizing agent **(Carunchio *et al.*, 2001)**. These differences in the oxidation mechanisms of laccase and peroxidase could influence the gelation process and, consequently, the characteristics of the gels formed. It was reported that laccase was a better option than peroxidase for oxidative gelation intended for food, pharmaceutical and biotechnological applications **(Martínez-López *et al.*, 2019).**

1.4.1. Peroxidase

Peroxidases are oxidoreductases with molecular mass ranging from 30000 to 150000 Da, which are produced by a number of microorganisms, plants and animals. They are a group of oxidoreductases which catalyses the reduction of peroxidase, such as hydrogen peroxide and the oxidation of a variety of organic and inorganic substrates in the presence of peroxidase **(Adam *et al.*, 1999).**

Peroxidases are haem proteins and contain iron (III) protoporphyrin IX (ferriprotoporphyrin IX) as the prosthetic group. In initial step the native ferric enzyme is oxidised by hydrogen peroxide to form an unstable intermediate, called compound I, which has a haem structure of FeIV = O-porphyrin π-cation radical, and the reduction of peroxide to water has happened. Then compound I oxidases the electron donor substrates to give compound II, releasing a free radical. Compound II is further reduced by a second substrate molecule, regenerating the iron (III) state and producing another free radical **(Conesa *et al.*, 2002; Hofrichter *et al.*, 2010).**

Peroxidases are useful in a number of industrial and analytical applications, such as the treatment of waste water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from food stuffs and industrial waste **(Agostini *et al.*, 2002).**

Lignin peroxidase and manganese peroxides are enzymes associated with lignin degradation and polymerization **(Ward *et al.*, 2001; Sakurai *et al.*, 2003).** Degradation of carotene by peroxidases from *Lepista irina* and *Marasmius scorodonius* was also investigated **(Zorn *et al.*, 2003; Schibner *et al.*, 2008).**

Horseradish peroxidase (HRP) is a known enzyme with many applications in industry including, degrading phenolic and other organic compounds, oxidative polymerization via free radical, degrading and precipitating azo dyes and decolourisation of textile dyes **(Tatsumi *et al.*, 1996; Bhunia *et al.*, 2002).** Cross-linking of water extractable polysaccharides through peroxidase treatment is a method to modify products with designed functional properties. Oxidative cross-linking of ferulated pectins and arabinoxylans by horseradish peroxidase formed gels with restricted swelling ratio as well as little flexibility **(Robertson *et al.*, 2008).**

There are some obstacles to apply peroxidase, including inactivation of peroxidases by peroxides through the oxidation and sensitivity of peroxidase to the temperature (limited application at relatively low temperature) **(Sahare *et al.*, 2014).**

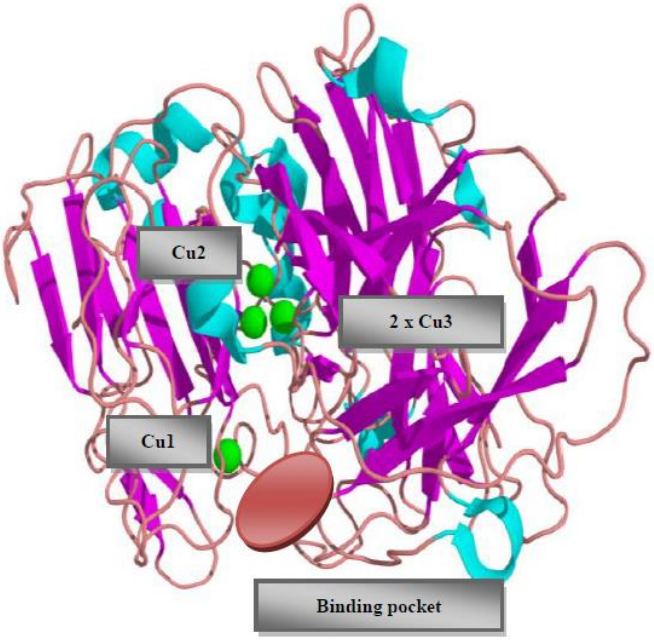
1.4.2. Laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multicopper oxidases. Typically, laccases are proteins with approximately 60-70 kDa and an acidic isoelectric point around pH 4.0. Laccases catalyse the reduction of oxygen to water accompanied by the oxidation of a substrate, typically a *p* -dihydroxy phenol or another phenolic compound **(Baldrian, 2006).** Nowadays, the interest of laccase has raised, due to the variety of the substrates, the use of the available oxygen as the electron acceptor, and no requirement for cofactors **(Strong and Claus, 2011).**

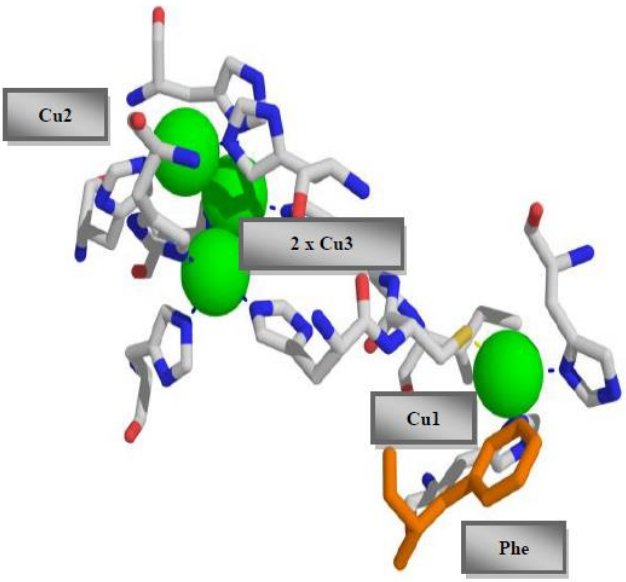
Laccase was discovered for the first time in plants by Yoshida (1883). Findings was based on the observation of rapid hardening of the latex in the Chinese or Japanese lacquer trees (*Rhus venicifera*) in the presence of air **(Yoshida 1883).** Gradually, numerous of reports focused on laccase enzymes in various plant tissues **(Bligny and Douce, 1983).** Afterwards, fungi and yeasts were introduced as an alternative source for more multicopper enzyme with phenol

oxidase activity **(Augustine *et al.*, 2008).** Some phenol oxidases laccase-like with enzymatic properties have been purified from insects **(Yamazaki, 1972).** Recently, classes of laccases have been found widespread in bacteria **(Claus, 2004; Sharma *et al.* 2007).** The first bacterial laccase was isolated from *Azospirillum lipoferum* **(Givaudan *et al.* 1993; Diamantidis *et al.* 2000).**

The comparison of the molecule structures of laccases from different sources has confirmed the fact that laccases generally need four copper atoms as functional catalytic groups. There are three major types of copper in a laccase catalyst. Type 1 is a blue copper with a maximum absorbance at 610 nm which corresponds to an intense blue colour. This absorption band is associated with the ligand-metal charge transfer from the sulphur atom of the cysteine ligand to the copper atom. This is the position where oxidation of the substrate takes place. The Type 2 centres containing conventional copper are similar to those observed for Cu (II) tetragonal complexes. The absorption spectrum of these centres frequently has a low intensity and is a non-blue copper. The Type 2 is connected by two histidines and one molecule of water. This position is very close to Type 3 and is connected by a strong hydrogen bridge. The Type 3 copper centre contains two copper ions bound to ligands and is called the binuclear site. The Type 3 sites are diamagnetic and exhibit an absorption band at 330 nm **(Thurston 1994; Solomon 1996 and 1998).** From a mechanistic point of view, the Type 1 copper is oxidized by substrate. This electron will then be transferred from Type 1 to Type 2 and Type 3, and in the last step the oxygen molecule is reduced to water. However, in this reaction, laccase uses oxygen as the electron acceptor to remove the proton from the substrate **(Gianfreda *et al.* 1999).** The Type 1 copper is connected with two histidines and one cysteine as ligands. Many studies have claimed that this axial ligand has influences on the oxidation potential, specificity and stability of the enzyme, however the catalytic efficiency (*kcat*/*Km*) of laccase has been related to their redox potential Type 1 copper and plays a major role in the overall performance of these enzymes **(Xu 1996a; Xu *et al.* 1996b)** (Figure 1.4).



A)



B)

Figure 1.4. A) Ribbon model of the X-ray crystal structure from *T. versicolor* Lac C. **B)** Cu- atom structure which shows Phe is responsible for redox potential **(Rodgers *et al.* 2010)**

Generally, the redox potential is defined as the energy that is necessary to capture one electron to reduce substrate and produce a cation radical **(Xu 1997; Lahtinen *et al.* 2009).** According to this definition and to compare laccase primary structures, laccases are divided in

3 major groups; laccases with higher redox potentials (730–780 mV; e.g. *Trametes versicolor*) occur when phenylalanine is the axial ligand of the enzyme **(Shleev *et al.* 2005)**, while laccases with middle -range redox potential (470–710 mV; e.g. *Ganoderma sp.*) carry leucine in the axial position **(Sharma *et al.* 2013)**, and lower redox potential laccases (340–490 mV; e.g. the bacterial CotA enzyme from *Bacillus subtilis*) have a methionine as the axial ligand **(Rodgers *et al.* 2010; Enguita *et al.* 2013).**

Although laccases are present in higher plants and fungi, the most studied group of enzymes to date is from fungal origin, including the genera of Ascomycetes, Deuteromycetes and especially Basidiomycete **(Brijwani *et al.*, 2010).**

1.4.2.1. Basidiomycetes as a source of laccases

Basidiomycota are a large family of fungi with more than 30,000 species that together with the Ascomycota, constitute the subkingdom Dikarya (Figure 1.5) **(Carlile and Watkinson, 1994).**

Figure 1.5. Basidiomycetes (*Pleurotus cornucopiae*) **(Carlile and Watkinson, 1994)**

The class of basidiomycetes contains some of the most common and familiar known fungi, including mushrooms, bracket fungi and puffballs. The majorities of this class are found

in woody plant materials and degrade plant residues **(Moore-Landecker, 1996).** There are about 12,000 species of fungi considered as mushrooms, with at least 2,000 edibles. More than 200 species have been used for different purposes. About 35 species have been cultivated commercially and 20 species are cultivated on an industrial scale **(Aida *et al.* 2009).**

Basidiomycete species act as lignocelluloses destroyers and include very different ecological groups such as white rot, brown rot, and leaf litter fungi **(Cho *et al.* 2009).** White rot basidiomycetes are the highest producers of laccase. Owing to the higher redox potential (+800 mV) of laccases, they are implicated in several biotechnological applications such as lignification, delignification, pathogenicity and detoxification **(Brijwani *et al.*, 2010).**

The main medicinal uses of laccase in Basidiomycetes reported so far, are as anti-oxidant, anti-diabetic, hypocholesterolaemia, anti-tumour, anti-cancer, immunomodulatory, anti-allergic, nephroprotective, and anti-microbial agents **(Jasim, 2019)**. Besides, fungal laccases have application in various industries **(Brijwani *et al.*, 2010).** Areas of the food industry that benefit from processing with laccase enzymes include baking, juice processing, wine stabilization, bioremediation of waste water and for improving sensory parameters of food **(Brijwani *et al.*, 2010; Ayala-Soto *et al.*, 2017; Flander *et al.*, 2008; Osma *et al.*, 2010).** The ability of laccase C to enhance the cross-linking in skimmed milk yogurt was also shown (**Struch *et al.*, 2015**). According to the recent research, laccases from *Funalia trogii* (LccFtr) and *Pleurotus pulmonarius* (LccPpu) successfully cross-linked ferulic acids in Fibrex and wheat bran arabinoxylan to form a hard gel **(Khalighi *et al.*, 2020a; Khalighi *et al.*, 2020b).**

1.5. The mechanism of enzymatic reaction

Enzymes are biocatalysts that reduce the required energy to convert a substance chemically into another one (Figure 1.6) **(Andres, 2008).** Principally, enzymes are proteins, but some of them contain additional non-protein compounds, such as lipids, metals, phosphate or some other organic moiety. The whole molecule of enzyme is called holoenzyme, while the protein part is known as apoenzyme, the rest of the molecule is called cofactor **(Copeland, 2000).**

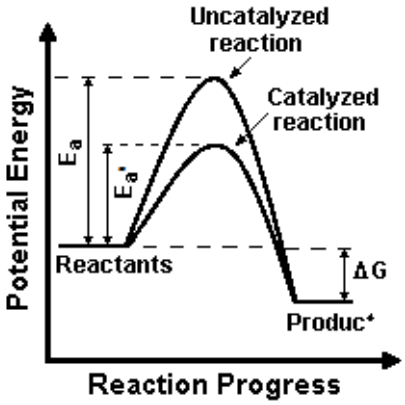


Figure 1.6. Catalyst mechanisms, Ea and Ea´ are the energies of activation of the non-catalyzed and catalyzed reaction. ΔG is the free energy change of the reaction **(Andres, 2008)**

Enzymes catalyze a biochemical reaction by binding a substrate at the active site. Once the reaction completes, the product leaves the active site and the enzyme can catalyze further reactions (Figure 1.7). The characteristics of enzymes as catalysts are derived from their molecular structure. Enzymes contain a number of amino acid residues that ranges from 100 to several hundreds. These amino acids are covalently bound through the peptide bond that is formed between the carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following. According to the nature of the R group, amino acids can be non-polar (hydrophobic) or polar (charged or uncharged) and their distribution along the protein molecule determines its behavior **(Lehninger, 1970).** According to this structure, some enzymes are so well designed for this purpose that they can accelerate the rate of chemical reactions by as much as 10^{12} times over the rate of the noncatalysed reaction **(Polaina and MacCabe, 2007).**

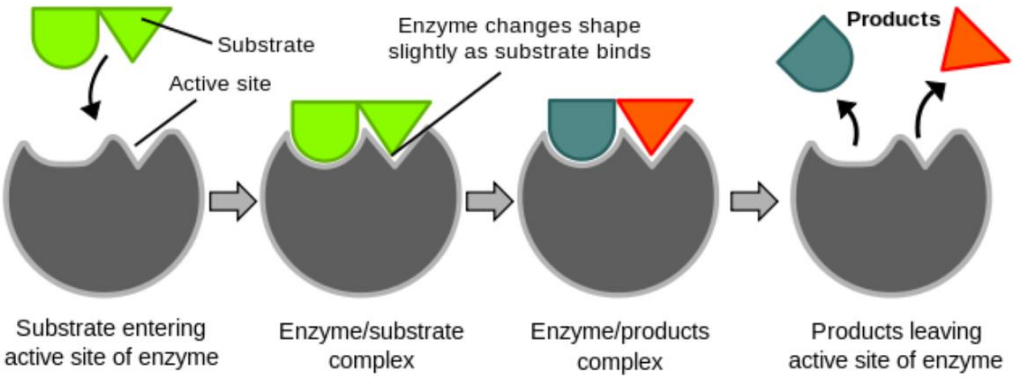


Figure 1.7. Model of enzyme catalysis action **(Lehninger, 1970)**

Enzymes have a number of advantages, such as high specificity, high activity under moderate conditions, high turnover number and biodegradability, but on the other hand they have disadvantages, such as high molecular complexity, denaturation at inappropriate pH and temperature **(Aehle, 2007).**

In some cases, enzymes cannot be directly involved in the reaction due to steric hindrance of the substrate, however small molecules which are called mediators and normally can be oxidised by enzyme, will participate in the reaction and can promote or facilitate enzyme action **(Elegir *et al.* 2005; Camarero *et al.* 2008).**

1.6. Mediators

Mediators can expand the catalytic activity of enzymes, such as laccase, by increasing the redox potential of the enzyme solution **(Kupski *et al.*, 2019).** When laccase is combined with a so-called mediator, oxidation of nonphenolic compounds is also possible. In such a laccase/mediator system, the mediator is first oxidized by the laccase, after which it can oxidize nonphenolic substrates via different mechanisms, such as electron transfer or radical hydrogen atom transfer (Figure 1.8) **(Riva, 2006; Hilgers *et al.*, 2018).**

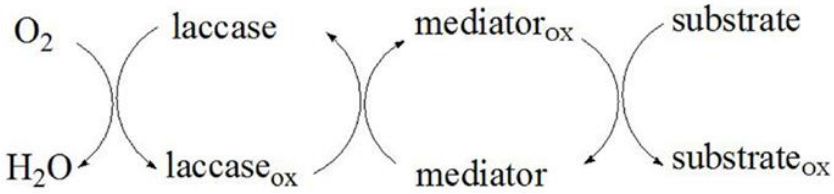


Figure 1.8. Proposed mechanism of substrate oxidation by laccase mediator system **(Lew Paul *et al.*, 2014)**

Synthetic mediator that have been widely used in laccase-catalyzed oxidation are 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,2,6,6-Tetramethylpiperidine-N-oxyl (TEMPO) and 1-hydroxybenzotriazole (HBT). Although the efficiency of laccase mediator systems to degrade aromatic compounds has been proven, the application of these mediators is hindered by the generation of toxic species. Therefore, mediators of natural origin are an excellent alternative due to their environmentally friendly approach **(Kupski *et al.*, 2019).** Ferulic acid, vanillic acid, caffeic acid and vanillin are examples of green mediators. Rheological properties of skim milk yogurt, in which caffeic acid and vanillin applied in the laccase mediator system, successfully enhanced (**Struch *et al.*, 2015**). In addition, impact of natural mediators, on developing rheological and structural properties of cross-linked polysaccharide gels, have recently been investigated **(Khalighi *et al.*, 2020a; Khalighi *et al.*, 2020b).**

1.7. Gels structural characteristics

A variety of techniques have been developed to analyze structural, microstructural and thermal properties of the gels. Rheological experiments, swelling ratio, Water Holding Capacity (WHC), Scanning Electron Microscopy (SEM) and FTIR spectroscopy are some common approaches to investigate structural properties of gels and polymers. There are also some techniques to measure thermal properties of gels, including: Melting point, Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA). In order to analyse microstructural properties of the gels, particularly detection of cross-linked bonds in the cross-linked gels, Liquid Chromatography Mass Spectrometry (LS-MS) is a known method **(Tokita and Nishinari, 2009; Takeno *et al.*, 2017; Basha, 2019; Khalighi *et al.*, 2020a; Khalighi *et al.*, 2020b).**

1.7.1. Rheology

Rheology is used to describe and assess the deformation and flow behaviour of materials. Fluids flow at different speeds and solids can be deformed to a certain extent. Oil, honey, shampoo, hand cream, toothpaste, sweet jelly, plastic materials, wood, and metals can be put in liquids, solids, and in between highly viscous, semi-solid substances (depending on their physical behaviour) **(Barnes, 2000)**.

1.7.1.1. Viscosity

Viscosity is a fluid's resistance to flow. It can also be explained in terms of a fluid's thickness. For example, water has very little resistance to flow, or very little viscosity. In other words, it isn't very thick. On the other hand, honey has more resistance to flow, more viscosity and is thicker.

The law of viscosity is defined as the ratio of shear stress to the shear rate.

$\eta = \tau/\dot{\gamma}$

where τ is shear stress (N/m^2 or Pa), $\dot{\gamma}$ is shear rate (1/s) and η is viscosity (Pa.s)

This law has some important practical applications such as formulations/injections, and food/beverage manufacturing **(Macosko, 1994).**

1.7.1.2. Fluid behavior

Fluids are characterized as Newtonian or non-Newtonian, depending on their viscosity behavior as a function of shear rate, stress and deformation history.

1.7.1.2.1. Newtonian fluids

Several types of flow behavior are generally recognized (Figure 1.9). The simplest is Newtonian behavior, with a linear relationship between stress and shear rate, which is the ideal fluid behavior. Water and honey are some examples of Newtonian fluids. Some fluids show plastic behavior (also called Bingham), in which flow only external stress exceeds the yield stress. Once the flow starts, they behave essentially as Newtonian fluids (i.e. shear stress is linear with shear rate). A great example of this kind of behavior is mayonnaise.

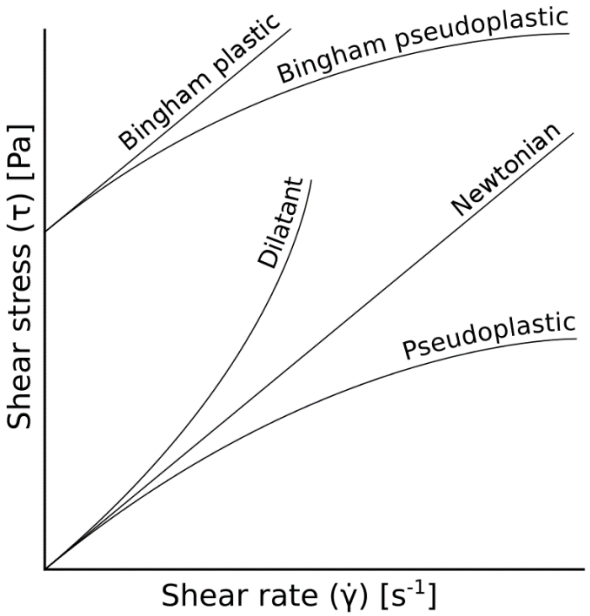


Figure 1.9. Types of rheological behaviour **(Struble and Ji, 2001)**

Newtonian fluids are normally comprised of small isotropic (symmetric in shape and properties) molecules that are not oriented by flow. However, it is also possible to have Newtonian behavior with large anisotropic molecules. For example, low concentration protein or polymer solutions might display a constant viscosity regardless of shear rate. It is also possible for some samples to display Newtonian behavior at low shear rates with a plateau known as the zero shear viscosity region **(Struble and Ji, 2001).**

1.7.1.2.2. Non-Newtonian behaviour

A non-Newtonian fluid is one whose flow curve (shear stress versus shear rate) is nonlinear and can even be time-dependent. Therefore, a constant coefficient of viscosity cannot be defined. In practice, many fluid materials exhibit non-Newtonian fluid behavior such as: salt solutions, molten, ketchup, custard, toothpaste, starch suspensions, paint, blood, and shampoo. The apparent viscosity of non-Newtonian fluids is not constant at a given temperature and pressure but is dependent on flow conditions such as flow geometry and shear rate. These material divides in three groups:

1. Fluids for which the rate of shear at any point is determined only by the value of the shear stress at that point. These fluids are known as “time independent”.

2. More complex fluids for which the relation between shear stress and shear rate depends, in addition, upon the duration of shearing and their kinematic history. They are called “time-dependent fluids”.
3. Substances exhibiting characteristics of both ideal fluids and elastic solids and showing partial elastic recovery, after deformation. These are categorized as “viscoelastic substances” **(Gan, 2012).**

1.7.1.2.2.1. Time independent non-Newtonian fluids

The most common type of time-independent non-Newtonian fluid behavior observed is Pseudo-plasticity or shear-thinning, in which viscosity decreases as shear rate increases. Materials that show this behavior are ketchup, paints and blood and fruit juice (Figure 1.9) **(Struble and Ji, 2001).**

Occasionally materials show thickening behavior (dilatant), in which the viscosity increases with increased shear rate, but this is not common (Figure 1.9). Materials that typically display such behavior include wet sand and concentrated starch suspensions **(Struble and Ji, 2001).**

Bingham pseudoplastic substances are similar to pseudoplastic fluids with the exception that they exhibit a yield stress level, i.e. the fluid behaves as a pseudoplastic fluid above the yield stress level (Figure 1.9). Some ketchups and tomato paste are examples of Bingham pseudoplastic substance **(Gan, 2012).**

1.7.1.2.2.2. Time dependent non-Newtonian fluids

Time-dependent fluids have more complex shear stress and shear rate relationships. In these fluids, the shear rate depends not only on the shear stress, but also on shearing time. These materials are usually classified into two groups, thixotropic fluids and rheopectic fluids, depending upon whether the shear stress decreases or increases in time at a given shear rate and under constant temperature (Figure 1.10) **(Joanna and Sabu, 2016).**

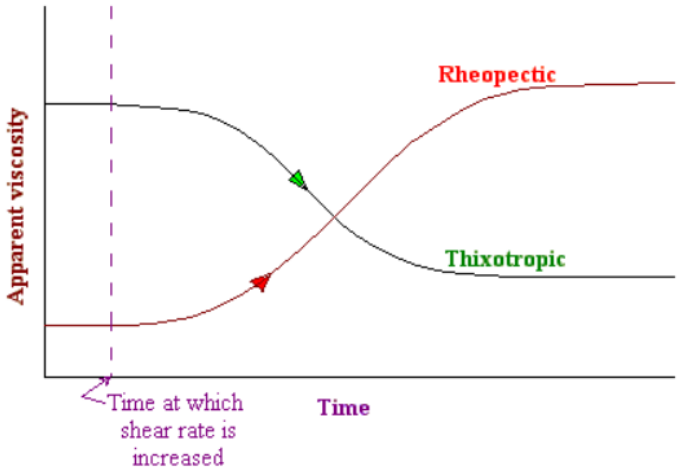
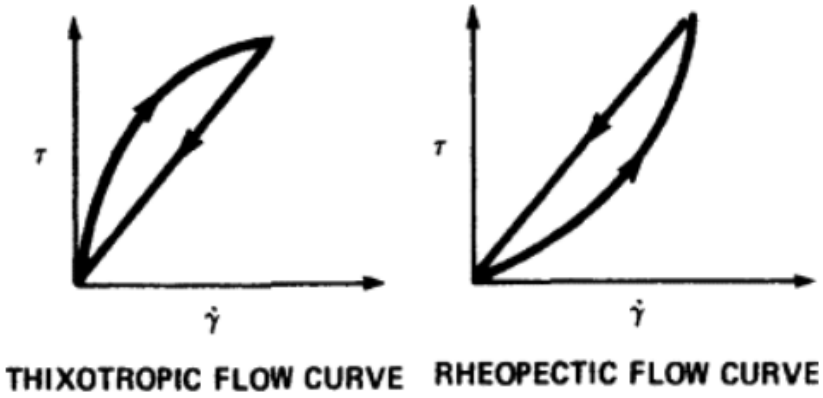


Figure 1.10. Time dependency of non-Newtonian fluids under a constant shear stress **(Gooch, 2007)**

Thixotropy is a time-dependent shear thinning property. Some gels or fluids, which are thick under static conditions, will flow over time under shear-stressed. Then, a gradual recovery of the structure occurs, when the stress is removed. The flow curve of a thixotropic fluid is shown in Figure 1.11 (A). A simple loop test with the increase in the share rate from zero to a maximum value and then a decrease to zero in the same way designates the flow curve. Many gels and colloids are thixotropic materials, exhibiting a stable form at rest but becoming fluid when agitated, for example yogurt. Thixotropy arises because particles or structured solutes require time to organize **(Joanna and Sabu, 2016).**

Rheopectic fluids are time-dependent non-Newtonian substances, which display shear-thickening behavior over time and under shear-stress. The longer the fluid undergo shear stress, the higher its viscosity. Gypsum pastes, printer ink and lubricants are some examples of Rheopectic fluids (Figure 1.11 B) **(Braun and Rosen, 1999).**



A) **B)**

Figure 1.11. Time-dependent rheological behaviour (τ: shear stress (Pa) plotted versus γ°: shear rate (1/s)) **(Joanna and Sabu, 2016)**

1.7.1.2.2.3. Viscoelasticity

As the name suggested, viscoelastic behaviour describes materials which show behaviour between of an ideal liquid (viscous) and ideal solid (elastic). Many materials display viscoelastic behaviour, such as gels, dough and gums **(Barnes, 2000)**.

In addition, the viscoelastic material has the distinctive characteristics when exposed to loading. As it is shown in Figure 1.12, the stress-strain curve of an ideal elastic is linear and the strain is recoverable.

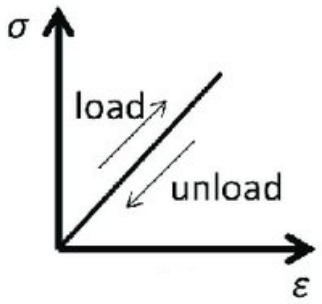


Figure 1.12. Loading and unloading curves of an ideal elastic model. (ε is the strain, σ is the stress) **(Wang and Pierscionek, 2018).**

While, a viscoelastic material under cyclic loading behaves differently during loading and unloading. In a sense, the stiffness of the material decreases during unloading when compared to the stiffness that material shows during loading. The area between loading and unloading curves called hysteresis that represents dissipated energy **(Naemi *et al.*, 2016).** A typical

hysteresis curve is shown in Figure 1.13, and the energy absorbed during one loading-unloading cycle is given by the area within the loop. The shape of the loop depends on the rates of loading and unloading (unlike normal time-independent elasticity) **(Dubruel and Vlierberghe, 2014).**

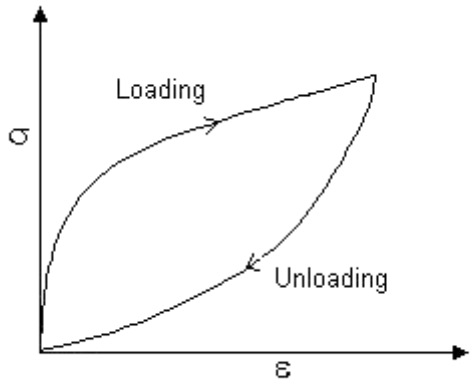


Figure 1.13. Loading and unloading curves of a viscoelastic model. (ε is the strain, σ is the stress) **(Zhang, 2005).**

Creep characterisation and stress relaxation are experiments to evaluate microstructure of viscoelastic materials. Creep is the variation of strain with time, when a constant stress is applied to the sample, and is a time-dependent mechanical behavior of materials. If the stress is instantaneously removed, the dependence of strain on time is defined as the recovery behavior **(Xu *et al.*, 2012).** Figure 1.14 presents creep and recovery phases of ideal elastic, ideal viscose and viscoelastic materials, under a constant stress.

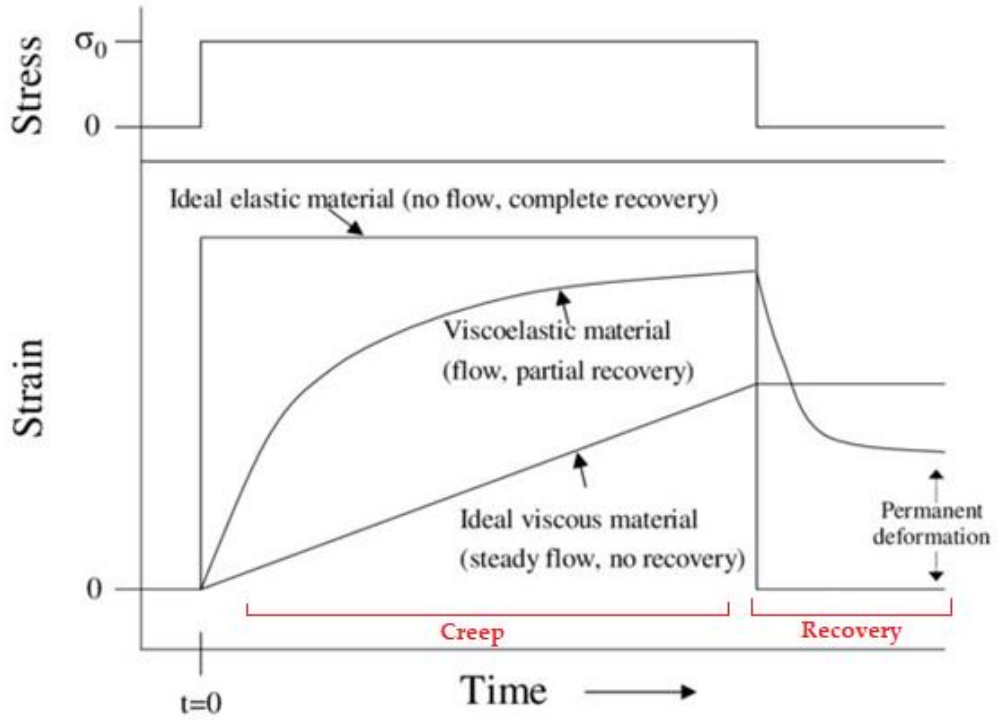


Figure 1.14. Creep and recovery curves of an ideal elastic, an ideal viscous and a viscoelastic material **(Van Bockstaele *et al.*, 2008).**

1.7.1.3. Approaches to measure Viscoelastic properties

Oscillatory rheology is a standard experimental tool to quantify both the viscus-like and the elastic-like properties of a material at different time scales. It is thus a valuable tool for understanding the structural and dynamic properties of viscoelastic systems.

1.7.3.1. Oscillatory rheology

The basic principle of an oscillatory rheometer is to induce a sinusoidal shear deformation in the sample and measure the resultant stress response, the time scale probed is determined by the frequency of oscillation (ω) of the shear deformation. The two-plates model, is a typical experiment, which can be used for explaining oscillatory tests (Figure 1.15). A sample is sheared while sandwiched between two plates, with the upper plate moving and the lower plate remaining stationary. A push rod mounted to a driving wheel moves the upper plate back and forth parallel to the lower plate, as long as the wheel is turning. The deflection path of the upper, movable plate is measured and rheologically evaluated as strain or deformation γ. When the driving wheel moves, a time dependent strain $\gamma(t) = \gamma.\sin(\omega t)$ imposed on the sample.

Simultaneously, the time dependent stress σ (t) is quantified by measuring the torque that the sample imposes on the bottom plate **(Wyss *et al.*, 2007).**

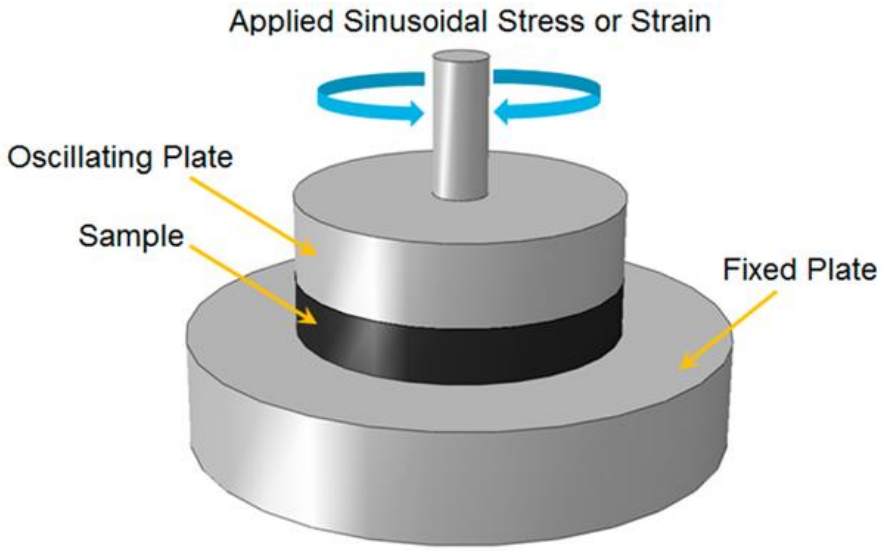


Figure 1.15. Schematic illustration of a two-plate rheometer model **(Majidi *et al.*, 2016).**

Measuring this time dependent stress response at a single frequency immediately reveals key differences between materials (Figure 1.16). If the material is an ideal elastic solid, then the sample stress is proportional to the strain deformation, and the proportionality constant is the shear modulus of the material. The stress is always exactly in phase with the applied sinusoidal strain deformation (with phase angel $\delta = 0$). In contrast, if the material is an ideal viscous fluid, the stress in the sample is proportional to the rate of strain deformation, where the proportionality constant is the viscosity of the fluid. The applied strain and the measured stress are out of phase, with a phase angle $\delta = \pi/2$ (Figure 1.16). Viscoelastic materials show a response that contains both in-phase and out-of-phase contributions (Figure 1.16), these contributions reveal the extents of solid-like (red line) and liquid-like (blue dotted line) behavior. As a consequence, the total stress response (purple line) shows a phase shift δ between solids and liquids, $0 < \delta < \pi/2$. The viscoelastic behavior of the system at ω is characterized by the storage modulus, G' (Pa), and the loss modulus, G'' (Pa), which respectively demonstrates the solid-like and fluid-like behavior **(Wyss *et al.*, 2007).**

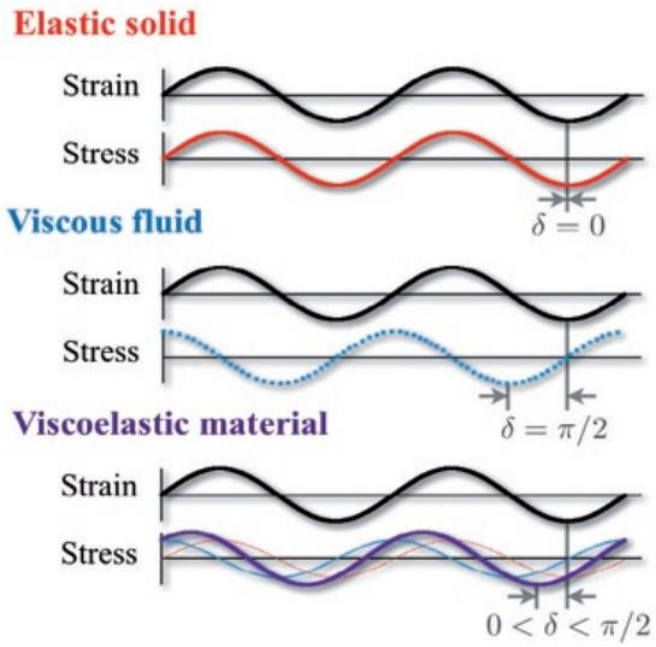


Figure 1.16. Schematic stress response to oscillatory strain deformation for an elastic solid, a viscus fluid and a viscoelastic material **(Wyss *et al.*, 2007).**

1.7.1.3.2. Storage modulus and loss modulus

The storage modulus gives information about the amount of structure present in a material. It represents the energy stored in the elastic structure of the sample. If it is higher than the loss modulus (G' > G") the material can be regarded as mainly elastic, in which the phase shift is below $\pi/4$ (45°). The loss modulus represents the viscous part or the amount of energy dissipated in the sample. if G" > G', the sample displays a fluid structure. In this case, the phase shift is higher than $\pi/4$ (45°). The 'sum' of loss and storage modulus is the so-called complex modulus G* **(Majidi *et al.*, 2016).**

Loss factor or damping factor is another parameter, which describes as:

$\tan \delta = G''/ G'$

where $\tan \delta$ is loss factor, G" is loss modulus (Pa) and G' is storage modulus (Pa).

For ideally elastic behavior, $\tan \delta = 0$ and for ideally viscose behavior $\tan \delta = 1$. If $\tan \delta > 1$ refers to liquid state, while $\tan \delta < 1$ present solid state **(Barnes, 2000).**

1.7.1.3.3. Amplitude sweeps

Amplitude sweeps aim at describing the deformation behavior of samples in the non-destructive deformation range. For amplitude sweeps, the amplitude of the strain is increased step-wise from one measuring point to the next, while keeping the frequency at a constant value (Figure 1.17).

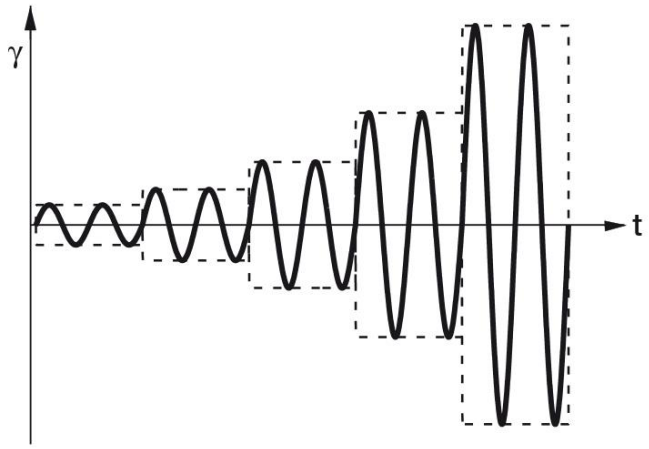


Figure 1.17. An amplitude sweep: Amplitude increased under a controlled strain and constant frequency **(Barnes, 2000)**

Results of the test present the limit of Linear Viscoelastic Region (LVE region), which indicate the range of strain that the structure of the sample does not destroy. In addition, the yield point, flow point and the flow transition index can be determined by an amplitude sweeps experiment **(Barnes, 2000)**. Figure 1.18 displays LVE region in an amplitude sweeps test. As it can be inferred from Figure 1.18, τ_y is the yield point at the limit of LVE region and τ_f is the flow point at the cross-over point (G'= G").

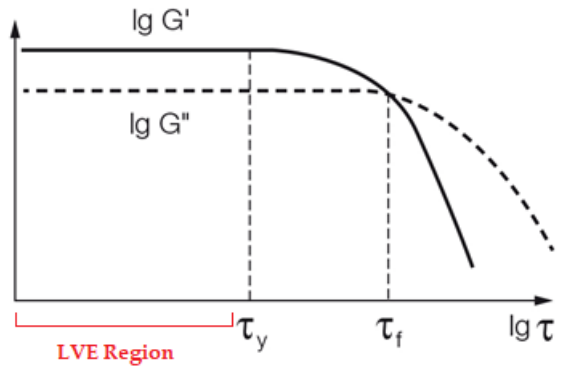


Figure 1.18. LVE region, yield point (τ_y) and flow point (τ_f) in an amplitude sweeps test **(Barnes, 2000)**

1.7.1.3.4. Frequency sweeps

Frequency sweeps describes the time-dependent behavior of a sample in the non-destructive deformation range. High frequencies are used to simulate fast motion on short timescales, whereas low frequencies simulate slow motion on long timescales or at rest. For frequency sweeps, with a controlled shear strain (in LVE region) the frequency is varied step-wise (Figure 1.19). Frequency sweeps are proven methods for gathering information on the behavior and inner structure of polymers as well as on the long-term stability of dispersions **(Barnes, 2000)**.

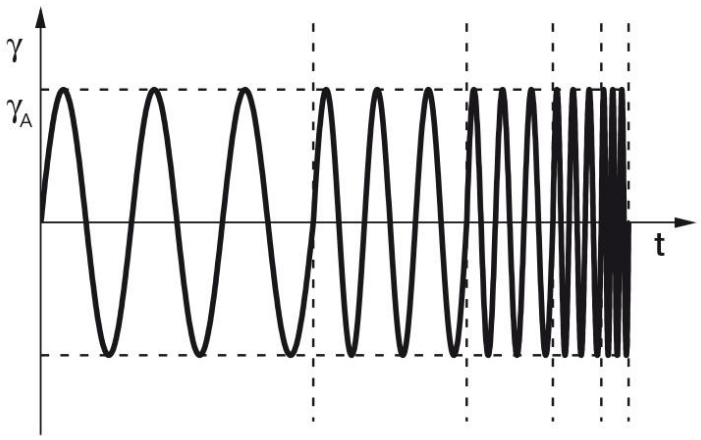


Figure 1.19. A frequency sweeps, under a controlled shear strain and constant strain amplitude, frequency is increased **(Barnes, 2000)**

1.7.2. Swelling ratio

The swelling ratio is defined as the increase in the weight of the hydrogel due to water absorption. The swelling properties of hydrogels are mainly related to the network elasticity,

the presence of hydrophilic functional groups, the cross-link density, and porosity. The swelling behaviors of the hydrogels, which is one of the fundamental properties of the gel to be known firstly, have been studied extensively. One of the important reasons of this is the fact that the solvent, that is water, is a common liquid for the biological systems. The information of the swelling behaviors of the hydrogel is of importance when the gels are used in the practical purposes. It has been proved that swelling behavior of the gel depends on the solvent mixture as well as the structure of the gel network **(Park *et al.*, 2009).** Swelling ratio is determined by placing a defined amount of sample in a solvent (which is usually water). Then, the swelling ratios of the gels are measured as a function of time. The swelling ratios are estimated from the ratio of the weight (or volume) of the swelled gel $W_{(t)}$ to that of the initial gel $W_{(t=0)}$ **(Takeno *et al.*, 2017):**

Swelling ratio $= W_t/W_{(t = 0)}$

1.7.3. Water Holding Capacity (WHC)

A major function of carbohydrate polymers is to stabilize a large amount of water. A knowledge of water holding capacity of hydrogels made from carbohydrate polymers is important in practical applications. Due to the intrinsic instability of hydrogels, water may be lost after extensive storage time as a result of passive diffusion (syneresis). Syneresis may also happen under external forces, or temperature fluctuation such as freeze-thawing. The loss of water may result in shrinking of the gels, changing texture and reducing quality **(Sanderson, 1990).**

Starch gels are the most widely used polysaccharide gels, which experience a sever syneresis after a long storage. Agar gels, which normally have a high storage modulus, as well as crunchy and brittle texture, demonstrate extensive syneresis over a long storage **(Mao *et al.*, 2001).** In addition, fresh agar gels posse relatively lower WHC than fresh gelatin gels **(Ryu *et al.*, 2012).** Alginates with large proportions of polyguluronate segments tend to form rigid, brittle gels that are also subject to syneresis, while alginates with predominantly polymannuronic acid units form gels that are more elastic, less brittle and do not exhibit much syneresis. In general, brittle polysaccharide gels have lower water holding capacity than elastic gels, and demonstrate higher syneresis during long storage **(Mao *et al.*, 2001).**

Water holding capacity is determined using the centrifugation method. It is calculated from the ratio of the weight (after centrifugation) to the initial weight of the sample **(Deniz and Martin, 1997)**:

$$WHC\ (\%) = (\text{Mass of gel after centrifugation}/\text{Mass of gel sample}) \times 100$$

1.7.4. Scanning Electron Microscopy (SEM)

The texture and morphological properties of hydrogels can be analysed by scanning electron microscopy (SEM). SEM reveals the porosity and nature of hydrogel structure. Moreover, the effect of modification on the size of the pore is explained by SEM images. SEM sample is generally prepared by first swelling the hydrogels, then freezing in liquid nitrogen, and finally freeze-drying and sputtering with gold prior to the SEM observation **(Rahman *et al.*, 2019).**

The main SEM component have been displayed in Figure 1.20. Electrons are produced at the top of the column (electron gum), accelerated down and passed through a combination of lenses and apertures to produce a focused beam of electrons which hits the surface of the sample. Since the microscope is designed to operate at low vacuums, both the column and the chamber are evacuated by a combination of pumps. As the electrons interact with the sample, they produce secondary electrons, backscattered electrons, and characteristic X-rays. These signals are collected by one or more detectors to form images **(Danilatos, 1988).** Figure 1.21 presents an SEM image of a surface of a thin gelatin film **(Rahman *et al.*, 2019).**

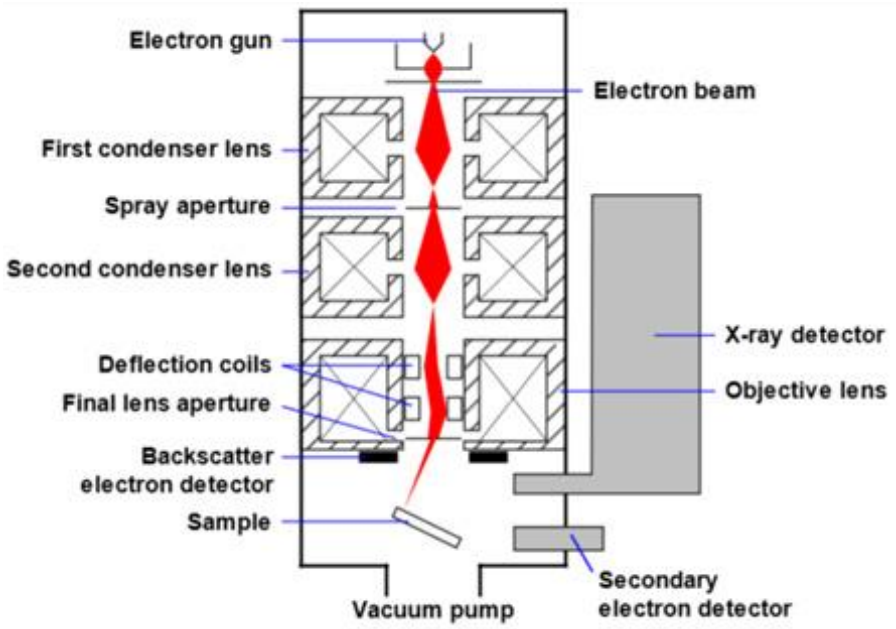


Figure 1.20. Schematic of a Scanning Electron Microscope **(Rahman *et al.*, 2019)**

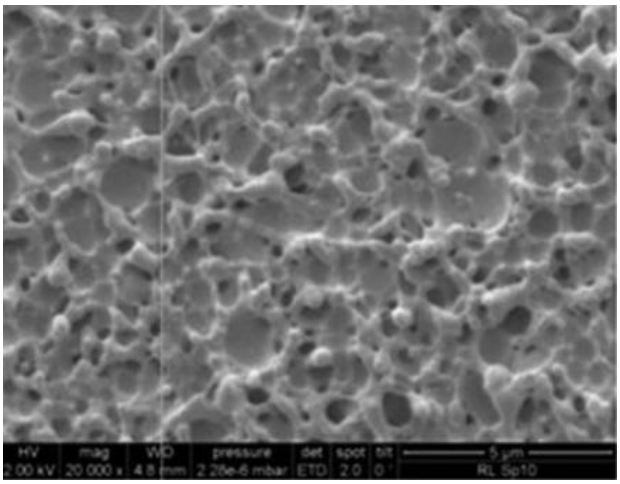


Figure 1.21. SEM image of a thin gelatin film (Resolution 20000 ×, irradiated at λ= 800 nm, T= 30 fs laser pulse and laser fluence F = 2.5 J/cm^2) **(Rahman *et al.*, 2019).**

1.7.5. FTIR spectroscopy

Fourier Transform Infrared or FTIR is an important technique in organic chemistry, in which IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. FTIR spectroscopy is useful to identify unknown materials, determine the quality or consistency of a sample, and recognize the amount of components in a mixture **(Ismail *et al.*, 1997).**

Figure 1.22 displays a diagram of an FTIR spectroscopy. An infrared spectrometer essentially consists of a source of continuous IR radiation, a means for resolving the IR radiation into its component wavelengths, and a detector. The source generates radiation which divided in two parts by a beam splitter. One beam strikes the fixed mirror and returns to the beam-splitter. The other beam goes to the moving mirror. The motion of the moving mirror makes the total path length variable versus that taken by the fixed mirror beam. When these two beams meet up again at the beam splitter, they recombine and passes through the sample and reaches detector. Then the signal is amplified and converted to digital signal by the amplifier and result in an interferogram containing all the spectral information related to the sample **(Van de Voort, 1992).**

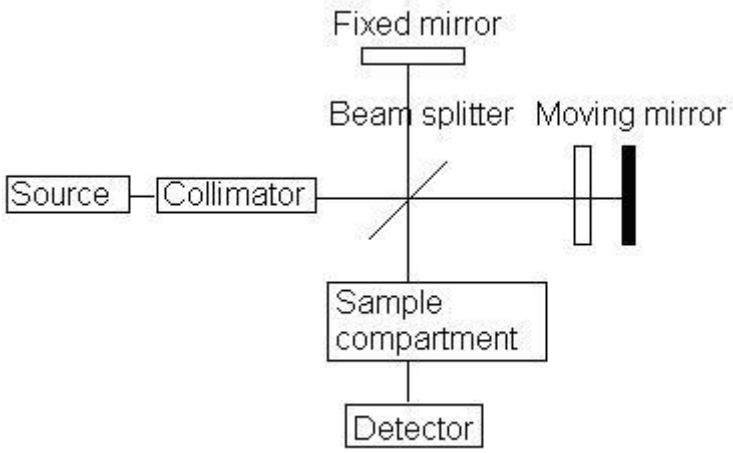


Figure 1.22. Schematic diagram of FTIR spectroscopy **(Van de Voort, 1992)**

FTIR spectroscopy is a non-destructive, reliable fast method in quality control of various food products, including: milk, oil, meat, sugary juice and food additives **(Van de Voort, 1992).** In addition, it has been applied in classification of corn starches, evaluation the composition of pectin and hemicellulose and characterisation of food additive polysaccharides and hydrocolloids in confectionery jellies **(Černá *et al.*, 2003).** The conformation of gelatin during the gel melting process has been also monitored by FTIR spectroscopy **(Prystupa and Donald, 1996).**

1.7.6. Melting point

Melting point is the temperature at which a given solid material changes from a solid state to a liquid, or melts **(Qiu and Scrivens, 2018).** Gels are sensitive materials to temperature and their three-dimensional structure collapses during thermal treatment, which leads to changes in

rheological behavior **(Yoon and Gunasekaran, 2007).** Therefore, temperature sweeps with oscillatory stress is a rheological method to determine the melting point and sol-gel transitions of gels. In a temperature sweep test, sample is subjected to a uniform frequency with constant strain amplitudes. In addition, a temperature sweep rate is preset, usually as a linear temperature increase, to evaluate rheological properties of the gels under a gradient rise of temperature. Both storage (G′) and loss (G″) modulus are recorded throughout the temperature sweeps and the melting point (T_m) determined by G′ = G″ or equivalently tanδ = 1 **(Somboon *et al.*, 2014).**

1.7.7. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry, or DSC, is a thermal analysis technique that looks at how a material's heat capacity is changed by temperature. A sample of known mass is heated or cooled and the changes in its heat capacity are tracked as changes in the heat flow. This allows the detection of transitions such as melts, glass transitions, phase changes, and curing. This technique is used in many industries, including: pharmaceuticals, polymers, food, paper, printing, manufacturing, agriculture, semiconductors, and electronics **(Groenwoud, 2001).**

DSC comprises the sample and reference holder, the heat resistor, the heat sink, and the heater (Figure 1.23). Heat of heater is supplied into the sample and the reference through heat sink and heat resistor. Heat flow is proportional to the heat difference of heat sink and holders. Heat sink has the enough heat capacity compared to the sample. In case the sample occurs endothermic or exothermic phenomena such as transition and reaction, this endothermic or exothermic phenomena is compensated by heat sink. Thus the temperature difference between the sample and the reference is kept constant. The difference the amount of heat supplied to the sample and the reference is proportional to the temperature difference of both holders. By calibrating the standard material, the unknown sample quantitative measurement is achievable **(Gabbott, 2007).**

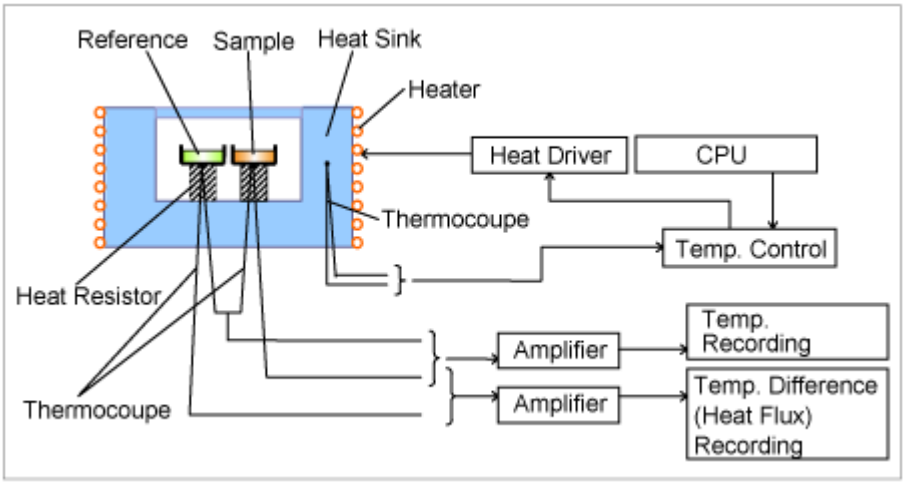


Figure 1.23. Schematic diagram of Differential Scanning Calorimetry **(Gabbott, 2007)**

Differential Scanning Calorimetry has a wide range of applications in food industry. For example, it provides information about melting and crystallization temperature of oils, as well as their polymorphic behavior and oxidation stability. The retrogradation and gelatinization of flour and rice starch can be evaluated by DSC. In addition, DSC determines the specific heat capacity and melting and crystallization temperature of pastes and gels containing polysaccharides and gums. Denaturation and aggregation of proteins are also analysed by DSC **(Heussen *et al.*, 2011).**

1.7.8. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) is a technique, in which the mass of a material is monitored as a function of temperature (or time), under a controlled atmosphere. Its principle uses include measurement of a materials thermal stability, moisture content of organic and inorganic materials, residual solvent content, decomposition temperature and oxidative stability **(Pomeranz and Meloan, 1994)**. It has been widely used for determination free water content of low moisture food, such as, starch, flour, coffee and milk powder **(Wang *et al.*, 2018).**

A schematic diagram of Thermogravimetric Analysis has been shown in Figure 1.24. A TGA consists of a sample pan that is supported by a balance controller. That pan resides in a furnace and is heated or cooled during the experiment. The mass of the sample is monitored during the experiment. A sample purge gas controls the sample environment. This gas may be inert or a reactive gas that flows over the sample and exits through an exhaust **(Thomas *et al.*, 2017).**

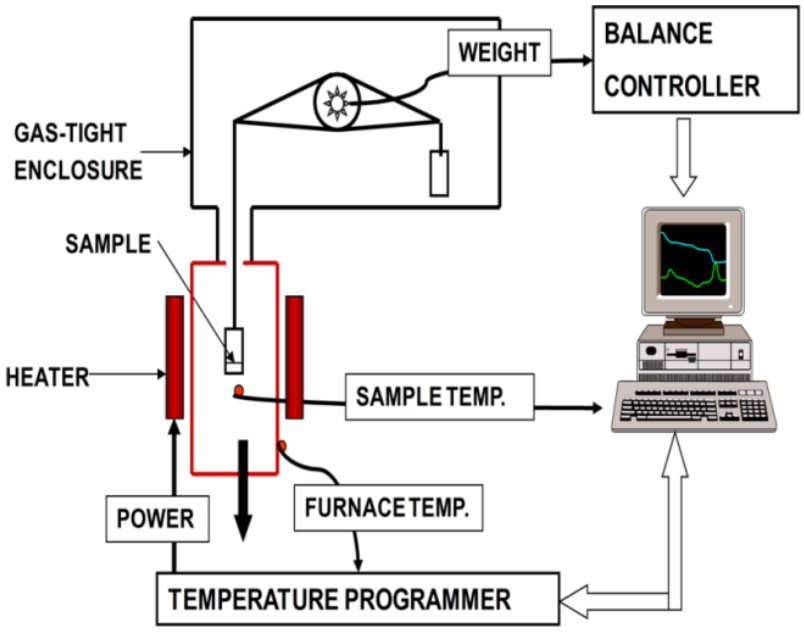


Figure 1.24. Schematic graph of Thermogravimetric Analysis **(Thomas *et al.*, 2017)**

1.7.9. Liquid Chromatography-Mass Spectrometry

Chromatography is a separation technique to separate the individual compound from a mixture using a stationary and mobile phase. There are different types of chromatography systems, based on the nature of stationary and mobile phase (gas-solid chromatography, gas-liquid chromatography, liquid-liquid chromatography and finally solid-liquid chromatography like: column chromatography, Thin Layer Chromatography, High Performance Liquid Chromatography, Liquid Chromatography-Mass Spectrometry). Chromatography is also categorized based on the principle of separation and type of the chromatographic method: Partition chromatography, Molecular exclusion chromatography (which is based on the molecular size), Affinity chromatography, Ion exchange chromatography (which can be classified as cationic exchange chromatography and ionic exchange chromatography), and Adsorption chromatography (which is based on the affinity towards stationery phase. Thin Layer Chromatography, High Performance Liquid Chromatography and Liquid Chromatography-Mass Spectrometry are the examples of adsorption chromatography) **(Subramani *et al.*, 2014).**

Liquid Chromatography-Mass Spectrometry (LC-MS) is a technique for quantitative and qualitative analysis of samples. Combination of chromatography with spectrometry is first reported in 1967 and first LC-MS system was introduced in 1980s. LC-MS is an analytical

chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis and mass spectrometry. Mainly the LC-MS contains liquid chromatography assembly, ion generation unit/ ionization source, mass analyzer and mass spectrometric data acquisition (Figure 1.25). The sample is separated by LC, and the separated sample species are sprayed into atmospheric pressure ion source, where they are converted into ions in the gas phase. The mass analyzer is then used to sort ions according to their mass to charge ratio and detector counts the ions emerging from the mass analyzer and may also amplify the signal generated from each ion. As a result, mass spectrum (a plot of the ion signal as a function of the mass-to-charge ratio) is created, which is used to determine the elemental or isotopic nature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules. Usually LC used in LC-MS is HPLC. The principle of separation in HPLC, is normal phase mode or reverse phase mode of adsorption. Normal phase constricts with polar stationary phase with non-polar solvent/mobile phase and reverse phase constricts with non-polar stationary phase with polar solvent/mobile phase **(Subramani *et al.*, 2014).**

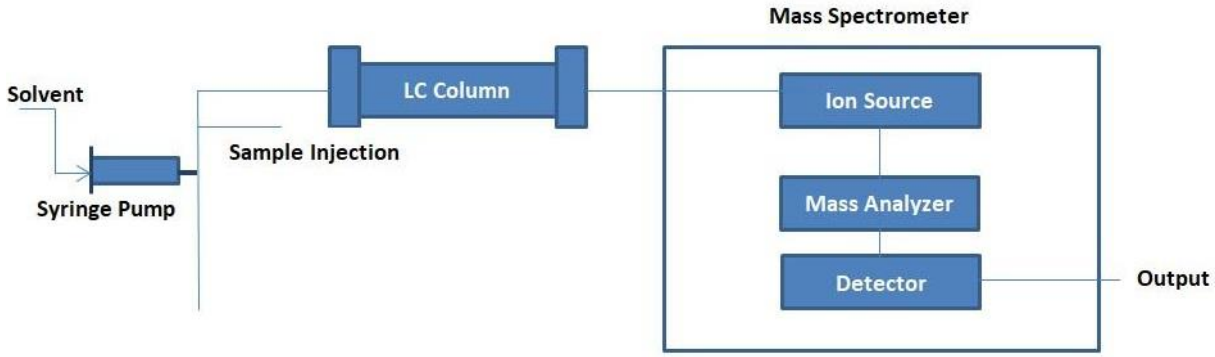


Figure 1.25. Simple schematic of LC-MS **(Subramani *et al.*, 2014)**

LC-MS has a wide range of application in most areas of analytical chemistry, molecular biology, food safety and quality control. The determination of organic trace compounds in food analysis is of major importance for food quality and food safety aspects. Both the separation of the analyte from potential inferences in the food matrix, as well as the qualitative and quantitative determination of the target compound, are vital steps in analytical food chemistry. Although LC-MS generates useful information about the molecular weight and the structure of analytes, combination of a mass analyzer (MS) to an LC-MS produces a powerful technique with a high sensitivity and specificity. Many analytical results from lipids, carbohydrates,

glycoproteins, flavonoids, vitamins, mycotoxins, pesticides, allergen and food additives have been published, owing to LC-MS/MS analysis **(Di Stefano *et al.*, 2012; Guillem *et al.*, 2019).**

Tandem mass spectrometry, also known as LC-MS/MS, is a method which represents further breakthrough in LC-MS analyte molecules. In this method, two quadrupole mass filters are combined and target analyte molecules from the first quadrupole are submitted to a controlled fragmentation in a collision cell. The entirety of ions formed by ion generator is transferred into the first quadrupole mass filter. Here, the m/z of the intact ionized target analyte is selected, all other ion species are filtered out. The selected ions sharing identical mass-to-charge ratio (m/z) are continuously transferred into the collision cell. In this part of the MS analyzer, a collision gas (in most cases argon or nitrogen) is present in trace amounts (10^{-3} mbar). Ions selected by the first quadrupole collide with these gas molecules and fragment into characteristic product ions. The entirety of these fragment ions are guided to the second quadrupole. The radiofrequency settings of the second analytical quadrupole are adjusted in a way that only one selected fragment ion will pass, while all other fragment ion species are filtered out. Thus, one defined "daughter ion" from one defined "parent ion" finally reaches the ion detector (Figure 1.26) **(Guillem *et al.*, 2019; Vogeser and Parhofer, 2007).**

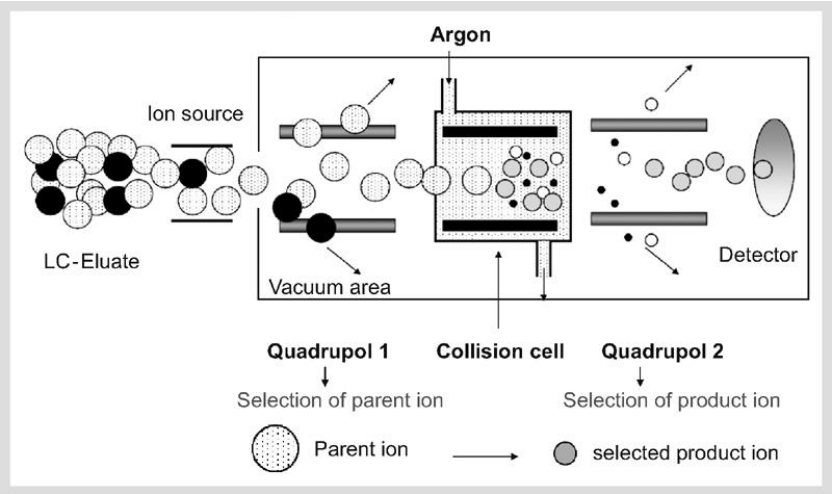


Figure 1.26. Principle of LC-MS/MS **(Vogeser and Parhofer, 2007)**

1.8. Aim of the work

The aim of this work can be divided in into four main parts:

1. Modifying extraction methods

Wheat bran, as a by-product of milling procedure, contains arabinoxylan. The ferulated AX can covalently cross-link, via enzymatic reaction, to form a gel. The more ferulic acid moieties are present on the AX backbone, the more covalent cross-links may be generated. Therefore, first and foremost is to modify an alkaline extraction method, in which a pure AX with the most intact ferulic acids is achieved from wheat bran. The incubation time and concentration of alkaline extracts are optimised to increase the remaining number of extracted ferulic acids for cross-linking.

Sugar beet fibre is an agricultural by-product of beet processing. It mainly contains pectin, which are feruloyl substituted. Feruloyl substitutions can be cross-linked through oxidative coupling reaction to form a gel. Thus, a hydrothermal extraction method is designed to extract more intact ferulic acid moieties.

2. Screening the most promising enzyme to cross-link ferulated substrates

Laccase from two specious of Basidiomycota (*Funalia trogii* and *Pleurotus pulmonarius*) are purified to demonstrate oxidative cross-linking reaction. In addition, commercial laccase C is applied to compare self-extracted enzymes with commercial enzyme for cross-linking enhancement. The aim of this stage is to pick the best enzyme, in order to form a solid cross-linked gel.

3. Analysing rheological and structural properties of cross-linked gels

Cross-linked AX gels, as well as cross-linked Fibrex gels, are formed and their properties are investigated in the presence of different additives (citrus pectin, sucrose, various mediators like vanillin and caffeic acid). Moreover, storage stability of the gels is also inspected. The major aims of this part is firstly, to determine the mechanical spectra of the gels. Secondly, analysing structural properties of the gels (melting point, swelling ratio and WHC), as well as detecting cross-linked diferulic acids. Fourthly, an attempt to enhance gel structural and rheological properties by adding different additives. Finally, investigating storage stability of the gels.

4. Comparing rheological and structural properties of cross-linked gels with gelatin gels

Among commercial hydrocolloids used in the food industry, gelatin has been regarded as special and unique, serving multiple functions with a wide range of applications in various industries. The issue of gelatin replacement has existed for many years for the vegetarian, halal and kosher food markets. The main aim of the entire work is to produce

cross-linked gels, which are formed with fungal laccases, and can be introduced as substitution for gelatine gels. Therefore, rheological, structural and storage stability of the enzymatically cross-linked gels are compared with gelatin gels, as a reference.

2. Intro for publication: Cross-linking of wheat bran arabinoxylan by fungal laccases yields firm gels.

Wheat bran is a by-product of the commercial wheat milling process and could become a potential source of added-value biomolecules, such as hydrocolloids. Wheat bran contains fibres, where 21% correspond to arabinoxylans **(Bergmans *et al.*, 1996)**. The ferulated arabinoxylans can be cross-linked through oxidative coupling reaction **(Carvajal-Millan *et al.*, 2005)**. Previous studies demonstrated a direct impact of extraction method, including incubation time and concentration of alkaline extracts, on the ferulic acid moieties. The more ferulic acid moieties are present on the AX backbone, the more covalent cross-links may be generated **(Berlanga-Reyes *et al.*, 2011)**.

The issue of gelatin replacement has existed for many years for the vegetarian, halal and kosher food markets. Various alternatives have been suggested as gelatin substitution. Arabinoxylan has the potential to form a cross-linked hard gel. Transformation of wheat bran arabinoxylan to an edible solid gel could introduce a new plant alternative for gelatin gels, which are derived from pork or cattle skins, split hides or bones.

In the present study, a modified arabinoxylan extraction method was designed, in which a pure AX with the most intact ferulic acids was achieved from wheat bran. Laccases from two specious of Basidiomycota (*Funalia trogii* and *Pleurotus pulmonarius*) were purified to demonstrate oxidative cross-linking. In addition, commercial laccase C was applied to compare self-extracted enzymes with commercial enzyme for cross-linking enhancement. Cross-linked AX gels were formed and their properties were investigated in the presence of different additives (citrus pectin, various mediators like ABTS and caffeic acid). Moreover, storage stability of the gels was inspected. It was aimed to determine the mechanical spectra of the gels, analyse structural properties of the gels (melting point, swelling ratio and WHC) and detect cross-linked diferulic acids.

In conclusion, it was an attempt to form a stable solid gel, which could be introduced as a substitution for gelatine gels.

3. Cross-linking of wheat bran arabinoxylan by fungal laccases yields firm gels

Sanaz Khalighi *, Ralf G. Berger, and Franziska Ersoy

Processes **2020**, *8*(1), 36; https://doi.org/10.3390/pr8010036, Received: 26 November 2019 / Accepted: 25 December 2019 / Published: 30 December 2019.

3.1. Abstract

The native extractable arabinoxylans (AX) from wheat bran were cross-linked by the commercial laccase C (LccC) and self-produced laccases from *Funalia trogii* (LccFtr) and *Pleurotus pulmonarius* (LccPpu) (0.04 U/µg FA, each). Dynamic oscillation measurements of the 6% AX gels demonstrated a storage modulus of 9.4 kPa for LccC, 9.8 kPa for LccFtr, and 10.0 kPa for LccPpu. A loss factor ≤ 0.6 was recorded in the range from 20 to 80 Hz for all three laccases, and remained constant for four weeks of storage, when LccFtr and LccPpu were used. Arabinoxylan gel characteristics, including high water holding capacity, swelling ratio in saliva, and heat resistance indicated a covalently cross-linked network. Neither the mediator compounds caffeic acid and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), nor citrus pectin, enhanced the elastic properties of the gels. Using laccases as an oxidant provided gels with a solid and stable texture, comparable in firmness to traditional gelatin gels. Thus, AX gels can be presented in the vegan, halal, and kosher food markets. They may also find use in pharmaceutical and other industrial applications.

Keywords: arabinoxylan gel; laccase; cross-linking; viscoelastic properties

3.2. Introduction

Among commercial hydrocolloids used in the food industry, gelatin has been regarded as special and unique, serving multiple functions with a wide range of applications in various industries **(Karim and Bhat, 2008)**. However, vegan, halal, and kosher food markets call for non-animal gels, particularly within Europe, with the emergence of bovine spongiform encephalopathy (“mad cow disease”) in the 1980s **(Morrison *et al.*, 1999)**.

Most of the common hydrocolloids lack the chemical functionalities for creating additional network nodes, which are required for forming solid gels. Arabinoxylans (AX), the major non-starch polysaccharides in wheat bran, are a by-product of the commercial wheat milling process

(Anderson and Simsek, 2019) and may provide a solution. Wheat bran contains approximately 13 mg/g ferulic acids and up to 50% non-digestible fibre, mostly AX **(Boz, 2015)**.

These hemicelluloses consist of *β*-(1→4)-linked D-xylopyranosyl residues decorated with α-(1→2)- and/or α-(1→3)-linked L-arabinofuranosyl units. Some of the arabinose residues are ester linked on (O)–5 to FA resulting in ferulated AXs **(Scalbert *et al.*, 1985; Mendis and Simsek, 2014)**. Diferulic (di–FA) and triferulic acids (tri–FA) have been identified as covalently cross-linked structures in gelled AX **(Fincher and Stone, 1974; Smith and Hartley, 1983)**. Figure 3.1 illustrates this oxidative reaction **(Carvajal-Millan *et al.*, 2005)**.

Besides, AX gels may form in the strong acidic range (pH 2) without addition of a crosslinking agent, and at low sugar levels through hydrogen bonds and hydrophobic interactions **(Zhang *et al.*, 2019)**.

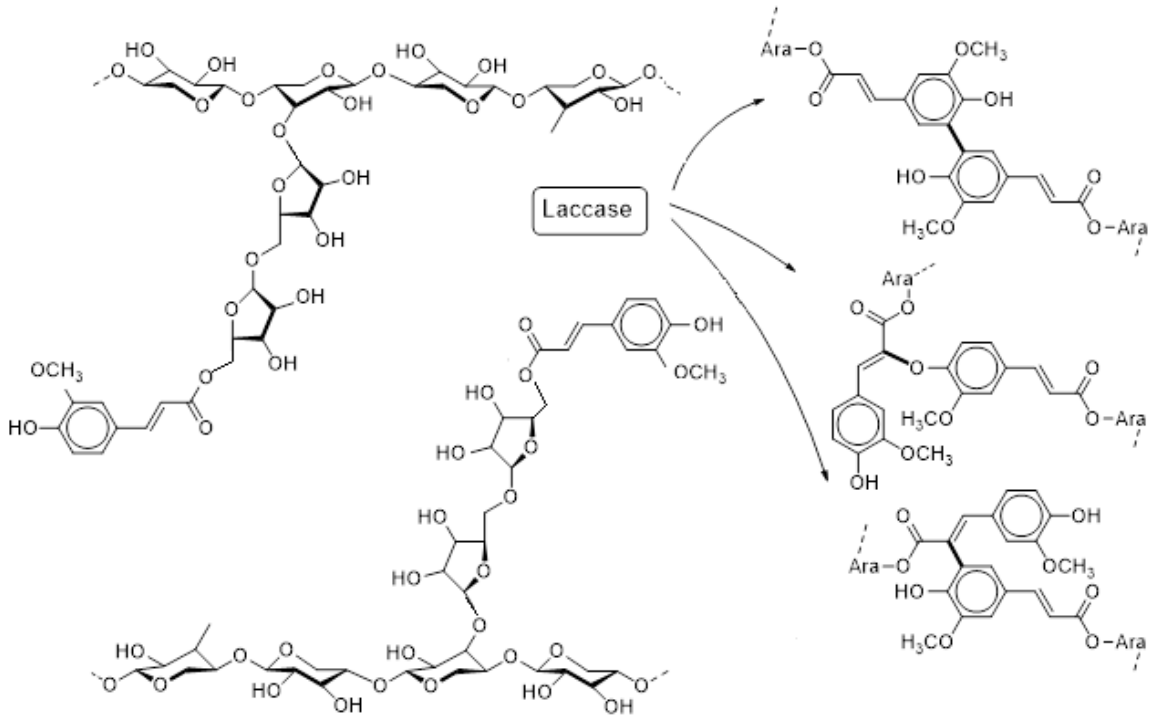


Figure 3.1. Oxidative gelation as catalysed by laccases (simplified): Feruloylated arabinoxylan in solution (left) and formation of network knots to constitute a gel (right); β-1,4-D-Xylopyranose backbone with α-1,3-linked L-arabinofuranosyl side chains carrying feruloyl substituents. Dimerization of adjacent feruloyl moieties occurs through intermediate carbon or oxygen centred radicals.

As wheat bran is an abundant industrial by-product generated during milling, it may be considered as a convenient source of AX, which, after cross-linking, may serve as a solid vegan

gel **(Bergmans *et al.*, 1996)**. The higher the number of ferulic acid moieties on the polysaccharide backbone, the more covalent cross-links may be generated **(Carvajal-Millan *et al.*, 2005)**.

AX are generally extracted by hot water and/or aqueous alkali and can be fractionated according to their solubility in different solvents, such as water/ethanol mixtures **(Kale *et al.*, 2015)**. The incubation time, concentration of alkaline extracted solution and initial mass of wheat bran has a direct impact on the number of ferulic acids remaining for cross-linking **(Berlanga-Reyes et al., 2011; Stavova *et al.*, 2017)**. A recent research on ferulic acid extraction on the laboratory scale indicated that the more wheat bran was used the less ferulic acid was extracted **(Stavova *et al.*, 2017)**. According to own analyses, little ferulic acid is left in commercial AX, which indicates high pH and/or long extraction times.

Various oxidative agents were applied for the cross-linking of AX gels, including hydrogen peroxide, manganese peroxidases, and laccases **(Ayala-Soto *et al.*, 2016; Carvajal-Millan *et al.*, 2005)**. Recent work compared laccase and peroxidase crosslinking activities and concluded that laccases would be preferable for AX gels **(Martínez-López *et al.*, 2019).** Laccases are part of a large group of multi-copper oxidases acting on polyphenols, methoxy-substituted phenols, aromatic diamines, and a range of other compounds **(Baldrian, 2006)**. They were suggested as processing aids for wine, beer, and bakery products, and for improving sensory parameters of food **(Flander *et al.*, 2008; Osma *et al.*, 2010; Bender *et al.*, 2018)**. Laccase catalysis occurs by reduction of oxygen to water accompanied by the oxidation of the substrate. The ability of laccase C to enhance the cross-linking in skimmed milk yogurt has been shown before (**Struch *et al.*, 2015**).

The objective of this study was to extract genuine AX from wheat bran with as many ferulic acid moieties still bound to the hemicellulose backbone as possible. The working hypothesis was that such a native substrate, in contrast to commercial AX, may be converted by laccases to plant based gels with a firmness similar to traditional gelatin gels.

3.3. Materials and Methods

3.3.1. Materials

Wheat bran was bought from Alnatura GmbH (Darmstadt, Germany). All chemicals were obtained from Carl Roth (Karlsruhe, Germany), Fluka (Buchs, Switzerland), and Sigma-Aldrich (Taufkirchen, Germany).

3.3.2. Enzymes

The commercial laccase C (EC 1.10.3.2), purified from a *Trametes* species, was purchased from ASA Spezialenzyme (Wolfenbüttel, Germany). The xylanase side-activity of the laccase C was determined using the 3,5-dinitrosalicylic acid (DNSA) test **(Cañtizares-Macias *et al.*, 2006)**. Based on previous work (**Struch *et al.*, 2015; Behrens *et al.*, 2017**), laccases were also purified from the basidiomycetes *Funalia trogii* (DSMZ 11919) and *Pleurotus pulmonarius* (DSMZ 5331). To obtain laccase Ftr from *Funalia trogii*, the fungus was cultivated in SNL medium (**Struch *et al.*, 2015**) and incubated at 25 °C and 180 rpm for three weeks. The purification was done by hydrophobic interaction chromatography on phenyl FF column and ion exchange chromatography on Q-sepharose XL material **(Kolwek *et al.*, 2018)**. The activity of the extracted and purified enzyme was 2.3 x 10^5 U/l. Laccase activity was measured at 25 °C and pH 4.5 using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (**Struch *et al.*, 2015**).

Laccase Ppu was isolated from a recombinant *Pichia pastoris* with a laccase gene from the fungus *Pleurotus pulmonarius* cultivated on BMM medium (**Behrens *et al.*, 2017**). *Pichia pastoris* was incubated at 25 °C and 180 rpm for one week. Purification of the laccase Ppu was achieved by Agarose Ni-NTA column **(Cañtizares-Macias *et al.*, 2006)**. The activity of the purified enzyme was 1.92 x 10^5 U/l.

3.3.3. General experimental setup

All data presented are averages of duplicate measurements. The standard deviation of replicates for the rheological measurements was typically below 5%.

3.3.4. Wheat bran arabinoxylan extraction

Wheat bran was manually milled to a 14-mesh particle size using liquid nitrogen. One hundred gram were suspended in 500 ml ethanol at 25 °C and 250 rpm overnight to eliminate lipophilic components. After filtration, the wheat bran was boiled in 700 ml water for 30 min to gelatinize starch and inactivate enzymes before centrifugation at 12,000 x g for 10 min at 20 °C (centrifuge 460R Hettich Rotanta, Taufkirchen, Germany). Afterwards, it was treated with 500 ml NaOH (0.5 mol/l) at 40 °C, 180 rpm for 40 min in the dark. Residual bran was removed by centrifugation (12,000 x g, 10 min and 20 °C). The supernatant was acidified to pH 4.5 with

HCl (3 mol/l) and then centrifuged at 12,000 x g and 20 °C for 15 min. The recovered supernatant was precipitated in ethanol at 4 °C overnight, freeze-dried, and milled **(Berlanga-Reyes *et al.*, 2011)**. The bound ferulic acid content was determined by incubating 2 ml of 10% AX solution with 2 ml KOH/NaOH (1:1; 4 mol/l each) at 50 °C and 220 rpm for 4 hours. The reaction was stopped with 1 ml 60% acetic acid and 5 ml methanol. After centrifugation at 15,000 x g and 4 °C for 15 minutes, ferulic acid (FA) was analysed by HPLC at λ = 323 nm (SPD-10A VP, Shimadzu Deutschland GmbH, Berlin, Germany) **(Nieter *et al.*, 2016)**.

3.3.5. Wheat bran arabinoxylan cross-linking

In order to cross link arabinoxylan gel, 6% (w/v) wheat bran AX solution in 0.05 M citrate phosphate buffer pH 5.5 was incubated with different laccases (0.04 U/µg FA, LccC, LccFtr, and LccPpu) overnight without shaking. The impact of caffeic acid ((E)-3-(3, 4-dihydroxyphenyl)-2-propenoic acid) and ABTS (1, 3, and 5 mM) as mediators and citrus peel pectin (1 and 3% (w/v)) instead of AX were likewise investigated. Commercial wheat bran AX (Megazyme, Bray, Ireland) without ferulic acid was used as negative control.

3.3.6. Rheology

To carry out rheological measurements, a Physica MCR 301 rheometer (Anton Paar, Graz, Austria) with plate geometry (25 mm diameter) and a gap width of 1 mm was used. Oscillation experiments (γ = 0.01 to 100%) were performed at a constant frequency of ω = 10 rad/s to ascertain the linear viscoelastic region (LVE) **(Francis, 1999)**. Frequency sweep test used a frequency of 0.01 to 100 Hz with an applied strain of 5%. All tests were carried out at 25 °C in duplicates. Rheoplus software was used to calculate storage modulus (G′), loss modulus (G″), and loss factor tanδ (= G″/G′).

Rheological properties of gel samples were analysed weekly over six weeks of storage at 25 °C. Injection of one unit xylanase activity into the gel and incubation at 30 °C for 2 h destroyed the structure of the gels and facilitated the ABTS assay to estimate the residual laccase activity during storage. Viscoelastic properties of the AX gels were compared with 6% gelatin gels (Dr. Oetker GmbH, Bielefeld, Germany) prepared with the same buffer, but without any other additives.

3.3.7. Analysing diferulic acids in arabinoxylan gels

3.3.7.1. Sample preparation

Arabinoxylan gel (cross-linked with LccPpu) was enzymatically hydrolyzed with a xylanase: ferulic acid esterase (1:1) mix for 3 hours at 40 °C, pH 6 and 220 rpm. Xylanase from *Trichoderma viride* was purchased from Fluka (Buchs, Switzerland). The recombinant ferulic acid esterase originated from *Streptomyces werraensis* (**Schulz *et al.*, 2018**). The hydrolyzed sample was purified on 20 ml XAD 2 resin column as described by Malunga and Beta (2016) **(Malunga and Beta, 2016)**. Adsorbed diferulic acids were eluted in 30 ml methanol:formic acid (9:1), concentrated under a nitrogen stream, lyophilised, and dissolved in 50 µl acetonitrile.

3.3.7.2. High performance liquid chromatography mass spectrometry

To detect cross-linked diferulic acids in the gel, 15 µl hydrolysate was loaded on a Varian 1200 LC-MS (Agilent, Santa Clara, USA) equipped with a C18 Pyramid column (Macherey-Nagel, Düren, Germany). As eluent A, water with 0.1% formic acid was used, whereas acetonitrile with 0.1% formic acid was applied as eluent B. A separation was achieved using a stepwise gradient at a flow velocity of 0.1 ml/min: sample loaded in 10% eluent B, followed by 10 to 100% eluent B in 20 min, 100% eluent B for 10 min, 100 to 10% in 5 min and ended with 10% eluent B for 3 min. Mass analysis was performed as follows: Needle voltage (+/-) 5000/ -4500 V; spray shield voltage (+/−) 600/− 600 V; spray chamber temperature 50 °C; drying gas temperature 350 °C; nebulizing gas pressure 379 kPa; drying gas pressure 124 kPa, and capillary voltage (+/−) 40/− 40 V. Mass spectrometry analysis was conducted in a scanning range from 100 to 800 m/z for 50 min. In the MS/MS experiments, 10 eV collision energy was chosen to fragment the molecules.

3.3.8. Melting point

Mechanical spectra of AX (gels formed with LccPpu) and gelatin gel (6%) were recorded using a Physica MCR 301 rheometer with plate geometry 25 mm diameter and a gap width of 1 mm. The measurement was performed at a temperature rate of 10 °C/min, angular frequency 10 1/s and strain 0.5%, during heating from 20 to 100 °C for the AX gel and 5 to 42 °C for the gelatin gel. Mechanical parameters were recorded every 0.1 minute.

3.3.9. Water-holding capacity WHC

In order to conduct the WHC test, 1 g gel sample (6%) was centrifuged at 700 g for 30 min at 10 °C. The water-holding capacity was calculated as **(Chen and Lin, 2002)**:

WHC (%) = (mass of gel after centrifugation/mass of gel sample) ×100

3.3.10. Swelling ratio

Arabinoxylan (gels formed with LccPpu) and gelatin gel (6%) with a defined volume were immersed in artificial saliva composed as described in Amal *et al.* (2015) **(Amal *et al.* 2015)** for 10 minutes at room temperature. The gel volume was measured every two minutes and the swelling ratio calculated as:

Swelling ratio = V_n / V_0

where V_n is the volume of the gels after every two minutes immersion (m^3) and V_0 is the initial volume of the gel before immersion in artificial saliva (m^3).

3.4. Results

3.4.1. Wheat bran arabinoxylan gel characterization

3.4.1.1. Wheat bran arabinoxylan

The yield of the extracted AX was 2.8% (mass of AX/mass of wheat bran) and contained 2.17 ± 0.06 µg ferulic acid /mgAX. Intact ferulic acid in the extracted arabinoxylan was cross-linked with laccase Ftr and Ppu to form a hard gel.

3.4.1.2. Oscillatory tests on gels from self-extracted and commercial AX formed using LccC

During a sweep test, the amplitude of the deformation was varied (γ = 0.01 to 100%), while the frequency was kept constant (ω = 10 rad/s). For the analyses, the storage modulus G′ and the loss modulus G″ were plotted against the deformation (γ). Gels from self-extracted and commercial AX (as negative control) had constant G′ and G″ at $0.1 \leq \gamma \leq 70\%$, which showed

that the sample structures were undisturbed. In the negative control, G'' was > G′, while in the AX gels G′ was > G″.

Viscoelastic properties derived from a frequency sweep test at ω = 10 rad/s presented G′ = 9.35 ± 0.017 and 6 ± 0.018 kPa and G″= 2.45 ± 0.024 and 8.03 ± 0.021 kPa for AX gels (6%) and the negative control (6%), respectively. The loss factors of AX gels and the negative control formed using laccase C were tan$\delta \leq 0.8$ and tan$\delta \geq 1$, respectively.

3.4.1.3. Different laccases from Basidiomycetes

Basidiomycete fungi are well known for their high-redox potential laccases. A possibly different effect of laccases from *Pleurotus pulmonarius* (Ppu) and *Funalia trogii* (Ftr) on the rheological properties of 6% AX gels compared with the action of commercial laccase C was investigated. The gels obtained using laccase Ftr and Ppu had a large linear viscoelastic region at $0.1 \leq \gamma \leq 70\%$ (data not shown). The viscoelastic properties deduced from a frequency sweep test at ω = 10 rad/s are shown in Table 3.1. The loss factors of the 6% AX gels formed by the different laccases can be found in Figure 3.2. Gels formed by laccase Ppu and Ftr possessed higher storage moduli and lower loss factors in comparison with laccase C. The xylanase activity in laccase C was determined by the DNSA test **(Miller, 1959)**. The used technical preparation contained 1.9 U/mg xylanase activity.

Table 3.1. Viscoelastic properties of the 6% arabinoxylan gels formed with different laccases

Arabinoxylan gel 6%	**Viscoelastic Properties**	
	G′ (kPa)	**G″ (kPa)**
LccC	9.3	2.4
LccPpu	10	2.2
LccFtr	9.8	2.5

SD ≤ 0.03 kPa

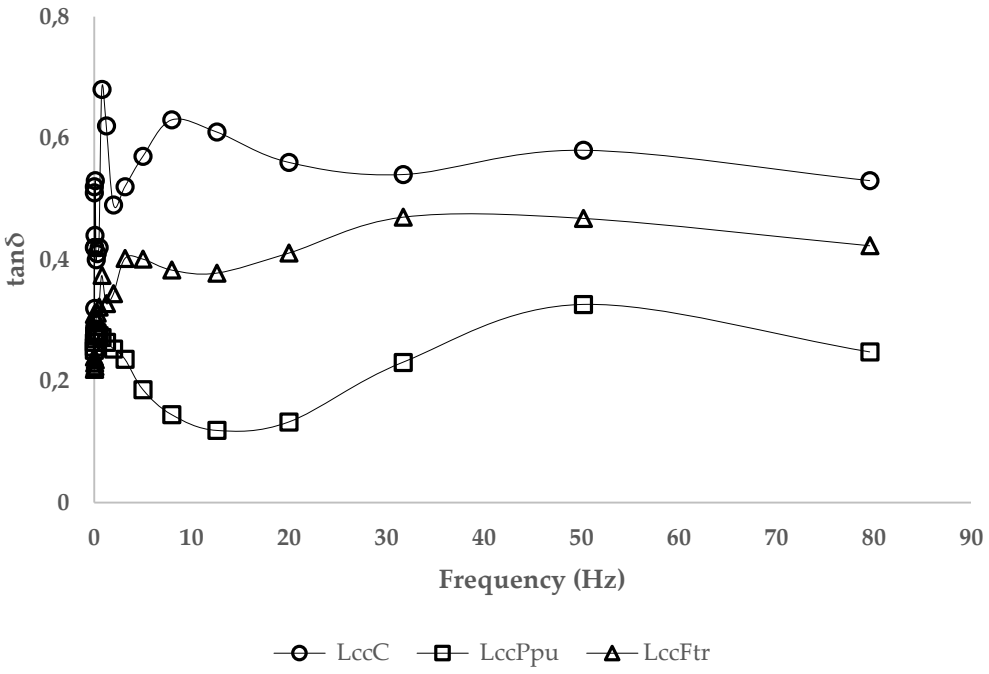


Figure 3.2. Loss factor of the arabinoxylan gels (6%), formed with laccase C (circles), Ppu (squares), and Ftr (triangles)

3.4.1.4. Effect of citrus pectin and mediators on arabinoxylan gels

Different additives were tried to enhance the firmness of the AX gels. Table 3.2 indicates the storage modulus, loss modulus, and loss factor of the gels formed in the presence of the reaction mediators caffeic acid (CA), ABTS, or citrus pectin.

Table 3.2. Viscoelastic properties of 6% AX gels, storage modulus G' (kPa), and loss modulus G" (kPa)

AX gel 6%	Mediators												Pectin C			
	1 mM CA[a]		3 mM CA		5 mM CA		1 mM ABTS		3 mM ABTS		5 mM ABTS		1%		3%	
	G'	G''	G'	G''	G'	G''	G'	G''	G'	G''	G'	G''	G'	G''	G'	G''
LccC	8.1	2.7	4.5	1.1	3.5	1	8.8	2.4	9.3	2	6.4	2.3	9.1	2.1	9.4	1.9
LccPpu	8.5	2.1	4.6	1.1	3.6	1	9.2	2.1	9.3	2.3	6.9	2.2	9.1	2.1	9.4	2.3
LccFtr	8.4	2.1	4.6	1.4	3.5	1.1	9.1	2.2	9	2.1	6.8	2.2	9.1	2.2	9.4	2.4

[a] Caffeic acid

SD ≤ 0.04 kPa

Caffeic acid, especially at concentrations of 3 and 5 mM, decreased the storage moduli of the gels and had a destructive effect on the final gel structure. Analysing loss factors of the gels showed much higher values than without CA or just 1 mM of the mediator, and the same was true for the viscosity.

Likewise, the non-food mediator ABTS significantly affected the viscoelastic properties of AX gels. Increasing the concentration of ABTS up to 5 mM resulted in lower storage moduli and higher loss factors of the gels in comparison with 3 mM ABTS. In addition, the loss factor of the gels formed with 3 mM ABTS was lower than 0.6 indicating elastic gels.

One and three percent pectin C were added to improve the viscoelastic properties, but no increase in storage modulus was observed. The rheological examination of the AX gels with pectin indicated that the storage moduli in the presence of 3% pectin were higher than in 1% samples, and their loss factor was < 0.5. As can be inferred from Tables 1 and 2, mediators and pectin C did not improve the storage and loss moduli and loss factors of AX gels.

3.4.2. Storage stability of arabinoxylan gels

The storage stability of the enzymatically cross-linked AX gels (6%) was investigated over six weeks at 25 °C. Besides, the residual activity of the laccases Ppu, Ftr, and C was determined. The storage modulus of the AX gels with laccase C showed a substantial decrease from 9.35 to 2.65 kPa after two weeks of storage. The storage moduli of the AX gels with laccase Ppu and Ftr are shown in Figure 3.3 over 42 days of storage. Gels formed with laccase Ftr showed a decreasing G′ after 28 days. By the end of day 35, the storage modulus reached around 6.32 kPa and lost its viscoelastic properties as reflected by a high loss modulus of 8.45 kPa (G″ > G′). AX gels formed with LccFtr reached a loss factor of 4.23 on day 42.

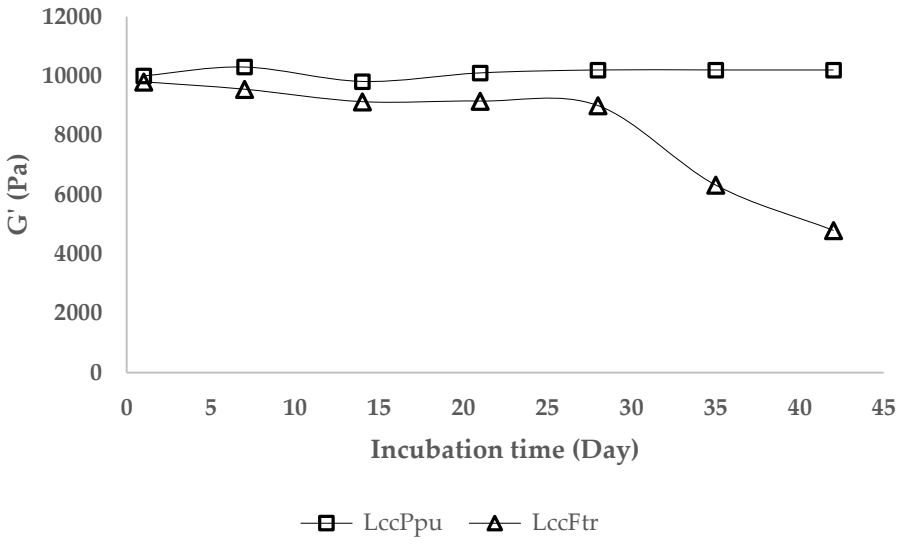


Figure 3.3. Storage modulus of the AX gels (6%) during six weeks incubation at 25°C

Gels formed using laccase Ppu showed a tan$\delta \leq 0.5$ after 28 days of storage. Although an increase in loss factor was observed after day 35 (tanδ ≤ 0.9), the storage modulus remained constant (Figure 3.3).

The activity of residual laccases during the incubation at 25 °C was determined using the ABTS assay (Figure 3.4). The activity of the laccase Ppu slightly declined from 0.04 to 0.0224 U/µg FA, whereas the activity of laccase Ftr strongly decreased to 0.0033 U/µg FA. An inverse correlation was observed between the activity of the residual laccases and the loss factor of the gels. A slight decrease in activity of residual laccase Ppu resulted in a gentle increase of the loss factor from 0.5 to ≤ 0.9 during the storage. The reduction of the residual laccase Ftr activity was accompanied by a distinct rise of the loss factor from 0.47 to 4.23 from day 28 to 42.

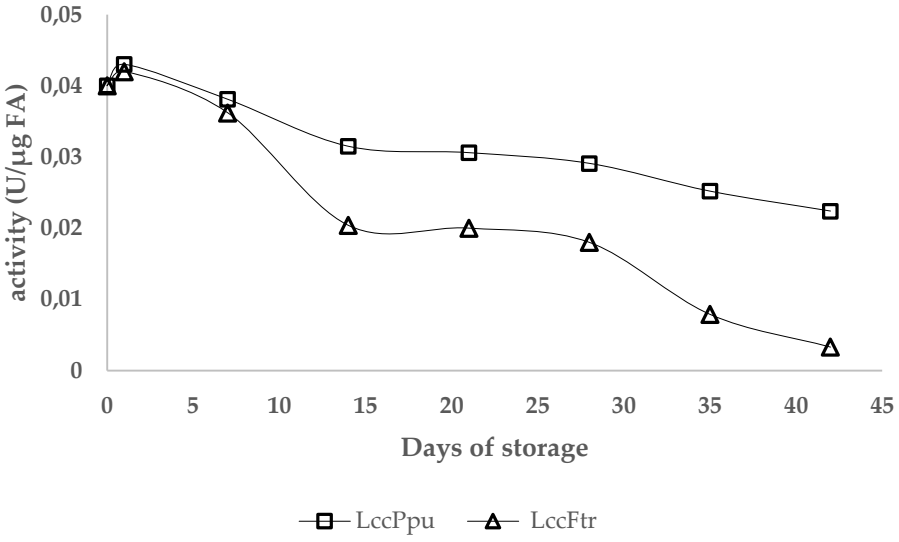


Figure 3.4. Activity of residual laccases in the AX gels during incubation at 25°C

3.4.3. Comparison of the viscoelastic properties of AX and gelatin gels

The viscoelastic properties of AX (6%) and gelatin gels (6%) were analysed by a frequency sweep test at ω = 10 rad/s (Figure 3.5). Storage and loss modulus of the gelatin gel were 9.75 and 2.24 kPa, respectively; the loss factor was < 0.3. At frequencies lower than 50 Hz, the loss factors of 6% AX gels formed using laccase Ppu were slightly higher than for gelatin gels. This difference was more significant with laccase Ftr, which obtained loss factors < 0.5. The storage moduli of AX gels and gelatin were on the same level (data not shown). They all showed loss factors < 1, which confirmed their elastic properties.

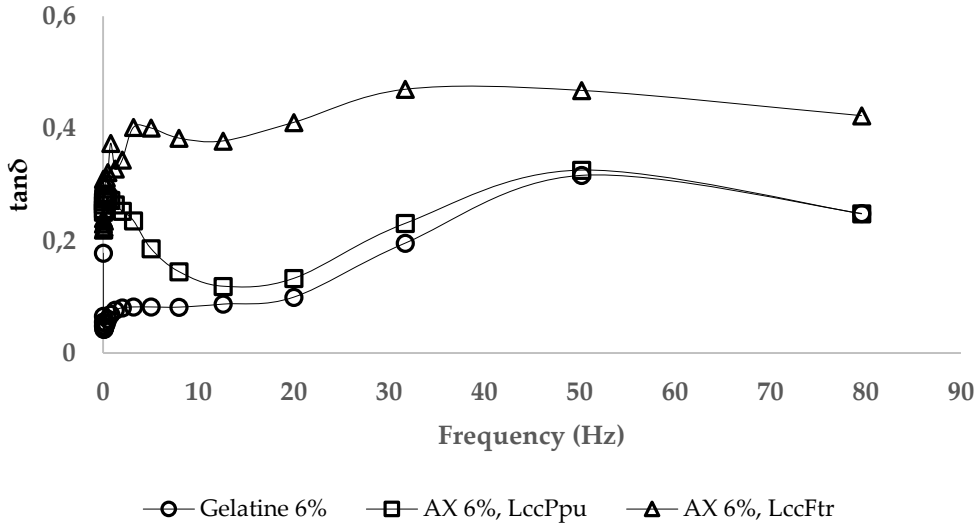


Figure 3.5. Comparing loss factors of AX (6%) and gelatin gels (6%)

3.4.4. Identifying diferulic acids in cross-linked arabinoxylan gel

The oxidative gelation of AX was supposed to result from the dimerization of FA residues on the polysaccharide chains. The FA dimerization mechanism starts with an abstraction of an H atom of the OH group at the ring position of FA leading to a phenoxy radical. Then, this radical is stabilized by resonance along the aromatic ring (C-4/ C-5) and the double bound (C-8) of the side chain. Subsequently, two phenoxy or alkoxy radicals cross-link and the coupling of unpaired electrons of two radicals forms a new covalent bond that connects two arabinoxylan chains. Consequently, the structure of the dimers formed during gelation depends on the radical position **(Mendez-Encinas *et al.*, 2018)** (Figure 3.1). Liquid chromatography mass spectrometry (LCMS) showed a peak (retention time about 14 minutes) with m/z 387 (+), indicative of a diferulic acid (Figure 3.6, peak A) **(Vismeh *et al.*, 2013)**. An MS/MS fragmentation experiment on this peak led to fragment ion masses m/z 369 (- H_2O), 343 (- COO-), 327 (- CH_3COOH), 263 (- hydroxy, methoxy-phenyl-), 193 (- feruloyl-), and 149 (- vinyl-guaiacyl-), which fit to 8-O-4′ diferulic acid (Figure 6A and 7). Moreover, a big peak at a retention time of about 11 min was observed with m/z 386.6 and 387.1 ions (Figure 3.6, peak B).

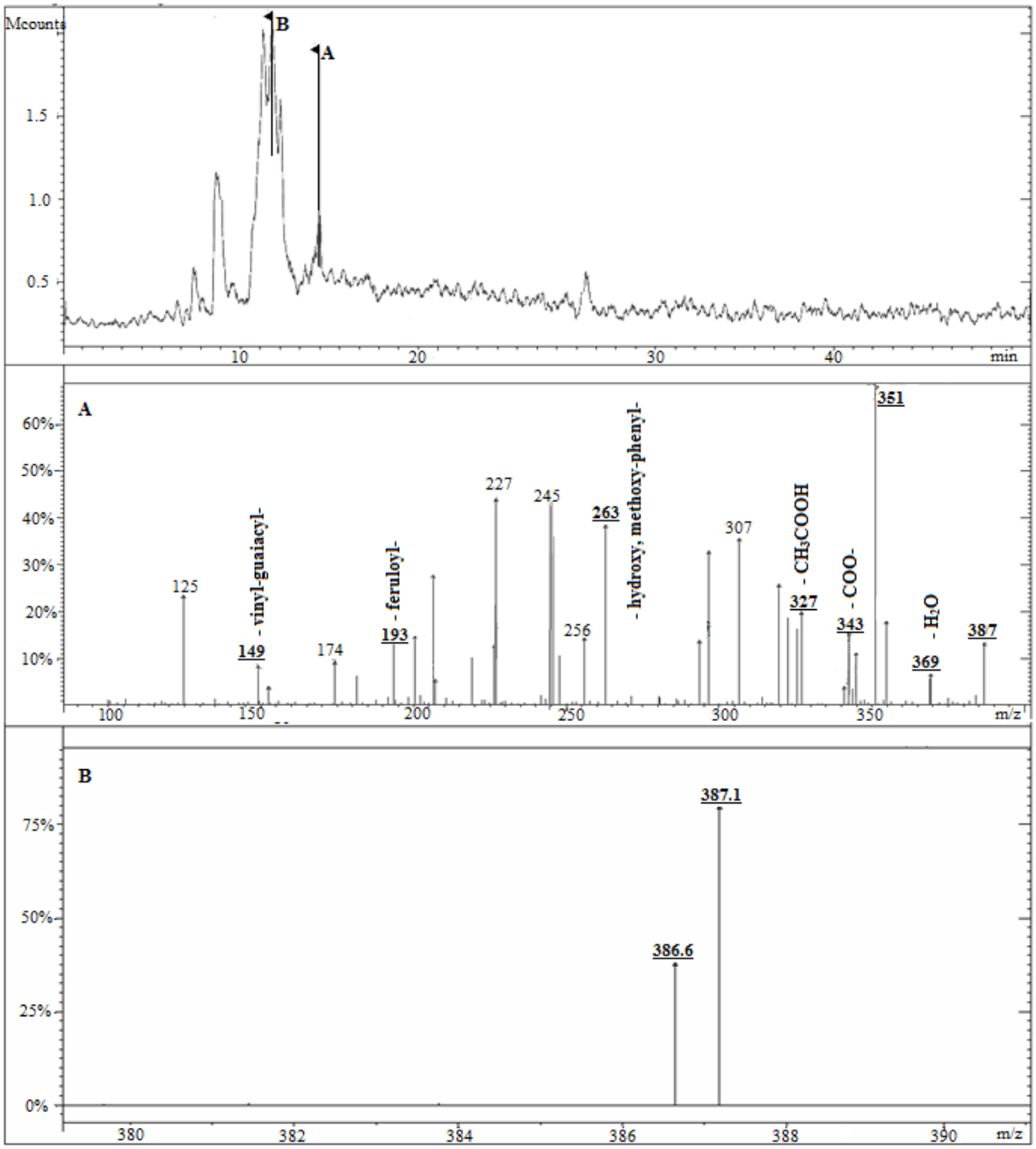


Figure 3.6. LCMS and MS/MS spectra of diferulic compounds in arabinoxylan gel

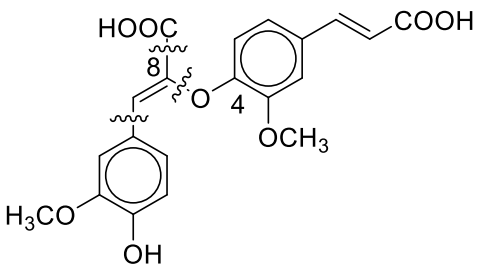


Figure 3.7. Molecular structure of 8-O-4 diferulic acid with MS/MS fragmentation sites

3.4.5. Melting point

The melting point of AX gels (formed with LccPpu) and gelatin gels was measured by a constant frequency oscillatory test. Figures 3.8 and 3.9 present the temperature dependence of dynamic modulus (G′ and G″) during isochronal temperature sweeps in heating from 5 to 42 °C and 20 to 100 °C for gelatin and AX gels, respectively. The starting temperature was chosen according to the gel formation temperature. Gelatin gel showed a high storage modulus during heating from 5 to almost 30 °C followed by a sudden drop at around 32 °C. The melting point of gelatin depends on its grade and concentration, but is typically ≤ 35 °C **(Francis, 1999)**. As shown in Figure 3.9, there was a gradual decrease of the storage modulus with increasing temperatures in AX gels, which presented a linear correlation between rising temperature and falling storage modulus with $R^2 = 0.96$.

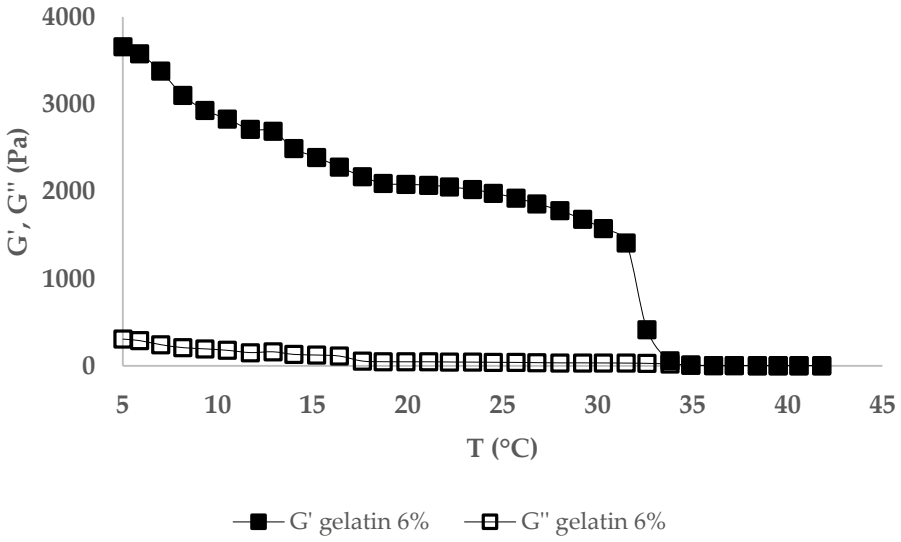


Figure 3.8. Melting point of gelatin gel (6%)

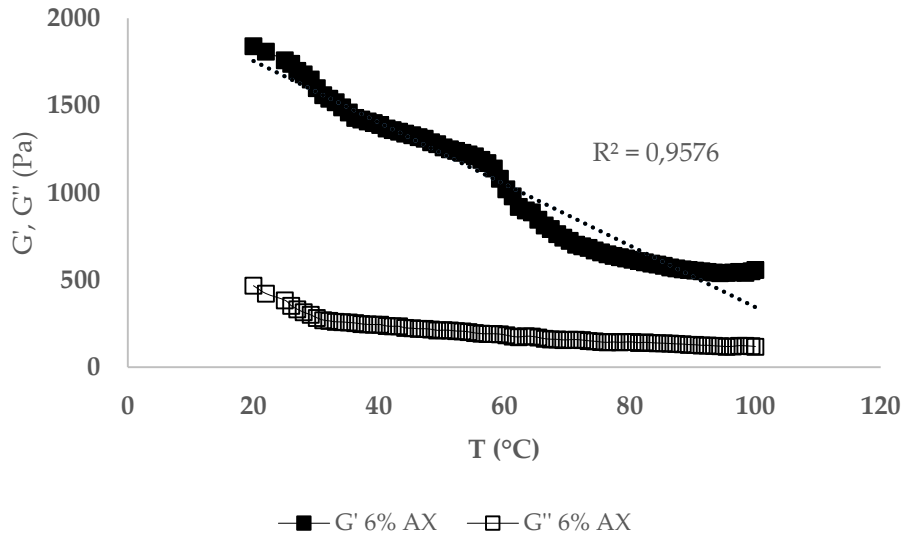


Figure 3.9. Melting point of arabinoxylan gel (6%)

3.4.6. Water-holding capacity

Water holding capacity is defined as the physical property, which prevents water from being released from the structure of the gel **(Zayas, 1997)**. The water holding capacities for arabinoxylan gels (formed with LccPpu) and gelatin gels were determined to be 98.2% and 98.6%, respectively.

3.4.7. Swelling ratio

Figure 3.10 presents the swelling ratio of gelatin and AX gels (formed with LccPpu). A linear correlation (R^2 = 0.98) between swelling ratio of the AX gel and immersing time in artificial saliva was recorded.

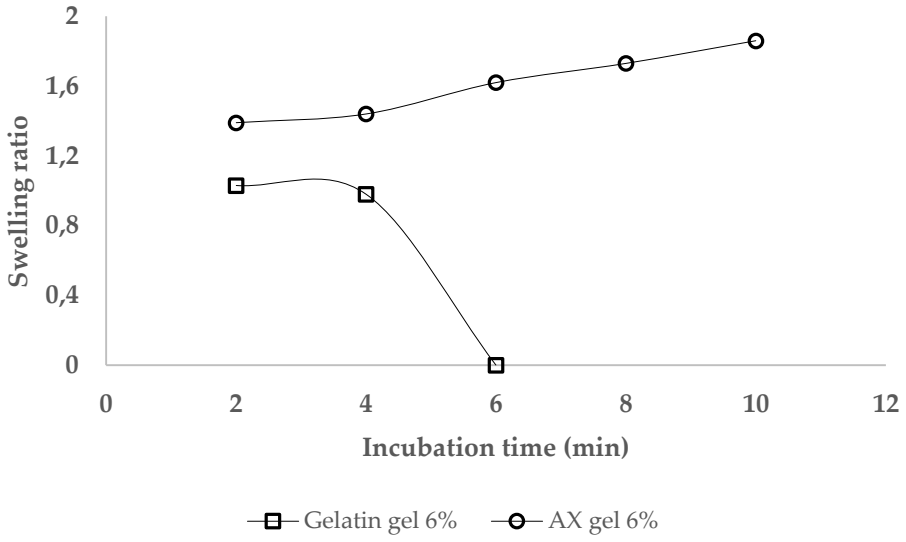


Figure 3.10. Swelling ratio of gelatin (6%) and arabinoxylan gel (6%) in artificial saliva at room temperature

3.5. Discussion

3.5.1. Wheat bran arabinoxylan

The most common method of separating arabinoxylan on a large scale is alkaline extraction, using sodium hydroxide, potassium hydroxide, or barium hydroxide solutions (1 M for 16 h) **(Börjesson *et al.*, 2018)**. Although alkaline treatment increases arabinoxylan yield, it hydrolyses ferulic acid bonds. In the current study, AX was treated with sodium hydroxide 0.5 M for 40 min only. The reduced pH and shorter treatment time led to a decrease of AX yield from 6% to 2.8% (mass of AX/mass of wheat bran), but increased the ferulic acid content from non-detectable to 2.17 ± 0.06 µg ferulic acid /mgAX. The self-extracted AX could therefore be used for gel formation, which is not possible with commercially available AX.

3.5.2. Wheat bran arabinoxylan gel characterization

Arabinoxylan gels have higher storage than loss moduli. This indicates viscoelastic behaviour of the gels **(Barnes, 2000)**. The higher loss (G″) than storage modulus (G′) in the negative control was attributed to a lack of ferulic acid moieties. The AX gelation process and gel properties are governed by the establishment of covalent (di-FA, tri-FA bridges) linkages **(Vansteenkiste *et al.*, 2004)**. The more intact ferulic acid moieties on the polysaccharide backbone, the more covalent cross-links may be formed **(Carvajal-Millan *et al.*, 2005)**. Carvajal-Millan *et al*. reported that an elevation in ferulic acid concentration from 0.93 to 1.4

µg/mg led to an increase in storage modulus for 1% AX gels from 6 to 43 Pa. **(Carvajal-Millan *et al.*, 2005)**.

According to the results, self-extracted AX with intact ferulic acid moieties formed a firm gel structure as shown by its high storage modulus (Table 3.1). This was not observed when the same conditions were applied to commercial AX samples (negative control) **(Hollmann and Lindhauer, 2005)**.

A loss factor lower than one indicates elastic while higher than one implies viscous properties **(Eidam *et al.*, 1995)**. The negative control, which contained no ferulic acid, did not form a firm gel, as proven by higher loss factors ($\tan\delta \geq 1$). In contrast, the self-extracted AX formed a solid gel.

3.5.3. Different laccases from Basidiomycetes

Laccase C had 1.9 U/mg xylanase activity, which may have led to the observed weaker gel network by slowly hydrolysing glycosidic bonds of the AX main chain (Figure 3.1) **(Van Den Brink and De Vries, 2011)**. In contrast, the high purity of the laccases Ppu and Ftr most likely contributed to gels with a firmer structure.

3.5.4. Effect of citrus pectin and mediators on arabinoxylan gels

A series of experiments was conducted to enhance the rheological properties of AX gels through mediators (Table 3.2). Although CA (5 mM) clearly improved the storage modulus of cross-linked yogurt formed with LccC (**Struch *et al.*, 2015**), it had a devastating impact on the storage moduli of AX gels. The inhibitory impact of caffeic acid was attributed to its oxidation while reducing the semiquinones of ferulic acid produced by laccases. Therefore, ferulic acid was no longer oxidized to dimers **(Figueroa-Espinoza and Rouau, 1999)**.

Increasing the concentration of ABTS to 5 mM was followed by a decrease in the storage modulus of AX gels (Table 3.2). Higher concentrations of ABTS competed with ferulic acids for laccase. In addition, it was reported that the laccase/ABTS system coupled ABTS to ferulated arabinoxylan, rather than causing dimerization of ferulic acids **(*Mendez-Encinas et al.*, 2018)**.

Citrus pectin, a common thickening and gelling agent, has the ability to link with various compounds to form a gel **(Saha & Bhattacharya, 2010)**. Nonetheless, as shown in Table 3.2, it did not have a significant impact on the viscoelastic properties of AX gels. Overall, addition

of pectin resulted in hydrogen bonds and hydrophobic interactions in the gel, instead of increasing covalently cross-linked bonds **(Gawkowska *et al.*, 2018)**. To sum up, the investigated mediators were not successful in improving the rheological properties of cross-linked AX gel.

3.5.5. Storage stability of arabinoxylan gels

Since the aim of the project was to form a hard gel, which was stable during storage, a rheological analysis was performed over six weeks of storage. AX gels formed with Laccase C showed a sudden drop of G′ after two weeks storage. Xylanase in the enzyme preparation may have started hydrolysing the AX main chain and have broken down the gel structure **(Carvajal-Millan *et al.*, 2005)**. Gels formed using laccase Ftr and Ppu were stable for four and six weeks, respectively (Figure 3.3). In contrast to earlier findings **(Morales-Burgos *et al.*, 2017)**, no syneresis was observed in gels over the course of this work. This may be attributed to the three times higher FA concentration of the AX used in the previous work. As a result, the network contracted more easily due to increasing polymer chain connectivity **(Morales-Burgos *et al.*, 2017)**. Some softening of the structure was observed after four weeks of storage. Laccase-produced radicals were probably responsible for this phenomenon. Radicals participating in secondary reactions may have led to a beginning degradation of the AX main chains **(Anderson and Simsek, 2018)**. It has been recently reported that a 90 min heat treatment at 85 °C significantly decreased the laccase activity. As a result, the storage modulus of sweetened cross-linked Fibrex gels formed with LccFtr stayed stable for over a month of storage after the above-mentioned heat treatment. Due to their highly similar chemical structure, these results should be transferable to the AX gels in this study **(Khalighi *et al.*, 2020a)**.

3.5.6. Viscoelastic properties of AX and gelatin gels

Cross-linking of AX with LccFtr and LccPpu formed elastic gels with viscoelastic properties comparable to gelatin gels. AX gels, like traditional gelatin gels, do not raise nutritional concerns as *Bifidobacterium* enzymes present in the human large intestine degrade them **(Anderson and Simsek, 2018)**. The covalent structure of cross-linked AX gels confers them some superior characteristics, such as high water absorption capacity and stability to pH, temperature, and ionic charges **(Mendez-Encinas *et al.*, 2018)**.

3.5.7. Identifying diferulic acids in cross-linked arabinoxylan gel

AX gels formed with LccPpu have been analysed with LCMS/MS, and 8-O-4′ dehydrodimers of ferulic acid were detected as cross-links. Dominating dimers of ferulic acids in cross-linked wheat bran AX were 8-O-4′ and 8-5′, both of which increased after gel formation **(Khalighi *et al.*, 2020a; Carvajal-Millan *et al.*, 2005)**. It has recently been reported that in the cross-linked gels, which were formed with LccFtr, 8-5′ dimers of ferulic acids were predominant **(Khalighi *et al.*, 2020a)**. The production of dimers of ferulic acids corresponded to the consumption of ester-bound ferulic acid **(Figueroa-Espinoza and Rouau, 1999)**. Obviously, due to different substrate specificities, laccase Ppu formed phenoxy radicals by absorption of the H atom of the OH group at the C-4 ring position of ferulic acid, while laccase Ftr preferred the C-5 ring position.

In addition, a big peak at a retention time of about 11 min with 386.6 and 387.1 m/z ions showed a clusteration. The difference of 0.5 amu between the detected masses means that an ion possessing a double charge was present. This is interpreted as a clustered diferulate of as of now unknown structure. Recently, formation of cross-links of ferulic acid of higher molecular masses than dimers and undefined superior structures was reported **(Mendez-Encinas *et al.*, 2018)**.

3.5.8. Arabinoxylan and gelatin gel structural characteristics

Melting point, water holding capacity and swelling ratio have been determined to investigate physical properties of the gels. Analysing the melting point of gelatin gels showed a drop in storage modulus at almost 35 °C, followed by a G'' overtaking G' (Figure 3.8). AX gels gradually decreased in G', and G' stayed greater than G'' during the heating process (Figure 3.9). The higher temperature stability of the AX gel must be attributed to the covalent bonds formed after oxidative coupling of the ferulic acids **(Mendez-Encinas *et al.*, 2018)**.

The high WHC of AX gels, which equalled that of the gelatin gel, resulted from the three-dimensional network that was able to retain a large aqueous phase and provided a structure stable against syneresis and shrinkage **(Mendez-Encinas *et al.*, 2018; Morales-Burgos *et al.*, 2017).**

Estimated swelling ratio of the AX gel (in water and for 36 h incubation at room temperature) was higher than theoretically determined from the diferulic acid content of AX gels. It was suggested that, in addition to di-FA and tri-FA, higher ferulated cross-linking and physical entanglements would contribute to the final arabinoxylan gel structure **(Carvajal-**

Millan *et al.*, 2005). In contrast, the hydrogen bonds of the gelatin gel were dissolved by the penetrating water molecules after five minutes storage at room temperature.

3.6. Conclusions

The present study showed that AX obtained by a modified alkaline extraction method was successfully cross-linked with purified laccases from the basidiomycetes *Funalia trogii* (LccFtr) and *Pleurotus pulmonarius* (LccPpu). The new AX gels resembled gelatin gels in terms of storage modulus, loss factor ($\tan\delta < 0.5$), high water holding capacity, and digestibility. The AX gels retained their viscoelastic properties during a storage period of up to six weeks at 25 °C (LccPpu, Figure 3.3) and may be classified vegan. However, temperature stability and swelling behaviour of the AX and gelatin gels (Figures. 3.8 to 3.10) were different and reflected the difference between covalent and hydrogen bond network knots. The thermo-reversible gelatin gels are known as an easy process product, which melt at body temperature. However, they have low stability to heat and are soluble only at higher temperatures **(Karim and Bhat, 2008)**. Arabinoxylan gels provide a heat resistant gel, formed at room temperature and as firm as gelatin, which could be applied in pharmaceutical and other industries.

Covalent cross-links were proven by 8-O-4 dimers of ferulic acids as well as clusters of diferulic acids, which will have to be analysed in more detail after analytical declusteration.

The refined extraction method and the enzymatic cross-linking concept appear promising to provide gels with a firm texture that is stable over an extended period of time. Sensory tests must be performed to investigate the organoleptic properties of the gels including the release of volatile and non-volatile ingredients typically contained in a wide range of foods, such as candies, confectionary, liquorice products, glaze, and desserts.

3.7. Author Contributions:

S.K. and R.G.B. conceived and designed the experiments; S.K. performed the experiments; S.K. analysed the data; R.G.B. and F.E. contributed materials and analysis tools; S.K. and F.E. wrote the paper.

3.8. Funding:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

3.9. Acknowledgments:

The authors are grateful to U. Krings at the Institute of Food Chemistry, LU Hannover, for his skilful mass spectrometric analyses and help with the interpretation of the MS/MS data. We also thank D. Linke for helpful assistance. The publication of this article was funded by the Open Access fund of Leibniz Universität Hannover.

3.10. Conflicts of Interest:

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

4. Intro for publication: Cross-Linking of Fibrex Gel by Fungal Laccase: Gel Rheological and Structural Characteristics.

Sugar beet fibre is an agricultural by-product of beet processing, which mainly contains insoluble hemicellulose and soluble pectin **(Sato *et al.*, 2013)**. The ferulated side chains of sugar beet pectin have the potential to be cross-linked through oxidative coupling reaction **(Levigne *et al.*, 2004)**. The gel-forming ability of cross-linked sugar beet fibre was previously investigated and proven that the extraction method can have a direct impact on the total intact ferulic acids **(Micard and Thibault, 1999).** In addition, the formation of cross-linked arabinoxylan gels has been recently studied and introduced a hard gel, comparable with gelatin gels **(Khalighi *et al.*, 2020b)**. Therefore, a new opportunity for the transformation of an agro-industrial by-product to an edible gel was offered. Moreover, investigating Fibrex as another plant polysaccharide substrate for cross-linked gels would extend the range of so far underutilized by-products and contribute to the understanding of the properties of oxidatively gelled polysaccharides.

The following publication describes a hydrothermal extraction method, in which extracted Fibrex contained many intact ferulic acid moieties. A self-produced laccase from *Funalia trogii*, was applied to form the cross-linked gels. Rheological and structural gel characteristics were analysed and compared with gelatin gels. The major aims of the study were: firstly, to determine the mechanical spectra of the gels. Secondly, analysing structural properties of the gels (melting point, swelling ratio and WHC), as well as detecting cross-linked diferulic acids. Thirdly, an attempt was made to enhance gel structural and rheological properties by adding vanillin as a mediator and flavour, and citrus pectin as a gel thickening. Fourthly, investigate the impact of D-sucrose, the most common industrial sweetener, on the properties of the Fibrex gels. Finally, inspecting storage stability of the gels and forming a solid sweetened gel, comparable with gelatin gels.

5. Cross-linking of Fibrex gel by fungal laccase: gel rheological and structural characteristics

Sanaz Khalighi *, Ralf G. Berger, and Franziska Ersoy

Processes **2020**, *8*(1), 16; https://doi.org/10.3390/pr8010016, Received: 26 November 2019 / Accepted: 17 December 2019 / Published: 20 December 2019.

5.1. Abstract

Sugar beet fibre (Fibrex) is an abundant side-stream from the sugar refining industry. A self-produced laccase from *Funalia trogii* (LccFtr) (0.05 U/µg FA) successfully cross-linked Fibrex to an edible gel. Dynamic oscillation measurements of the 10% Fibrex gels showed a storage modulus of 5.52 kPa and loss factors ≤ 0.36 in the range from 20 to 80 Hz. Comparing storage stability of sweetened 10% Fibrex gels with sweetened commercial 6% gelatin gels (10% and 30% D-sucrose) indicated a constant storage modulus and loss factors ≤ 0.7 during four weeks of storage in Fibrex gels. Loss factors of sweetened gelatin gels were ≤0.2, and their storage modulus decreased from 9 to 7 kPa after adding D-sucrose and remained steady for four weeks of storage. Fibrex gel characteristics, including high water holding capacity, swelling ratio in saliva, and heat resistance are attributed to a covalently cross-linked network. Vanillin, as a mediator, and citrus pectin did not enhance covalent cross-links and elastic properties of the Fibrex gels. Thus, laccase as an oxidative agent provided gels with a solid and stable texture. Fibrex gels may find uses in pharmaceutical and other industrial applications, which require a heat-resistant gel that forms easily at room temperature. They also represent an ethical alternative for manufacturing vegan, halal, and kosher food.

Keywords: Fibrex gel; laccase; cross-linking; rheology; viscoelastic properties

5.2. Introduction

Fibrex is a dietary fibre, which represents an abundant side-stream from the sugar refining industry (approximately 200 kg of beet pulp per ton of sugar beet root) **(Sato *et al.*, 2013; Michel *et al.*, 1987)**. The main fibre types in Fibrex are soluble pectin and insoluble hemi cellulose, but also small amounts of cellulose and lignin **(Pacheco *et al.*, 2019)**. Sugar beet pectin contains arabinan (α-(1, 5)-linked **L**-arabinofuranosyl residues), and/or galactan (β-(1, 4)-linked **D**-galactose residues) as side chains of the rhamnogalacturonan main chain **(Saulnier & Thibault, 1999)**. These side chains can be feruloyl substituted either on O-2 in the main

backbone of rhamnogalacturonan-(1, 5)-linked arabinan, on O-5 in the terminal arabinose, or on O-6 in the main backbone of galactan **(Holck *et al.*, 2011)**. Most of the ferulic acid ester linked to arabinan and/or galactan can be recovered as ferulate ester of their oligosaccharides under hydrothermal conditions **(Sato *et al.*, 2013)**.

Feruloyl substitutions can be cross-linked through oxidative coupling reaction (Figure 5.1) **(Levigne *et al.*, 2004; Liu *et al.*, 2003)**. Recently, 8-5′ and 8-O-4′ were noted as the main ferulate dehydrodimers in covalently cross-linked structures in gelled sugar beet pectin **(Gawkowska *et al.*, 2018)**.

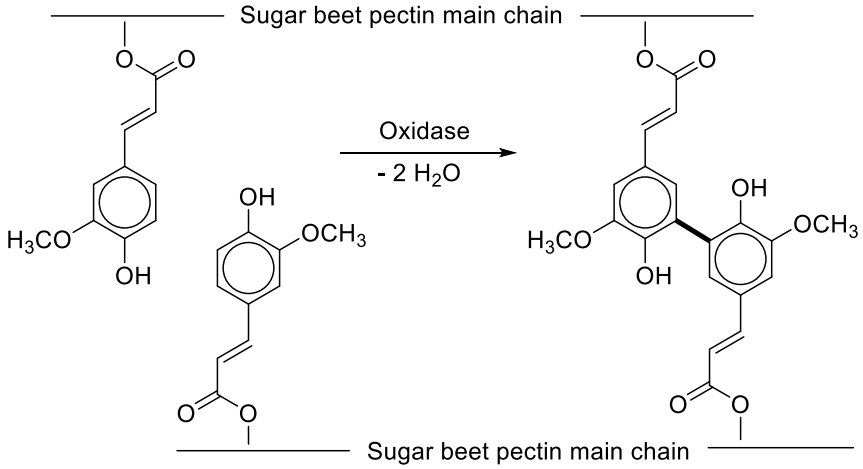


Figure 5.1. Cross-linking reaction of sugar beet pectins

Various oxidative agents were applied for the cross-linking of sugar beet pectin gels, including hydrogen peroxide, manganese peroxidases, and laccases **(Kuuva *et al.*, 2003)**. Laccases are polyphenol oxidases containing copper atoms in the catalytic centre and reducing oxygen to water accompanied by the oxidation of a phenol substrate **(Baldrian, 2006).** They were suggested as processing aids for wine and beer stabilisation, bakery products, to improve sensory parameters of food, and recently contributed to cross-linked gel formation **(Osma *et al.*, 2010; Khalighi *et al.*, 2020b; Ayala-Soto *et al.*, 2016)**.

Formation of a vegan and halal hard gel is a growing issue that has existed for many years, particularly within Europe, with the emergence of bovine spongiform encephalopathy ("mad cow disease") in the 1980s **(Morrison *et al.*, 1999)**. In the present study, sugar beet fibre, with as many ferulic acid moieties still bound to the sugar chain as possible, was extracted under hydrothermal conditions and cross-linked with a new purified high-redox laccase from the basidiomycete *Funalia trogii*. The working hypothesis was to convert the abundant side-stream

from the sugar refining industry to plant based gels with a firmness comparable to traditional gelatin gels.

5.3. Material and Methods

5.3.1. Materials

Dried and ground sugar beet pulp was bought from SternEnzym (Ahrensburg, Germany). All chemicals were obtained from Carl Roth (Karlsruhe, Germany), Fluka (Buchs, Switzerland), and Sigma-Aldrich (Taufkirchen, Germany).

5.3.2. Enzymes

Laccase were purified from the basidiomycete *Funalia trogii* (DSMZ 11919). For this, the fungus was cultivated in SNL medium (**Struch *et al.*, 2015**) and incubated at 25 °C and 180 rpm for 3 weeks. The purification was done by hydrophobic interaction chromatography on phenyl FF column and ion exchange chromatography on Q-sepharose XL material **(Kolwek *et al.*, 2018)**. The activity of the extracted and purified enzyme was 2.3 x 10^5 u/l. Laccase activity was measured at 25 °C and pH 4.5 using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (**Struch *et al.*, 2015**).

5.3.3. General experimental setup

All data presented are averages of duplicate measurements. The standard deviation of replicates for the rheological measurements was typically below 5%.

5.3.4. Sugar beet fibre extraction

Sugar beet pulp (10 g) was autoclaved at 121 °C for 20 min (Laboratory autoclave Biomedis, Giessen, Germany) (**Guillon *et al.*, 1992)**. Then, a 5% suspension of sugar beet pulp in water was formed and heated at 95 °C for 5 h. After cooling to room temperature and filtration (0.45 µm), the suspension was centrifuged at 12,000 x g for 10 min at 20 °C (centrifuge 460R Hettich Rotanta, Taufkirchen, Germany) **(Yapo *et al.*, 2007)**. The recovered supernatant was concentrated at 50 °C and vacuum 121 mbar (Heidolph rotary evaporator Laborota 4000, Schwabach, Germany). The concentrated sample was freeze dried (Alpha 1-4 LSCbasic, Martin Christ, Osterode, Germany) and milled manually. The bound ferulic acid content was determined by incubating 2 ml of 10% Fibrex solution with 2 ml KOH/NaOH (1:1; 4 mol/l

each) at 50 °C and 220 rpm for 4 hours. The reaction was stopped with 1 ml 60% acetic acid and 5 ml methanol. After centrifugation at 15,000 x g and 4 °C for 15 minutes, ferulic acid (FA) was analysed by HPLC at λ = 323 nm (SPD-10A VP, Shimadzu Deutschland GmbH, Berlin, Germany) **(Nieter *et al.*, 2016)**.

5.3.5. Sugar beet fibre cross-linking

In order to crosslink sugar beet fibre (Fibrex) gel, a 10% (w/v) Fibrex solution in 0.05 M citrate phosphate buffer pH 5.5 was incubated with laccase (0.05 U/µg FA, LccFtr) overnight without shaking. The impact of vanillin (4-hydroxy-3-methoxybenaldehyde) (2 and 4 mM) as mediator, and citrus peel pectin (1, 3, and 5% (w/v)) instead of Fibrex were likewise investigated.

5.3.6. Forming sweetened Fibrex gels

Fibrex gels (10%) were sweetened by adding 10 and 30% D-sucrose and laccase was deactivated under 90 min heat treatment at 85 °C. Injection of one unit xylanase activity into the gel and incubation at 30 °C for 2 h destroyed the structure of the gels and facilitated the ABTS assay to estimate the residual laccase activity after heat treatment.

In parallel, 6% gelatin gels (Dr. Oetker, Bielefeld, Germany, as standard), were formed and also heated at 85 °C for 90 min. All the samples were cooled and stored at 4 °C. Viscoelastic properties of the Fibrex gels were compared with gelatin gels prepared with the same buffer and D-sucrose concentrations (10 and 30%) during 4 weeks storage.

5.3.7. Rheology

To carry out rheological measurements, a Physica MCR 301 rheometer (Anton Paar, Graz, Austria) with plate geometry (25 mm diameter) and a gap width of 1 mm was used. Oscillation experiments (γ = 0.01 to 100%) were performed at a constant frequency of ω = 10 rad/s to ascertain the linear viscoelastic region (LVE) **(Barnes, 2000)**. Frequency sweep test used frequencies of 0.01 to 100 Hz with an applied strain of 5%. All tests were carried out at 25 °C in duplicates. Rheoplus software was used to calculate storage modulus (G′), loss modulus (G″), and loss factor tan δ (= G″/G′).

5.3.8. Analysing diferulic acids in Fibrex gels

5.3.8.1. Sample preparation

Fibrex gel was enzymatically hydrolyzed with a xylanase: ferulic acid esterase (1:1) mix for 3 hours at 35 °C, pH 6 and 220 rpm. The recombinant ferulic acid esterase originated from *Streptomyces werraensis* (**Schulz *et al.*, 2018**). The hydrolyzed sample was purified on 20 ml XAD 2 resin column as described by Malunga and Beta **(Malunga and Beta, 2016)**. Adsorbed diferulic acids were eluted in 30 ml methanol: formic acid (9:1), concentrated, lyophilised, and dissolved in 50 µl acetonitrile.

5.3.8.2. High performance liquid chromatography mass spectrometry

To detect cross-linked diferulic acids in the gel, 15 µl hydrolysate was loaded on a Varian 1200 LC-MS (Agilent, Santa Clara, USA) equipped with a C18 Pyramid column (Macherey-Nagel, Düren, Germany). As eluent A, water with 0.1% formic acid was used, whereas acetonitrile with 0.1% formic acid was applied as eluent B. A separation was achieved using a stepwise gradient at a flow velocity of 0.1 ml/min: sample loaded in 10% eluent B, followed by 10 to 100% eluent B in 20 min, 100% eluent B for 10 min, 100 to 10% in 5 min and ended with 10% eluent B for 3 min. Mass analysis was performed as follows: Needle voltage (+/-) 5000/ -4500 V; spray shield voltage (+/−) 600/− 600 V; spray chamber temperature 50 °C; drying gas temperature 350 °C; nebulizing gas pressure 379 kPa; drying gas pressure 124 kPa, and capillary voltage (+/−) 40/− 40 V. Mass spectrometry analysis was conducted in a scanning range from 300 to 400 m/z for 50 min. In the MS/MS experiments, 10 eV collision energy was chosen to fragment the molecules.

5.3.9. Melting point

Mechanical spectra of Fibrex (10%) and gelatin gel (6%) were recorded using a Physica MCR 301 rheometer with plate geometry of 25 mm diameter and a gap width of 1 mm. The measurement was performed at a temperature rate of 10 °C/min, angular frequency 10 1/s and strain 0.5%, during heating from 20 to 100 °C for the Fibrex gel and 5 to 42 °C for the gelatin gel. Mechanical parameters were recorded every 0.1 minute.

5.3.10. Water-holding capacity WHC

In order to conduct the WHC test, 1 g gel sample (10%) was centrifuged at 700 g for 30 min at 10 °C. The water-holding capacity was calculated as **(Chen and Lin, 2002)**:

$$\text{WHC (\%)} = (\text{Mass of gel after centrifugation}/\text{Mass of gel sample}) \times 100$$

5.3.11. Swelling ratio

Fibrex (10%) and gelatin gel (6%) with a defined volume were immersed in artificial saliva composed as described by Amal *et al.* **(Amal *et al.* 2015)** for 10 minutes at room temperature. The gel volume was measured every two minutes and the swelling ratio calculated as:

$$\text{Swelling ratio} = V_n / V_0$$

where V_n is the volume of the gels after every two minutes immersion (m^3) and V_0 is the initial volume of the gel before immersion in artificial saliva (m^3).

5.4. Results

5.4.1. Fibrex gel characterization

5.4.1.1. Oscillatory tests on Fibrex formed with LccFtr and standard gelatin gels

During a sweep test, the amplitude of the deformation was varied (γ = 0.01 to 100%), while the frequency was kept constant (ω = 10 rad/s). For the analyses, the storage modulus G' and the loss modulus G″ were plotted against the deformation (γ). Gels from Fibrex (10%) and gelatin (6% as standard) had constant G′ and G″ at $0.1 \leq \gamma \leq 70\%$, which showed that the sample structures were undisturbed (data not shown). In both, Fibrex and gelatin gels, G′ was > G″.

Viscoelastic properties derived from a frequency sweep test at ω = 10 rad/s presented G′ = 5.52 ± 0.023 and 9.05 ± 0.015 kPa and G″= 1.26 ± 0.014 and 2.24 ± 0.011 kPa for Fibrex (10%) and gelatin gels (6%), respectively.

As can be inferred from Figure 5.2, the loss factors of both, Fibrex and the standard gelatin gels, were ≤ 0.36, which proved the formation of a solid gel.

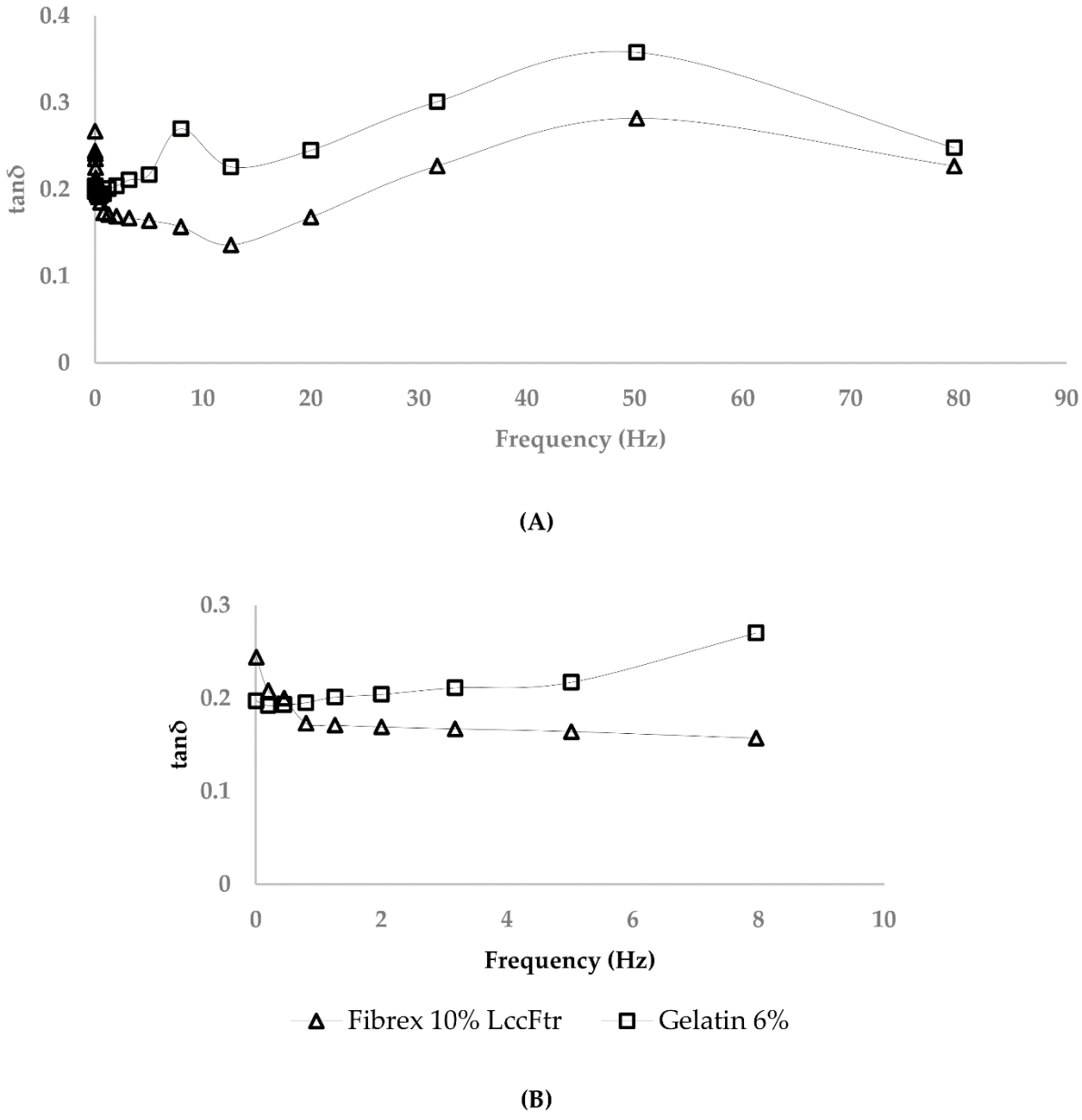


Figure 5.2. Loss factors of Fibrex gel (10%) formed with LccFtr, and gelatin gel 6% (**A**: in frequencies < 100 Hz, **B:** magnifying frequencies < 10 Hz)

5.4.1.2. Effect of citrus pectin and vanillin on Fibrex gels

Different additives were tested to enhance the firmness of the Fibrex gels. Table 5.1 indicates the storage modulus, loss modulus, and loss factor of the gels formed in the presence of the presumed reaction mediator vanillin or citrus pectin.

Vanillin decreased the storage moduli of the gels and had a destructive effect on the final gel structure at concentrations of two and four mM. Analysing loss factors of the gels showed much higher values than without vanillin, and the same was true for the viscosity.

Pectin C (1, 3, and 5%) was added to improve the viscoelastic properties, but no increase in storage modulus was observed. The rheological examination of the Fibrex gels with pectin indicated that the storage moduli were slightly higher in the presence of 5% pectin than in 1 and 3% samples, and all loss factors were ≤ 0.3. As can be inferred from Table 5.1, mediators and pectin C did not improve storage and loss moduli and loss factors of Fibrex gels.

Table 5.1. Viscoelastic properties of 10% Fibrex gels, storage modulus G′ (kPa), loss modulus G″ (kPa) and loss factor tanδ

Rheological parameters	No additives	Vanillin		Pectin C		
		2 mM	4 mM	1%	3%	5%
G′	5.52	1.83	1.34	5.44	5.23	5.57
G″	1.26	0.41	0.38	1.26	1.28	1.40
tanδ	≤ 0.36	≤ 0.76	≤ 0.80	≤ 0.31	≤ 0.29	≤ 0.30

SD ≤ 0.06 kPa

5.4.2. Viscoelastic properties of sweetened Fibrex gels

An ABTS assay was performed on sweetened Fibrex gels to measure the residual activity of laccase prior to rheological experiments. Approximately 0.03% of LccFtr activity was left after 90 min heat treatment at 85 °C (activity of laccase decreased from 19,800 to 5.4 U/l).

The viscoelastic properties of sweetened Fibrex (10%) and gelatin gels (6%) are presented in Table 5.2. Although neither 10 nor 30% D-sucrose had a significant impact on storage modulus of Fibrex gels, it caused an increase in loss factor of Fibrex gels. As can be seen in Table 5.1, tanδ of Fibrex gels with 10% D-sucrose in week 0 was higher than 1, which indicated a destruction of the gel structure. Gels containing 30% D-sucrose were able to maintain their structure. A decrease in storage moduli was observed for sweetened gelatin (mean G′ = 7.40 ± 0.50 kPa) in comparison with non-sweetened gels (G′ = 9.05 ± 0.015 kPa). Loss factors of both sweetened gelatin gels were lower than those of non-sweetened samples with tanδ ≤ 0.1, especially at frequencies between 0.1 to 10 Hz (Figure 5.3).

Table 5.2. Storage modulus G′ (kPa), loss modulus G″ (kPa) and loss factor tanδ of 10% Fibrex gels and 6% gelatin gels (containing D-Saccharose) during 4 weeks storage at 4 °C

Gels	additives	Week #	G′	G″	tanδ
Fibrex gel 10% formed with LccFtr	**10% D-sucrose**	0	5.48	1.34	≤ 1.73
		2	5.36	1.43	≤ 0.53
		4	5.75	1.50	≤ 0.52
	30% D-sucrose	0	5.27	1.33	≤ 0.72
		2	5.23	1.25	≤ 0.48
		4	5.86	1.49	≤ 0.66
Gelatin gel 6%	**10% D-sucrose**	0	7.90	1.51	≤ 0.19
		2	8.22	1.40	≤ 0.17
		4	6.16	1.19	≤ 0.20
	30% D-sucrose	0	6.86	1.44	≤ 0.20
		2	6.85	1.36	≤ 0.19
		4	6.46	1.23	≤ 0.19

SD ≤ 0.08 kPa

Storage moduli of sweetened Fibrex (10%) and gelatin (6%) gels were investigated during four weeks storage. Although there was a slight reduction (less than 0.12 kPa) in storage moduli of Fibrex gels after two weeks, loss factors have decreased (Table 5.2). Particularly, loss factor of Fibrex gels with 10% D-sucrose declined from almost 1.8 to 0.5 during the first two weeks of storage, which indicates recovery of the gel structure. Fibrex gels with 30% of D-sucrose followed the same trend as Fibrex gels with 10% sugar, by a decline in tanδ from 0.72 to 0.48 (Table 5.2 and Figure 5.3). Storage moduli of sweetened Fibrex gels experienced a slight raise by the end of week four, which confirmed gel stability during four weeks of storage.

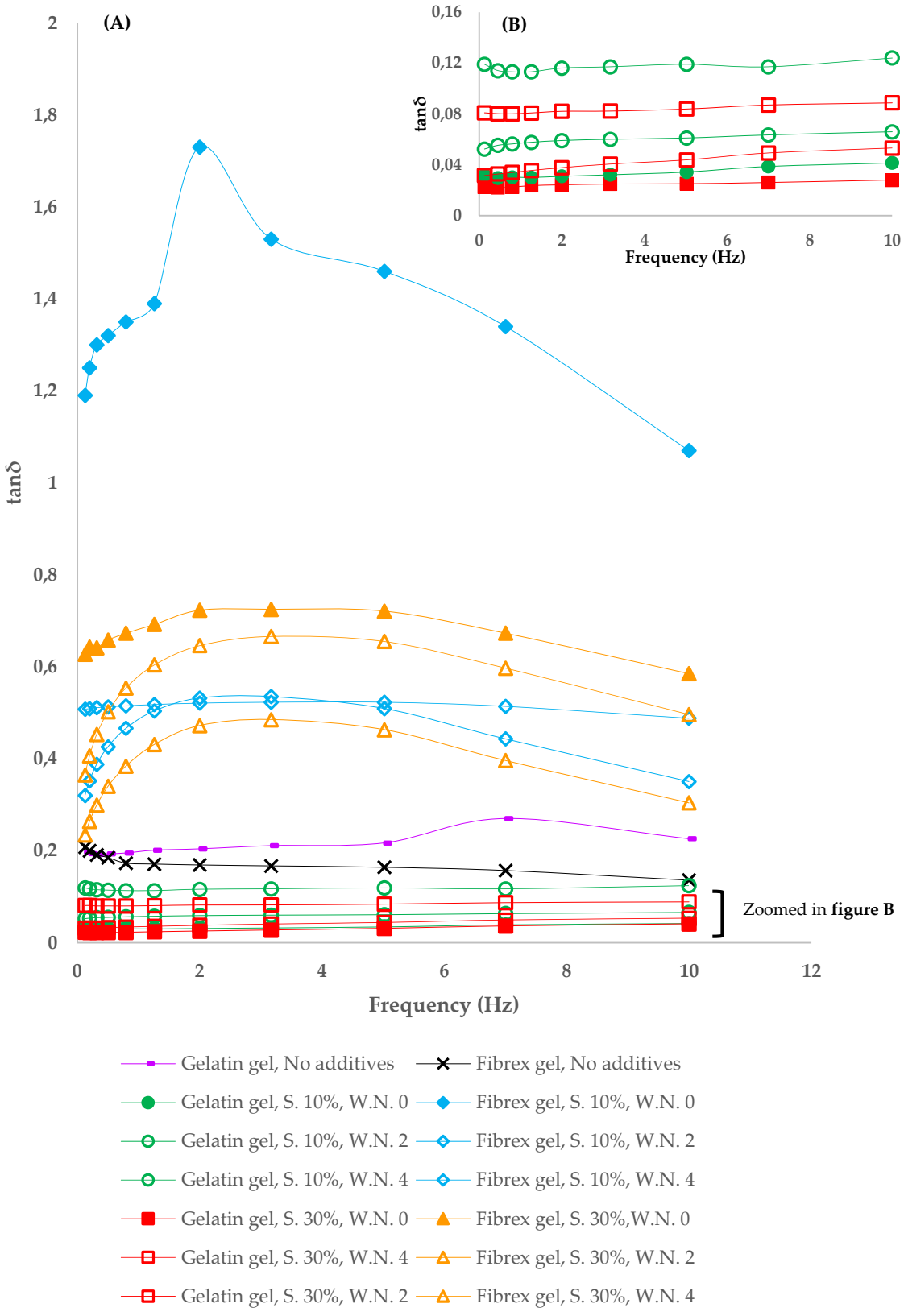


Figure 5.3. A: Loss factors of Fibrex (10%) formed with LccFtr, and gelatin gels (6%), containing different concentrations of D-sucrose during four weeks of storage (S.: D-sucrose, W.N.: week number), **B:** Magnifying loss factor of gelatin gels

As it can be inferred from Table 5.2 and Figure 5.3, viscoelastic properties of sweetened gelatin gels with 10% D-sucrose increased after 2 weeks of storage. Loss factors of all sweetened gelatin gels remained steady during four weeks storage, although a slight decline of storage moduli was observed in week four.

5.4.3. Identifying diferulic acids in cross-linked Fibrex gel

The oxidative gelation of Fibrex was supposed to result from the dimerization of FA residues on the polysaccharide chains. The FA dimerization mechanism starts with an abstraction of an H atom of the OH group at the ring position of FA leading to a phenoxy radical. Then, this radical is stabilized by resonance along the aromatic ring (C-4/ C-5) and the double bound (C-8) of the side chain. Subsequently, two phenoxy or alkoxy radicals cross-link, and the coupling of the unpaired electrons forms a new covalent bond that connects two sugar chains. Consequently, the structure of the dimers formed during gelation depends on the radical position **(Mendez-Encinas *et al.*, 2018; Khalighi *et al.*, 2020b)**.

Liquid chromatography mass spectrometry (LC-MS) showed a peak (retention time about 14.3 minutes) with m/z 387 (+), indicative of a diferulic acid **(Vismeh *et al.*, 2013)**. An MS/MS fragmentation experiment on this peak led to fragment ion masses m/z 351 (- $2H_2O$), 343 (-COO-), 325 (- H_2O-COO -), 307 (- $2H_2O$-COO -), 293 (- H_2O-COO-CH_3OH -), 265 (- H_2O-CO-COO-CH_3OH -), 219 (- H_2O-CO-COO-COO-CH_3OH -) and 201 (- $2H_2O$-CO-COO-COO-CH_3OH -), which fit to 8-5′ diferulic acid (Figure 5.4).

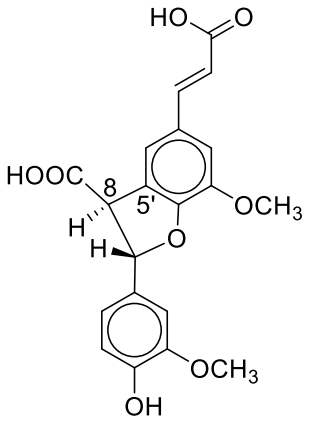
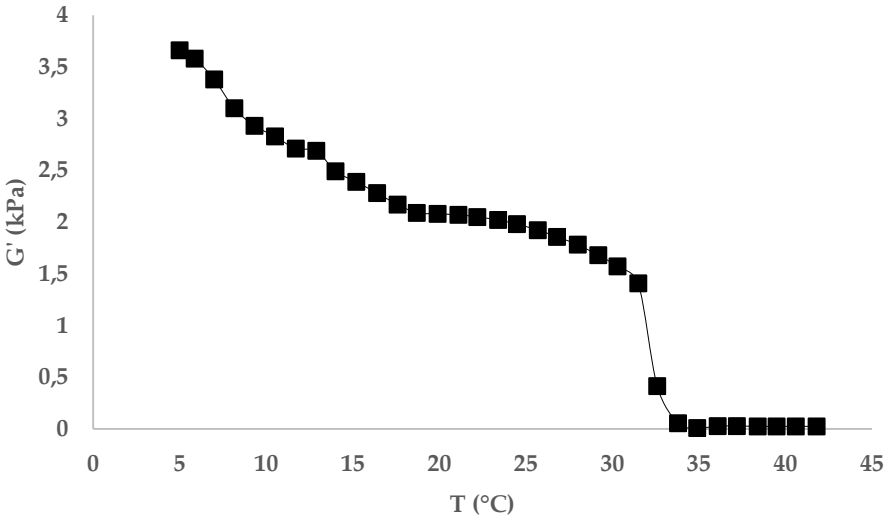


Figure 5.4. Molecular structure of 8-5′ diferulic acid

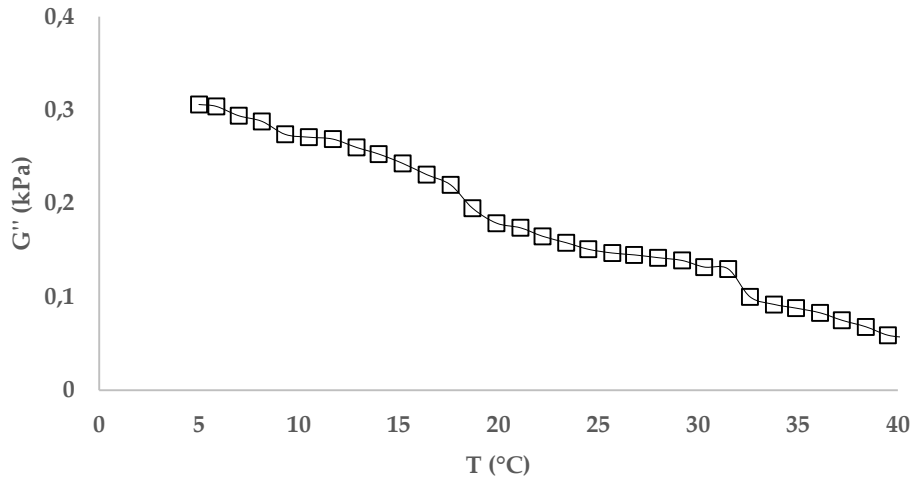
5.4.4. Melting point

Figures 5.5 and 5.6 present the temperature dependence of the dynamic modulus (G′ and G″) during isochronal temperature sweeps from 5 to 42 °C and 20 to 100 °C for gelatin and Fibrex gels, respectively. The starting temperature was chosen according to the gel formation temperature. Measurements were conducted by a constant frequency oscillatory test.

Storage modulus of gelatin gels gradually decreased by increasing temperature from 5 to 30 °C, followed with a sudden drop at around 32 °C (Figure 5.5). The melting point of gelatin is ≤ 35 °C, however it depends on its grade and concentration **(Francis, 1999)**. As shown in Figure 5.6, the storage modulus of Fibrex gels slightly raised with increasing temperatures (0.003 kPa/°C), and there was a correlation between rising temperature and storage modulus alteration with $R^2 = 0.89$.

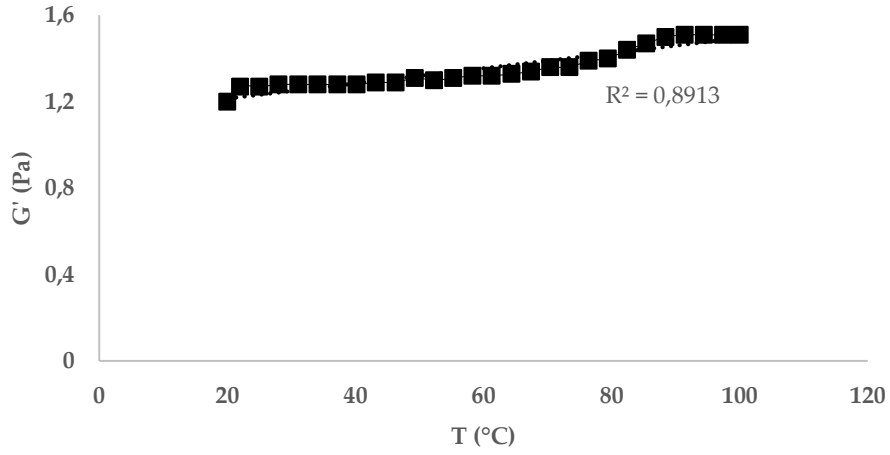


(A)

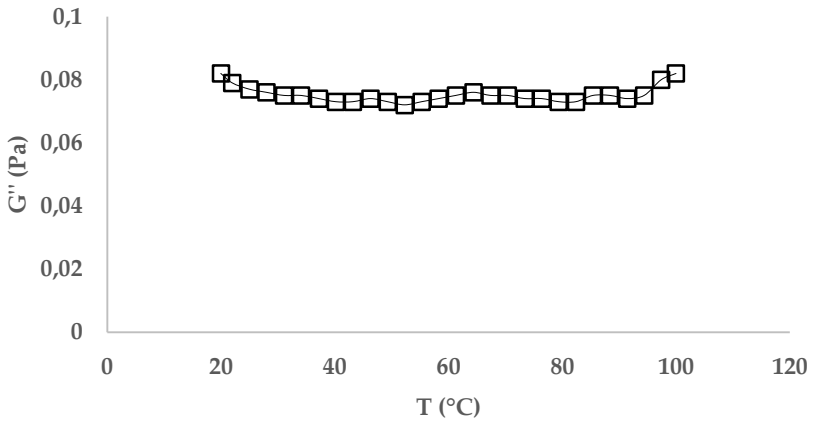


(B)

Figure 5.5. Melting point of gelatin gel (6%) (A. Storage modulus, B. Loss modulus)



(A)



(B)

Figure 5.6. Melting point of Fibrex gel (10%) (A. Storage modulus, B. Loss modulus)

5.4.5. Water-holding capacity

Water holding capacity is a physical property that prevents water from being released from the structure of the gel **(Zayas, 1997)**. The water holding capacities for Fibrex (10%) and gelatin (6%) gels were determined to be 98.5% and 98.6%, respectively.

5.4.6. Swelling ratio

Figure 5.7 presents the swelling ratio of gelatin (6%) and Fibrex (10%) gels. A linear correlation ($R^2 = 0.98$) between swelling ratio of the Fibrex gel and immersion time in artificial saliva was recorded. In contrast, the gelatin gel structure was broken after five minutes storage at room temperature.

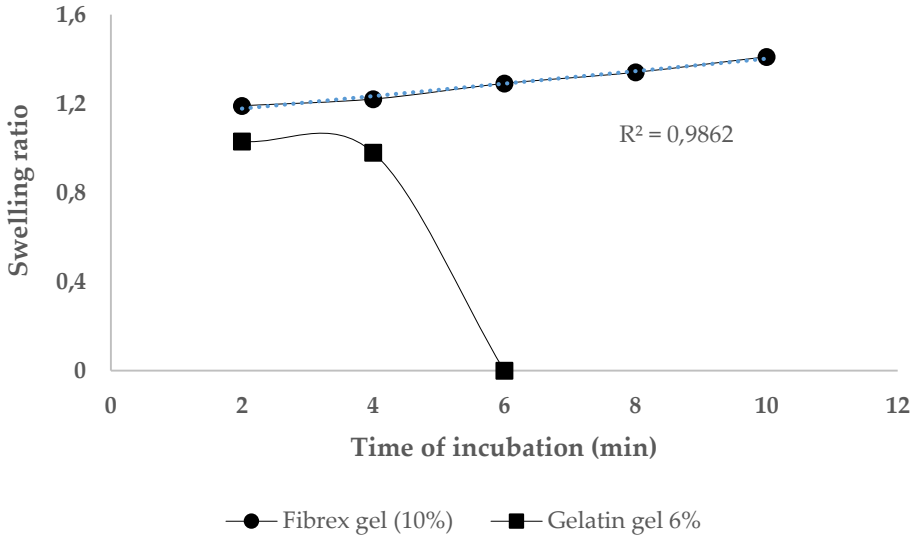


Figure 5.7. Swelling ratio of gelatin (6%) and Fibrex gel (10%) in artificial saliva at room temperature

5.5. Discussion

5.5.1. Fibrex and gelatin gel characterization

Rheological experiments on Fibrex and gelatin gels showed that G′ > G″. A larger storage modulus than loss modulus indicates viscoelastic behaviour of the gels **(Barnes, 2000)**. According to the results, although the storage modulus of Fibrex gels was lower than that of gelatin gels, a firm and stable cross-linked gel was formed owing to bound ferulic acid moieties **(Oosterveld *et al.*, 1997)**. Ferulic acid content of the self-extracted Fibrex in the current study was 1.98 ± 0.04 µg/mg, which was 0.19 µg/mg lower than the ferulic acid content in the self-extracted arabinoxylan in a previous study **(Khalighi *et al.*, 2020b)**. The higher storage modulus of cross-linked arabinoxylan gel than Fibrex gel can be attributed to the lower ferulic acid content of the extracted Fibrex **(Khalighi *et al.*, 2020b)**. In addition, laccases from *Pleurotus pulmonarius* (LccPpu) as well as *Funalia trogii* successfully cross-linked arabinoxylan gels, while laccase Ppu did not form a hard Fibrex gel **(Khalighi *et al.*, 2020b)**, an indication of a different enzyme specificity. Both Fibrex and standard gelatin gels had loss factor lower than one (tanδ≤ 0.36), which proved viscoelastic properties (Figure 5.2) **(Eidam *et al.*, 1995)**.

5.5.2. Effect of citrus pectin and vanillin on Fibrex gels

Although citrus pectin is known as a common thickening and gelling agent to link with various compounds to form a gel **(Saha & Bhattacharya, 2010)**, it did not significantly affect the rheological properties of Fibrex gels (Table 5.1). It appeared that the addition of pectin

resulted in hydrogen bonds and hydrophobic interactions in the gel, instead of increasing covalently cross-linked bonds **(Gawkowska *et al.*, 2018).**

Vanillin is a flavouring agent and effectively contributed to some laccase mediator system **(Weiss *et al.*, 2016).** However, it had a destructive impact on the mechanical spectra of the cross-linked Fibrex gel. The phenolic compound may have impeded gelation by quenching ferulic acid radicals **(Figueroa-Espinoza and Rouau, 1999).**

5.5.3. Viscoelastic properties of sweetened Fibrex and gelatin gels

A heat treatment and cooling procedure was applied to deactivate the laccase prior to viscoelastic analysis of sweetened gels. Although a significant decrease in laccase activity was observed, a residual activity of of 3.1 x 10^{-5} U/µg FA remained. Laccases from white rot fungus are typically stable within temperatures of 20-50 °C. The half-life of crude LccFtr was reported to be about five minutes at 70 °C **(Yesilada *et al.*, 2014).** However, the residual activity of the enzyme revealed that LccFtr resisted higher temperatures (85 °C) when enclosed in the gel structure.

Analysing viscoelastic properties of sweetened gels indicated that the tanδ of Fibrex gels with 10% D-sucrose in week zero was higher than in one, which indicates a destruction of the gel structure (Table 5.2). This was attributed to the competition between D-sucrose and hydrocolloids for the available water in the system. It was reported that higher concentrations of D-sucrose removed this phenomenon in some gel matrices **(Tabilo-Munizaga *et al.*, 2018)**. This would explain the unbroken gel structure in Fibrex gels containing 30% D-sucrose. Krnic *et al.* found that the common concentration of sugar in jelly confectionary was 30% of D-sucrose, which is usually combined with other sweeteners, such as glucose **(Krnic *et al.*, 2014)**. As it can be inferred from the results, 30% of D-sucrose will produce a firm gel.

Sweetened Fibrex gels with 10 and 30% D-sucrose followed the same trend after two weeks of storage (Table 5.2). Loss factors of the gels at frequencies between 0.1 to 10 Hz decreased in week two (Figure 5.3). This behaviour may result from the slow immobilization of free water and rearrangement of cross-linked junction zones **(Cheng and Yang, 2015)**. Table 5.2 shows an enhancement in storage modulus of the sweetened Fibrex gels after four weeks of storage. However, some softening of the structure was observed, owing to an increase in loss factors of the Fibrex gels (30% D-sucrose) in week four (Figure 5.3). Residual radicals produced by the laccase were probably responsible for this phenomenon. Participating in secondary reactions,

these may have led to a beginning degradation of the Fibrex main chains **(Morales-Burgos *et al.*, 2017)**.

The loss factors of sweetened gelatin gels were lower than that of non-sweetened samples with tanδ ≤ 0.1, especially at frequencies between 0.1 to 10 Hz (Figure 5.3). This represented a more elastic behaviour of the sweetened gelatin gels **(Fu and Rao, 2001)**. Added sugar led to smaller but more numerous junction zones, thus producing a more extended gel network and increasing the rigidity of the gel **(Oakenfull and Scott, 1986)**. Loss factors of all sweetened gelatin gels remained steady after four weeks of storage, despite a slight decline in storage moduli in week four.

There are two important steps during the gelatin gelation process: setting and ageing. Setting involves network formation throughout the gelatin solution. During ageing, gel strength develops at a constant temperature and only the strongest bonds survive **(Burey *et al.*, 2010)**. Reduction of the storage modulus in gelatin gels containing D-sucrose lower than 20% can result from the increased distance between molecular chains during ageing, which decrease the number of entangles points in the gelatin networks **(Kuan *et al.*, 2016)**. On the other hand, textural analysis of gelatin gels in presence of 0-40% D-sucrose and glucose syrup indicated an increase in gumminess and hardness, when compared to samples with no sugars. In the study, sugars were shown to have a stronger effect on textural properties than the gelatin concentration **(Porayanee *et al.*, 2015)**.

As it becomes evident from Figure 5.3, sweetened Fibrex gels had higher loss factors than fresh non-sweetened Fibrex gels, in contrast to sweetened gelatin gels, which showed lower loss factor than fresh non-sweetened gel samples. D-sucrose may shrink cross-linking enhancement by trapping available water in the system, which caused a minor rise in tanδ. Otherwise, added sugar declined tanδ in gelatin gels by growing junction zones, producing more extended gel networks, and increasing the rigidity of the gel **(Kamer *et al.*, 2019)**. Both Fibrex and gelatin gels stayed stable during four weeks of storage. They all showed loss factors < 1 at week 4, which confirmed their elastic properties. Moreover, it was reported that sweetened gelatin gels have stable viscoelastic and physical properties during 30 days cold storage (4 °C) **(Cheng and Yang, 2015)**.

5.5.4. Identifying diferulic acids in cross-linked Fibrex gel

The Fibrex gelation process and gel properties were governed by the establishment of covalent (di-FA, tri-FA bridges) linkages **(Norulfairuz *et al.*, 2012)**. Analysing diferulic acids

in the Fibrex gels cross-linked with LccFtr proved the presence of 8-5′ ferulic acid dehydrodimers. Dominating dimers of ferulic acids in cross-linked Fibrex gel were 8-O-4′ and 8-5′, which increased after gel formation **(Oosterveld *et al.*, 1997)**. Recently, formation of cross-links of ferulic acid of higher molecular masses than dimers and undefined superior structures was also reported **(Mendez-Encinas *et al.*, 2018).**

In a recent study, another fungal laccase, laccase Ppu, was applied to cross-link arabinoxylan gels. LCMS/MS analysis of the cross-linked arabinoxylan gel demonstrated that 8-O-4′ dimers of ferulic acids predominated **(Khalighi *et al.*, 2020b)**. In contrast, laccase Ftr, which has been applied to cross-link Fibrex gels, produced 8-5′ diferulic acids. This is most likely based on different substrate specificities: Laccase Ftr formed phenoxy radicals by absorption of the H atom of the OH group at the C-5 ring position of ferulic acid, while laccase Ppu preferred the C-4 ring position.

5.5.5. Fibrex and gelatin gel structural characteristics

Melting point, water holding capacity, and swelling ratio were the investigated structural characteristics in Fibrex and gelatin gels.

Gelatin gels are known to be melted at body temperature **(Karim and Bhat, 2008)**, and as is shown in Figure 5.5, loss modulus overtakes storage modulus at temperature higher than 35 °C. In contrast, cross-linked Fibrex gels demonstrated a heat resistant property (Figure 5.6). The higher temperature stability of the Fibrex gel resulted from the covalent bonds formed after oxidative coupling of the ferulic acids. The covalent structure gave some unique characteristics to the gels, such as high water absorption capacity and stability to pH, temperature, and ionic charges **(Mendez-Encinas *et al.*, 2018).**

High water holding capacity is a unique characteristic in oxidative cross-linked gels. The high WHC of Fibrex gels, which equalled that of the gelatin gel, resulted from the three-dimensional network that was able to retain a large aqueous phase and provided a structure stable against syneresis and shrinkage **(Mendez-Encinas *et al.*, 2018).** In a recent study, high WHC of arabinoxylan gels, which are also based on the cross-linking of diferulic acids, was also proven **(Khalighi *et al.*, 2020b)**.

Fibrex gels demonstrated a swelling ratio with a linear correlation ($R^2 = 0.98$) (Figure 5.7). In contrast, gelatin gels experience a collapse in gel structure owing to dissolving hydrogen bonds during immersion in saliva. This was attributed to the penetration of water molecules

into the gel network and dissolving hydrogen bonds. Cross-linked gels are known for their higher stability towards swelling **(Singh *et al.*, 2007)**. It was reported that swelling ratio in some cross-linked gels was higher than theoretically determined from the diferulic acid content. It was suggested that, in addition to di-FA and tri-FA, higher ferulated cross-linking and physical entanglements would contribute to the final gel structures **(Carvajal-Millan *et al.*, 2005)**.

5.6. Conclusion

The present study showed that sugar beet fibre obtained by a modified hydrothermal extraction method was successfully cross-linked with purified laccases from *Funalia trogii*. Although the storage modulus of the final gel was not as high as in gelatin gels, it had loss factors < 0.4, which proved the elastic properties of the Fibrex gels. An enhancement of the cross-linking effect, using vanillin as a food-grade mediator and flavour, or citrus pectin, was unsuccessful.

Adding D-sucrose as a sweetener not only decreased the storage modulus of Fibrex gels, but also doubled the loss factor. This was in contrast to gelatin gels, which showed lower loss factors than non-sweetened gelatin gels. Sweetened gels retained their rheological properties during a storage period of 28 days at 5 °C.

Fibrex, as a high amount side-stream of the refining sugar industry, formed a hard edible gel via covalent cross-links. Formation of 8-5′ dehydrodimers of ferulic acids governed the formation of the cross-linked Fibrex gels. The gels demonstrated viscoelastic gel characteristics, including heat resistance, high swelling ratio, and water holding capacity. Cross-linking of Fibrex effectively altered an industrial side-stream to a firm, heat resistant gel, formed at room temperature, which could be applied in pharmaceutical and other industries.

The enzymatic cross-linking concept appears promising to provide gels with special textural features. Sensory tests must be performed to investigate the organoleptic properties of the gels including the release of volatile and non-volatile ingredients typically contained in foods, such as candies, confectionary, liquorice products, glaze, and desserts.

5.7. Author Contributions:

S.K. and R.G.B. conceived and designed the experiments; S.K. performed the experiments; S.K. analysed the data; R.G.B. and F.E. contributed materials and analysis tools; S.K. and F.E. wrote the paper.

5.8. Funding:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

5.9. Acknowledgments:

The authors are grateful to U. Krings at the Institute of Food Chemistry, LU Hannover, for his skilful mass spectrometric analyses and help with the interpretation of the MS/MS data. We also thank T. Detering for helpful assistance. The publication of this article was funded by the Open Access fund of the Leibniz Universität Hannover.

5.10. Conflicts of Interest:

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

6. Outlook

Beside various cross-linking agents, fungal laccases are able to cross-link phenolic compounds, especially ferulic acids, to form cross-linked gels. In this thesis, a modified extraction method, as well as new enzymatic route, for the production of solid cross-linked gels was investigated. Although, the products were promising but further investigation for the production of these products in the industrial scale is needed.

In addition to this work which focused on the extraction health beneficial polysaccharides from industrial by-products and transformation of cheap substrates to a substitution for gelatin gels, high redox potential laccases from basidiomycetes, were found as promising candidate for cross-linking reaction. Besides, fungal laccases demonstrated different substrate specificities to produce dimers of ferulic acids, which can be further investigated.

A better understanding of the fungal biochemistry, screening of new isolated enzymes, optimizing reaction condition, supplementing with cheap and natural substrates for enzymatic cross-linking and modifying extraction methods to achieve the highest amount of intact ferulic acid moieties could open the way to high-yielding processes for the future work.

Meanwhile, for the future scientific interest, production of flavored cross-linked gels with using the same method and isolated enzyme is suggested. Therefore, the released properties of the cross-linked gels, as well as volatile and non-volatile compounds can be determined.

7. References

Adam, W., Lazarus, M., Saha-Möller, C.R., Weichhold, O., Hoch, U., Häring, D. And Schreier, P. *Biotransformattion with Peroxidases.* 1999. Springer, Berlin Heidelberg.

Aehle, W. *Enzymes in Industry*. 2007. Wiley-VCh, Weinheim.

Ağar, B., Gençcelep, H., Gruppen, H., Saricaoğlu, F. T., and Turhan, S. *Effect of sugar beet fibre concentrations on rheological properties of meat emulsions and their correlation with texture profile analysis.* Journal of Food and Bioproducts Processing, 2016. **100**: pp. 118 – 131.

Agostini, E., Hernandez-Ruiz, J., Arnao, M.B., Milrand, S.R., Tigier, H.A. and Acosta, M. *A peroxidase isoenzyme secreted by turnip* (*Brassica napus*) *hairy-root culture inactivation by hydrogen peroxide and application in diagnostic kits.* Applied Biochemistry and Biotechnology, 2002. pp. 1 – 7.

Aida, F.M., Shuhaimi, M., Yazid, M. and Maaruf, A.G. *Mushroom as apotential source of prebiotics: a review.* Trends in Food Science and Technology, 2009. pp. 567 – 575.

Amal, A., Hussain, S. and Jalaluddin M. *Preparation of artificial saliva formulation.* International Conference ICB Pharma II, 2015. **A002**: pp. 6 – 12.

Anderson, C. and Simsek, S. *What are the characteristics of arabinoxylan gels? Food and Nutrition Science,* 2018. **9**: pp. 818 – 833.

Anderson, C. and Simsek, S. *Mechanical profiles and topographical properties of films made from alkaline extracted arabinoxylans from wheat bran, maize bran, or dried distillers grain.* Food Hydrocolloids, 2019. **86**: pp. 78 – 86.

Andres, I. *Enzyme Biocatalysis Principles and Applications*. 2008. Springer, Berlin, Germany.

Augustine, A.J., Kragh, M.E., Sarangi, R., Fujii, S., Liboiron, B.D., Stoj, C.S., Kosman, D.J., Hodgson, K.O., Hedman, B., and Solomon, E.I. *Spectroscopic studies of perturbed T1 Cu sites in the multicopper oxidases Saccharomyces cerevisiae and Rhus vernicifera l*accase*: allosteric coupling between the T1 and trinuclear Cu sites.* Biochemistry, 2008. pp. 2036 – 2045.

Ayala-Soto, F.E., Serna-Saldivar, S.O. and Welti-Chanes, J. *Effect of processing time, temperature and alkali concentration on yield extraction, structure and gelling properties of corn fiber arabinoxylans.* Food Hydrocolloids, 2016. **60**: pp. 21 – 28.

Ayala-Soto, F.E., Serna-Saldivar, S.O. and Welti-Chanes, J. *Effect of arabinoxylans and laccase on batter rheology and quality of yeast-leavened gluten-free breads.* Journal of Cereal Science, 2017. **73**: pp. 10 – 17.

Baldrian, P. *Fungal laccases - occurrence and properties.* FEMS Microbiology Reviews, 2006. **30**: pp. 215 – 242.

Basha, M.A.F. *Optical properties and colorimetry of gelatine gels prepared in different saline solutions.* Journal of Advanced Research, 2019. **16**: pp. 55 – 65.

Barnes, H.A. *A handbook of elementary rheology.* 2000. The University of Wales: Institute of Non-Newtonian Fluid Mechanics, Department of Mathematics. ISBN 92-5-101137-0.

Bataillon, M., Mathaly, P., Nunes Cardinali, A.P. and Duchiron, F. *Extraction and purification of arabinoxylan from destarched wheat bran in a pilot scale.* Industrial Crops and Products, 1998. **8**: pp. 37 – 43.

Behrens, C., Linke, D., Allister, A.B., Zelena, K. and Berger, R.G. *Variants of PpuLCC, a multi-dye decolorizing laccase from Pleurotus pulmonarius expressed in Pichia pastoris.* Protein Expression and Purification, 2017. 137: pp. 34 – 42.

Bender, D., Nemeth, R., Wimmer, M., Götschhofer, S., Biolchi, M., Török, K., Tömösközi, S., D'Amico, S. and Schoenlechner, R. *Optimization of Arabinoxylan Isolation from Rye Bran by Adapting Extraction Solvent and Use of Enzymes.* Journal of Food Science, 2017a. **82**(11): pp. 2562 – 2568.

Bender, D., Schmatz, M., Novalin, S., Nemeth, R., Chrysanthopoulou, F., Tömösközi, S., Török, K., Schoenlechner, R. and D'Amico, S. *Chemical and rheological characterization of arabinoxylan isolates from rye bran.* Chemical and Biological Technologies in Agriculture, 2017b. **4**: pp. 1 – 14.

Bender, D., Regner, M., D'Amico, S., Jäger, H., Tömösközi, S. and Schoenlechner, R. *Effect of differently extracted arabinoxylan on gluten-free Sourdough-Bread Properties.* Journal of Food Quality, 2018. DOI 10.1155/2018/5719681: pp. 1 – 10.

Bergmans, M.E.F., Beldman, G., Gruppen, H. and Voragen, A.G.J. *Optimisation of the selective extraction of (glucurono) arabinoxylans from wheat bran: Use of barium and calcium hydroxide solution at elevated temperatures.* Journal of Cereal Science, 1996. **23** (3): pp. 235 – 245.

Berlanga-Reyes, M., Carvajal-Millan, C., Lizardi-Mendoza, E., Islas-Rubio, J.R., and Rascón-Chu, A. *Enzymatic cross-linking of alkali extracted arabinoxylans: Gel rheological and structural characteristics.* International Journal of Molecular Science, 2011. **12**: pp. 5853 – 5861.

Bhunia, A., Durani, S. and Wangikar, P.P. *Horseradish peroxidase catalysed degradation of industrially important dyes.* Biotechnology and Bioengineering, 2002. pp. 7 – 18.

Bligny, R., Douce, R. *Excretion of laccase by sycamore* (*Acer pseudoplatanus* L) *cells. Purification and properties of the enzyme.* Journal of Biochemistry, 1983. pp. 489 – 496.

Börjesson, M., Härdelin, L., Nylander, F., Karlsson, K., Larsson, A. and Westman, G. *Arabinoxylan and nanocellulose from a kilogram-scale extraction of barley husk. BioResearch, 2018.* **13** (3): 6201 – 6220.

Boz, H. *Ferulic acid in cerials – A review.* Czech Journal of Food Science, 2015. **33**: 1 – 7.

Braun, D.D. and Rosan, M.R. *Rheology Modifiers Handbook.* 1999. William Andrew, New York, US. Hardcover ISBN: 9780815514411.

Brijwani, K., Rigdon, A. and Vadlani, P.V. *Fungal Laccases: Production, Function, and Applications in Food Processing.* Enzyme Research, 2010. doi: 10.4061/2010/149748.

Burey, P., Bhandari, B.R., Howes, T. and Gidley, M.J. *Hydrocolloid gel particles: formation, characterisation, and application.* Journal of critical reviews in food science and nutrition, 2008. **48** (5): pp. 361 – 377.

Burey, P., Bhandari, R., Rutgers, R.P.G., Halley, P.J. and Torley, P.J. *Confectionery Gels: A Review on Formulation, Rheological and Structural Aspects.* International Journal of Food Properties, 2010. **12**: pp. 176 – 210.

Camarero, S., Cañas, A.I., Nousiainen, P., Record, E., Lomascolo, A., Martínez, M.J. and Martínez, Á.T. *p-Hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds.* Environmental Science and Technology, 2008. pp. 6703 – 6709.

Cańtizares-Macias, P., Hernandez-Garciadiego, L. and Gomez-Ruiz, H. *An automated flow injection analysis procedure for the determination of reducing sugars by DNSA methods.* Journal of Food Science, 2006, **66** (3): pp. 407 – 411.

Carlile, M.J., Watkinson, S.C. *Fungal diversity In: The Fungi. 1994.* Academic Press, UK.

Carunchio, F., Crescenzi, C., Girelli, A. M., Messina, A. and Tarola, A. M. *Oxidation of ferulic acid by laccase-induced: identification of the products and inhibitory effects of some dipeptides.* Talanta, 2001. **55**: pp. 189 – 200.

Carvajal-Millan, E., Landillon, V., Morel, M.H., Rouau, X., Doublier, J.L. and Micard, V. *Arabinoxylan gels: Impact of the feruloylation degree on their structure and properties. Bio macromolecules, 2005a.* **6** (1): pp. 309 – 317.

Carvajal-Millan, E., Guigliarelli, B., Belle, V., Rouau, X. and Micard, V. *Storage stability of laccase induced arabinoxylan gels.* Carbohydrate Polymers, 2005b. **59** (2): pp. 181 – 188.

Černá, M., Barros, A.S., Nunes, A., Rocha, S.M., Delgadillo, I., Čopíková, J. and Coimbra, M.A. *Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives.* Carbohydrate polymers, 2003. 51 (4): pp. 383 – 389.

Chen, M.J. and Lin C.W. *Factors Affecting the Water-Holding Capacity of Fibrinogen/Plasma Protein Gels Optimized by Response Surface Methodology.* Journal of Food Science, 2002. **67** (7): pp. 2579 – 2582.

Cheng, L. and Yang, H. *Effect of salt and sugar addition on the physicochemical properties and nanostructure of fish gelatin.* Food Hydrocolloids. 2015. **45**: pp, 72 – 82.

Cho, N.S., Wilkolazka, A.J., Staszczak, M., Cho, H.Y. and Ohga, S. *The role of laccase from white rot fungi to stress conditions.* Journal of the Faculty of Agriculture Kyushu University, 2009. pp. 81 – 83.

Claus, H. *Laccases: Structure, reactions, distribution.* Micron Technology, 2004. pp. 93 – 96.

Conesa, A., Punt, P.J. and Van den Hondel, C.A.M.J.J. *Fungal peroxidases: molecular aspects and applications.* Journal of Biotechnology, 2002. pp. 143 – 158.

Copeland, R.A. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2000. Wiley-VCH, New York, USA.

Danilatos, G. D. *Foundations of environmental scanning electron microscopy. Advances in Electronics and Electron Physics.* ESEM Research Laboratory North Bondi (Sydney), 1988. Australia. *Doi: 10.1016/S0065-2539(08)60902 6. ISBN 9780120146710.*

Di Stefano, V., Avellone, G., Bongiorno, D., Cunsolo, V., Muccilli, V., Sforza, S., Dossena, A., Drahos, L. and Vékey, K. *Applications of liquid chromatography-mass spectrometry for food analysis.* Journal of Chromatography A, 2012. **12**: pp. 74 – 85.

Diamantidis, G., Effosse, A., Potier, P. and Bally, R. *Purification and characterization of the first bacterial laccase in the rhizospheric bacterium Azospirillum lipoferum.* Soil Biology and Biochemistry, 2000. pp. 919 – 927.

Diniz, F.M. and Martin, A.M. *Effect of the extent of enzymatic hydrolysis on the functional properties of shark protein hydrolysate.* Lebensmittel-Wissenschaft und-Technology, 1997. ***30****:* pp. 66 – 272.

Dubruel, P. and Vlierberghe, S.V. *Biomaterials for Bone Regeneration,* 2014. Woodhead publishing, Cambridge, England. Hardcover ISBN: 9780857098047.

Eidam, D., Kulicke, W.M., Kuhn, K. and Stute, R. *Formation of maize starch gels selectively regulated by the addition of hydrocolloids biosynthesis.* Starch – Stärke**,** 1995. **47** (10): pp. 378 – 384.

Elegir, G., Daina, S., Zoia, L., Bestetti, G. and Orlandi, M. *Laccase mediator system: oxidation of recalcitrant lignin model structures present in residual kraft lignin.* Enzyme and Microbial Technology, 2005. pp. 340 – 346.

Enguita, F.J., Martins, L.O., Henriques, A.O. and Carrondo, M.A. *Crystal structure of bacterial endospore coat component. A laccase with enhanced theromstability properties.* Journal of Biological Chemistry, 2013. pp. 19416 – 19425.

Figueroa-Espinoza, M. C. and Rouau, X. *Oxidative Cross-Linking of Pentosans by a Fungal Laccase and Horseradish Peroxidase: Mechanism of Linkage between Feruloylated Arabinoxylans.* Cereal Chemistry, 1998. **75** (2): pp. 259 – 265.

Figueroa-Espinoza, M.C. and Rouau, X. *Effect of cysteinyl caffeic acid, caffeic acid, and L-dopa on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase.* Journal of Agricultural and Food Chemistry, 1999. **47** (2): pp. 497 – 503.

Fincher, G.B. and Stone, B.A. *A water-soluble arabinogalactan peptide from wheat endosperm by fractional precipitation of their acetates.* Austrian Journal for Science. 1974. **27** (2): pp. 117 – 132.

Flander, L., Rouau, X., Morel, M.H., Autio, K., Seppanen-Laakso, T., Kruus, K. and Buchert, J. *Effects of laccase and xylanase on the chemical and rheological properties of oat and wheat doughs.* Journal of Agricultural and Food Chemistry, 2008. **56** (14): pp. 5732 – 5742.

Francis, *F.J. Wiley Encyclopaedia of Food Science and Technology, 1999. 2nd ed.; John Wiley & Sons Inc. London, United Kingdom. ISBN 978-0-471-19285-5.*

Fu, J. and Rao, M. *Rheology and structure development during gelation of low-methoxyl pectin gels: the effect of sucrose.* Food Hydrocolloids, 2001. **15**: pp. 93 – 100.

Gabbott, P. The *Principles and Applications of Thermal Analysis.* 2007. Wiley-Blackwell: London, England.

Gan, Y. X. *Continuum Mechanics – Progress in Fundamentals and Engineering Applications.* 2012. In Tech**,** *Rijeka, Croatia.*

Gawkowska, D., Cybulska, J. and Zdunek, A. *Structure-related gelling of pectins and linking with other natural compounds: A review.* Polymers, 2018. **10** (7) DOI 10.3390/polym10070762.

Gianfreda, L., Xu, F. and Bollag, J.M. *Laccases: a useful group of oxidoreductive enzymes.* Bioremediation Journal, 1999. pp. 1 – 25.

Givaudan, A., Effosse, A., Faure, D., Potier, P., Bouillant, M. L. and Bally, R. *Polyphenol oxidase in Azospirillum lipoferum isolated from rice rhizosphere: Evidence for laccase activity in nonmotile strains of Azospirillum lipoferum*. FEMS. Microbiology Letter, 1993. pp. 205 – 210.

Gooch J.W. *Encyclopedic Dictionary of Polymers.* 2007. Springer, New York, US. Print ISBN: 978-0-387-31021-3.

Groenwoud, W.M. *Characterization of Polymers by Thermal Analysis.* 2001. Elsevier Science, Hertogenbosch, Netherland. ISBN: 978-0-444-50604-7.

Guillem, C., Nerea, N. and Oscar, N. *The Role of Liquid Chromatography-Mass Spectrometry in Food Integrity and Authenticity, Mass Spectrometry - Future Perceptions and Applications,* 2019. Ganesh Shamrao Kamble, IntechOpen, DOI: 10.5772/intechopen.85087.

Guillon, F., Barry, J., and Thibault, J. *Effect of autoclaving sugar- beet fibre on its physico-chemical properties and its in-vitro degradation by human faecal bacteria.* Journal of the Science of Food and Agriculture, 1992. **60** (1): pp. 69 – 79.

Hanson, M., and Wypych, A. *Data book of Curatives and cross linkers.* 2019. ChemTec Publishing, Toronto, Canada. ISBN 978-1-927885-49-9.

Hemdane, S., Jacobs, P. J., Dornez, E., Verspreet, J. and Delcour, J.A. *Wheat (Triticum aestivum L.) Bran in Bread Making: A Critical Review.* Compre-hensive Reviews in Food Science and Food Safety, 2016. **15**: pp. 28 – 42.

Heussen, P.C.M., Menard, K. and Courtney, P. *Practical Food Applications of Differential Scanning Calorimetry.* Unilever Research and Development, 2011. Report number: 009742_01 APP. Affiliation: PerkinElmer.

Hilgers, R., Vincken, J. P., Gruppen, H., and Kabel, M. A. *Laccase/Mediator Systems: Their Reactivity Toward Phenolic Lignin Structures.* ACS sustainable chemistry & engineering, 2018. **6** (2): pp. 2037 – 2046. doi:10.1021/acssuschemeng.7b03451.

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C. and Lundell, T. *New and classic families of secreted fungal heme peroxidases.* Applied Microbiology and Biotechnology, 2010. pp. 871 – 897.

Holck, J., Lorentzen, A., Vigsnaes, L. K., Licht, T. R., Mikkelsen, J. D., and Meyer, A. S. *Feruloylated and Nonferuloylated Arabino-oligosaccharides from Sugar Beet Pectin*

Selectively Stimulate the Growth of Bifidobacterium spp. in Human Fecal in Vitro Fermentations. Journal of Agricultural and Food Chemistry, 2011. **59**: pp. 6511 – 6519.

Hollmann, J. and Lindhauer, M.G. *Pilot-scale isolation of glucuronoarabinoxylans from wheat bran.* Carbohydrate Polymers, 2005. **59** (2): pp. 225 – 230.

Ismail, A.A., Van de Voort, F.R. and Sedman, J. Techniques and Instrumentations in Analytical Chemistry. 1997. Elsevier. ISBN: 978-0-444-81868-3.

Izydorczyk M.S. and Biliaderis, C.G. *Arabinoxylans: technologically and nutritionally functional plant polysaccharides, in functional food carbohydrates,* 2007. CRC Press, Boca Raton, FL, USA.

Jacquemin, L., Mogni, A., Zeitoun, R., Guinot, C., Sablayrolles, C., Saulnier, L. and Pontalier, P.Y. *Comparison of different twin- screw extraction conditions for production of arabinoxylans.* Carbohydrate Polymers, 2015. **116**: pp. 86 – 94.

Jasim, A. *Medicinal properties of laccase from Basidiomycetes mushroom: a review.* Advance in Life Science and Technology, 2019. **54**: ISSN 2224-7181.

Joanna, I., Sabu, T. *Printing on Polymers.* 2016. William Andrew, New York, US. Hardcover ISBN: 9780323374682.

Kale, M.S., Yadav, M.P., Hicks, K.B. and Hanah, K. *Concentration and shear rate dependence of solution viscosity for arabinoxylans from different sources.* Food Hydrocolloids, 2015. **47**: pp. 178 – 183.

Kamer, D.D., Palabiyik, I., Isik, N.O., Akyuz, F., Demiric, A.S. and Gumus, T. *Effect of confectionery solutes on the rheological properties of fish (Oncorhynchus mykiss) gelatin. LWT-Food Science and Technology,* 2019. **101**: pp. 499 – 505.

Karim, A.A. and Bhat, R. *Gelatin alternatives for the food industry: recent developments, challenges and prospects.* Trend in Food Science and Technology, 2008. **19** (12): pp. 644 – 656.

Khalighi, S., Berger, R.G. and Ersoy, F. *Cross-linking of Fibrex gel by fungal laccase: gel rheological and structural characteristics.* Process, 2020a. **8** (1): 16; doi: 10.3390/pr8010016.

Khalighi, S., Berger, R.G. and Ersoy, F. *Cross-linking of wheat bran arabinoxylan by fungal laccases yields firm gels.* Process, 2020b. **8** (1): 36; doi: 10.3390/pr8010036.

Kolwek, J., Behrens, C., Linke, D., Krings, U. and Berger, R.G. *Cell-free one-pot conversion of (+)-valencene to (+)-nootkatone by a unique dye-decolorizing peroxidase combined with a laccase from Funalia trogii.* Journal of Industrial Microbiology and Biotechnology, 2018. **45** (2): pp. 89 – 101.

Krnic, P., Truman, P., Bates, D. and Vilkhu, K. *Method for the production of jelly confectionary*. FPO. 2014. United States Patent 8895093.

Kuan, Y., Mohammadi, A., Huda, N., Arif, F. and Karim, A. *Effect of sugars on the gelation kinetics and texture of duck feet gelatin.* Food Hydrocolloids, 2016. **58**: pp. 267 – 275.

Kupski, L., Salcedo, G. M., Caldas, S. S., de Souza, T. D., Furlong, E. B. and Primel, E. G. *Optimization of a laccase-mediator system with natural redox-mediating compounds for pesticide removal.* Environmental Science and Pollution Research, 2019. **26**: pp. 5131 – 5139.

Kuuva, T., Lantto, R., Reinikainen, T., Buchert, J. and Autio, K. *Rheological properties of laccase-induced sugar beet pectin gels,* Food Hydrocolloids, 2003. **17**: pp. 679 – 684.

Lahtinen, M., Kruus, K., Heinonen, P. and Sipila, J. *On the Reactions of Two Fungal Laccases Differing in Their Redox Potential with Lignin Model Compounds: Products and Their Rate of Formation.* Journal of Agricultural and Food Chemistry, 2009. pp. 8357 – 8365.

Lehninger, A. *Biochemistry. The molecular basis of cell structure and function. 1970.* Worth Publ Inc, New York, USA.

Levigne, S., Ralet, M.C., Quemener, B. and Thibault, J.F. *Isolation of diferulic bridges ester linked to arabinan in sugar beet cell walls.* Journal of Carbohydrate Research, 2004. **339** (13): pp. 2315 – 2319.

Lew Paul, C., Bin, Y. and Yun, J. *Lignin Biodegradation with Laccase-Mediator Systems.* Frontiers in Energy Research, 2014. **2**: pp. 12, https://doi.org/10.3389/fenrg.2014.00012.

Liu, L., Fishman, M., Kost, J. and Hicks, K. *Pectin based systems for colon specific drug delivery via oral route.* Biomaterials, 2003. **24**: pp. 3333 – 3343.

Macosko, C.W. *Rheology, principles, measurements, and applications.* 1994. Wiley, New York.

Maes, C. and Delcour, J.A. *Structural characterisation of water-extractable and water-unextractable arabinoxylans in wheat bran.* Journal of Cereal Science, 2002. **35**: pp. 315 – 326.

Maitra, J., and Shukla, V. *Cross-linking in Hydrogels – A review.* American Journal of Polymer Science, 2014. **4** (2): pp. 25 – 31.

Majidi, B., Taghavi, S.M., Fafard, M., Zeigler, D.P. and Alamdari, H. *Discrete Element Method Modeling of the Rheological Properties of Coke/Pitch Mixtures.* Materials, 2016. **9** (5): 334; https://doi.org/10.3390/ma9050334.

Malunga, L.N. and Beta, T. *Isolation and identification of feruloylated arabinoxylan mono- and oligosaccharides from undigested and digested maize and wheat.* Heliyon, 2016. **2** (5): e00106.

Mao, R., Tang, J. and Swanson, B.G. *Water Holding Capacity and Microstructure of Gallan Gels.* Carbohydrate Polymers, 2001. **46** (4): pp. 365 – 371.

Martínez-López, A.L., Carvajal-Millan, E., Marquez-Escalante, J., Campa-Mada, A.C., Rascón-Chu, A., López-Franco, Y.L. and Lizardi-Mendoza, J. *Enzymatic cross-linking of ferulated arabinoxylan: effect of laccase or peroxidase catalysis on the gel characteristics.* Food Science Biotechnology, 2019. **28** (2): pp. 311 – 318.

Mendez-Encinas, M.A., Carvajal-Milan, E., Rascon-Chu, A., Astiazaran-Garcia, H.F. and Valencia-Rivera, E. *Ferulated arabinoxylan and their gels: functional properties and potential application as antioxidant and anticancer agent.* Hindawi Oxidative Medicine and Cellular Longevity, 2018. Article ID 2314759.

Mendis, M. and Simsek, S. *Arabinoxylan and human health.* Food Hydrocolloids, 2014. **42** (P2): pp. 239 – 243.

Micard, V., Thibault, F. *Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins,* Carbohydrate Polymers. 1999. **39** (3): pp. 265 – 273.

Micard, V., Rosa-Sibakov, N. and Poutanen, K. *How does wheat grain, bran and aleurone structure impact their nutritional and technological properties?* Journal of Trends in Food Science and Technology, 2015. **41**: pp. 118 – 134.

Michel, F., Thibault, J. and Barryb, J. *Preparation and characterisation of dietary fibre from sugar beet pulp*. Journal of the Science of Food and Agriculture, 1987. **42:** pp. 77 – 85.

Miller, G.L. *Use of dinitrosalicylic acid reagent for determination of reducing sugar*. Analytical Chemistry, 1959. **31** (3): pp. 426 – 428.

Moorehouse, R. *Ubiquitous hydrocolloid.* The World of Food Ingredients, 2004_September, pp. 24 – 30.

Moore-Landecker, E. *Kingdom fungi: Division Basidiomycota In*: *Fundamentals of the Fungi, 1996.* 4th ed. Chapter 6, Simon & Schuster Press, USA.

Morales-Burgos, A.M., Carvajal-Millan, E., Lopez-Franco, Y., Rascon-Chu, A., Lizardi-Mendoza, J., Sotelo-Cruz, N., Brown-Bojorquez, F., Burgara-Estrella, A. and Pedroza-Montero, M. *Syneresis in gels of highly ferulated arabinoxylans: characterization of covalent cross-linking, rheology, and microstructure.* Polymers, 2017. **9** (5): pp. 164/1 – 164/11.

Morrison, N.A., Clark, R.C., Chen, Y.L., Talashek, T. and Sworn. G. *Physical chemistry and industrial application of gellan gum.* 1999. Springer-Verlag, Heidelberg, Germany.

Naemi, N., Behforootan, S., Chatzistergos, P. and Chockalingam, N. *Viscoelasticity in Foot-Ground Interaction.* Viscoelastic and Viscoplastic Materials, 2016. DOI: 10.5772/64170.

Nazir, A., Asghar, A., Aslam Maan., A. *Advances in Food Rheology and Its Applications*. 2017. Woodhead Publishing Series in Food Science, Technology and Nutrition, Cambridge, England.

Nieter, A., Kelle, S., Linke, D. and Berger, R.G. *Feruloyl esterases from Schizophyllum commune to treat food industry side-streams.* Bioresource Technology, 2016. **220**: pp. 38 – 46.

Nino-Medina, G., Carvajal-Millan, E., Rascon-Chu, A., Marquez-Escalante, J.A., Guerrero, V. and Salas-Munoz, E. *Feruloylated arabinoxylans and arabinoxylan gels: structure, sources and applications.* Phytochemistry Reviews, 2010. **9**: pp. 111 – 120.

Norulfairuz, D., Zaidel, A., Chronakis, I. and Meyer, A. *Food hydrocolloids enzyme catalysed oxidative gelation of sugar beet pectin: kinetics and rheology.* Food Hydrocolloids, 2012. **28** (1): pp. 130 – 140.

Oakenfull, D. and Scott, A. *Stabilization of gelatin gels by sugars and polyols.* Food Hydrocolloids, 1986. **1**: 163 – 175.

Oosterveld, A., Grabber, J., Beldman, G., Ralph, J, and Voragen, A. *Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin.* Carbohydrate research, 1997. **300**: pp. 179 – 181.

Oosterveld, A., Beldman, G., and Voragen, A. *Oxidative cross-linking of pectin polysaccharides from sugar beet pulp.* Carbohydrate Research, 2000. **328** (2): pp. 199 – 207.

Osma, J.F., Toca-Herrera-J.L. and Rodríguez-Couto S. *Uses of Laccases in the Food Industry.* Enzyme Research, 2010. **10**, doi:10.4061/2010/918761.

Pacheco, M., Mar Villamiel, R.M. and Javier Moreno, F. *Structural and Rheological Properties of Pectins Extracted from Industrial Sugar Beet By-Products.* Molecules, 2019, **24**(3): 392 – 408.

Park, H., Guo, X., Temenoff, J. S., Tabata, Y., Caplan, A.I., Kasper, F.K. and Mikos, A.G. *Effect of Swelling Ratio of Injectable Hydrogel Composites on Chondrogenic Differentiation of Encapsulated Rabbit Marrow Mesenchymal Stem Cells In Vitro.* Biomacromolecules, 2009. **10** (3): pp. 541 – 546.

Polaina, J., MacCabe, A.P. *Industrial Enzymes: Structure, Function and Applications*. 2007. Springer, Dordrecht.

Pomeranz Y., Meloan C.E. *Thermal Analysis of Foods. In: Food Analysis.* 1994. Springer, Boston, MA.

Porayanee, M., Katemake, P. and Duangmal, K. *Effect of gelatin concentrations and glucose syrup to sucrose ratios on textural and optical properties of gelatin gel.* International Journal of Food Science and Technology, 2015. **1**: pp. 26 – 30.

Prystupa, D.A. and Donald, A.M. *Infrared study of gelatin conformations in the gel and sol states.* Polymer Gels and Networks, 1996. 4 (2): pp. 87 – 110.

Qiu, F. and Scrivens, G. *Fundamentals and Pharmaceutical Industry Practices.* 2018. Academic press, Massachusetts, US. Hardcover ISBN: 9780128027868.

Rahman M.S., Islam, M., Islam, S., Zaman, A., Ahmed, T., Biswas, S., Sharmee,S. and Rashi, T.U. *Morphological Characterization of Hydrogels. In: Mondal M. (eds) Cellulose-Based Superabsorbent Hydrogels. Polymers and Polymeric Composites: A Reference Series.* 2019. Springer, Cham, Print ISBN: 978-3-319-77829-7.

Riva, S. *Laccases: Blue enzymes for green chemistry.* Trends in Biotechnology, 2006. pp. 219 – 226.

Robertson, J. A., Faulds, C. B. Smith, A. C. and Waldron, K. W. *Peroxidase-Mediated Oxidative Cross-Linking and Its Potential to Modify Mechanical Properties in Water-Soluble Polysaccharide Extracts and Cereal Grain Residues.* Journal of Agricultural and Food Chemistry, 2008. **56**: 1720 – 1726.

Rodgers, C.J., Blanford, C.F., Giddens, S.R., Skamnioti, P., Armstrong, F.A. and Gurr, S.J. *Designer laccases: a vogue for high-potential fungal enzymes?* Trends in Biotechnology, 2010. pp. 63 – 72.

Ryu, J.N., Jung, J.H., Lee, S.Y. and Ko, S.H. *Comparison of Physicochemical Properties of Agar and Gelatin Gel with Uniform Hardness.* Food Engineering Progress, 2012. **16**: pp. 14 – 19.

Saha, D. and Bhattacharya, S. *Hydrocolloids as thickening and gelling agents in food: a critical review.* Journal of Food science and technology, 2010. **47** (6): pp. 587 – 597.

Sahare, P., Ayala, M., Vazquez-Duhalt, R. and Agrawal, V. *Immobilization of peroxidase enzyme onto the porous silicon structure for enhancing its activity and stability.* Nanoscale Research Leter, 2014. pp. 409 – 414.

Sakurai, A., Masuda, M. and Sakakibara, M. *Effect of surfactants on phenol removal by the method of polymerization and precipitation catalysed by Coprimus cinereus peroxidase.* Journal of Chemical Technology and Biotechnology, 2003. pp. 952 – 958.

Sanderson, G.R. *Gellan gum. Food gels.* Elsevier Science, 1990. New York, US.

Sato, N., Takano, Y., Mizuno, M., Nozaki, K., Umemura, S., Matsuzawa, T., Amano, Y., and Makishima, S. *Production of feruloylated arabino-oligosaccharides (FA-AOs) from beet fiber by hydrothermal treatment.* The Journal of Supercritical Fluids. 2013. **79**: pp. 84 – 91.

Saulnier, L., and Thibault J. F. *Ferulic acid and diferulic acid as components of sugar beet pectins and maize bran heteroxylan.* Journal of the Science of Food and Agriculture, 1999. **79**: pp. 396 – 402.

Scalbert, A., Monties, B., Lallemand, J.Y., Guittet, E. and Rolando, C. *Ether linkage between phenolic acids and lignin fractions of wheat straw.* Phytochemistry**,** 1985. **24** (6): pp. 1359 – 1362.

Scheibner, M., Hülsdau, B., Zelena, K., Nimty, M., de Boer, L., Berger, R.G. and Zorn, H. *Novel peroxidase of Marasmius scorodnius degrade β-carotene.* Applied Microbiology and Biotechnology, 2008. pp. 1241 – 1250.

Schulz, K., Nieter, A., Scheu, A.K., Copa-Patiño, J. L., Popper, L. and Berger, R.G. *A type D ferulic acid esterase from Streptomyces werraensis affects the volume of wheat dough pastries.* Applied Microbiology and Biotechnology, 2018. **102** (3): 1269 – 1279.

Sharma, P., Goel, R. and Capalash, N. *Bacterial laccases.* World Journal of Microbilogy and Biotechnology, 2007. pp. 823 – 832.

Sharma, K.K., Shrivastava, B., Sastry, V.R., Sehgal, N. and Kuhad, R.C. *Middle-redox potential laccase from Ganoderma sp.: its application in improvement of feed for monogastric animals.* SCIENTIFIC REPORTS, 2013. pp. 1 – 9.

Shleev, S., Tkac, J., Christenson, A., Ruzgas, T., Yaropolov, A.I., Whittaker, J.W. and Corton, L. *Direct electron transfer between copper-containing proteins and electrodes.* Biosensors and Bioelectronics, 2005. pp. 2517 – 2554.

Singh, J., Kaur, L. and McCarthy, O.J. *Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications – a review.* Food Hydrocolloids, 2007. **21**, pp. 1 – 22.

Skendi, A. and Biliaderis, C.G. *Gelation of Wheat Arabinoxylans in the Presence of Cu (+2) and in Aqueous Mixtures with Cereal Beta-Glucans.* Food Chemistry, 2016. **203**: pp. 267 – 275.

Smith, M.M. and Hartley, R.D. *Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants.* Carbohydrate Polymer Research, 1983. **118** (C): 65 – 80.

Solomon, E.I., Sundaram, U.M. and Machonkin, T.E. *Multicopper oxidase and oxygenases.* Chemical Reviews, 1996. pp. 2563 – 2605.

Solomon, E.I. *Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile.* Biochemical Journal, 1998. pp. 63 – 70.

Somboon, N., Karrila, T.T., Kaewmanee, T. and Karrila, S.J. *Properties of Gels from Mixed Agar and Fish Gelatin.* International Food Research Journal, 2014. **21** (1): pp. 485 – 492.

Stavova, E., Porizka, J., Stursa, V., Enev, V. and Divis, P. *Extraction of ferulic acid from wheat bran by alkaline hydrolysis.* MendelNet, 2017. **24** (1): 2017.000.

Strong, P.P. and Claus, H. *Laccase a review of its past and its future in bioremediation.* Critical Reviews in Environmental Science and Technology, 2011. pp. 373 – 434.

Struble, L.J. and Ji, X. *Handbook of Analytical Techniques in Concrete Science and Technology.* 2001. William Andrew, US. ISBAN 978-0-8155-1437-4.

Struch, M., Linke, D., Mokoonlall, A., Hinrichs, J. and Berger, R.G. *Laccase catalyzed crosslinking of skimmed milk yoghurt enhanced by food-grade mediators.* International Dairy Journal, 2015. **49**: pp. 89 – 94.

Subramani, P., Anish, R., Subramani, B., Selvadurai, M. Kalaimani, J.K. and Venugopal, V. *An Overview of Liquid Chromatography-Mass Spectroscopy Instrumentation.* Pharmaceutical Methods, 2014. **5** (2): pp. 47 – 55.

Tabilo-Munizaga, G., Saenz-Hernandez, C. and Herrera-Lavados, C. *Influence of temperature, calcium and sucrose concentration on viscoelastic properties of Prosopis Chilensis seed gum and nopal mucilage dispersions.* International Journal of Food Science and Technology, 2018. **53**: 1781 – 1788.

Takeno, H., Kimura, Y., Nakamura, W. *Mechanical, Swelling, and Structural Properties of Mechanically Tough Clay-Sodium Polyacrylate Blend Hydrogels.* Gels, 2017. **3** (1), 10; https://doi.org/10.3390/gels3010010.

Tatsumi, K., Wada, S. and Ichikawa, H. *Removal of chlorophenols from waste water by immobilized horseradish peroxidase.* Biotechnology and Bioengineering, 1996. pp. 126 – 130.

Thomas, S., Thomas, R., Zachariah, A.K. and Mishra, R.K. *Thermal and Rheological Measurements Techniques for Nanomaterials Characterization,* 2017. Elsevier, ISBN: 978-0-323-46139-9.

Thurston, C.F. *The structure and function of fungal laccases.* Microbiology, 1994. pp. 19 – 26.

Tokita, M., Nishinari, K. *Gels: Structures, Properties, and Functions.* 2009. Springer, Berlin, Heidelberg. Print ISBN: 978-3-642-00864-1.

Van Bockstaele, F., De Leyn, I, Eeckhout, M. and Dewettinck, K. *Rheological properties of wheat flour dough and the relationship with bread volume. I. Creep-recovery measurements.* Cereal Chemistry, 2008. **85** (6): pp. 753 – 761.

Van de Voort, F.R. Fourier transform infrared spectroscopy applied to food analysis. Food Research International, 1992. 25 (5): pp. 397 – 403.

Van Den Brink, J. and De Vries, R. *Fungal enzyme sets for plant polysaccharide degradation.* Applied Microbiology and Biotechnology, 2011. **91** (6): pp. 1477 – 1492.

Vansteenkiste, E., Babot, C., Rouau, X. and Micard, V. *Oxidative gelation of feruloylated arabinoxylan as affected by protein. Influence on protein enzymatic hydrolysis.* Food Hydrocolloids, 2004. **18** (4): pp. 557 – 564.

Vismeh, R., Lu, F., Shishir, P. S., Chundawat, S., Humpula, J.F., Azarpira, A., Balan, V., Dale, B. E., Ralph, J. and Jones, A.D. *Profiling of diferulates (plant cell wall cross-linkers) using ultrahigh-performance liquid chromatography mass spectrometry.* Analyst**,** 2013. **138** (21): pp. 6683 – 6692.

Vogeser, M. and Parhofer, K.G. *Liquid Chromatography Tandem-mass Spectrometry (LC-MS/MS) - Technique and Applications in Endocrinology.* Experimental and Clinical Endocrinology and Diabetes, 2007. **115** (9): pp. 559 – 570.

Wang, J., Sun, B., Liu, Y. and Zhang, H. *Optimisation of ultrasound-assisted enzymatic extraction of arabinoxylan from wheat bran.* Food chemistry, 2014. **150**: pp. 482 – 488.

Wang, K. ande Pierscionek, B. *Biomechanics of the human lens and accommodative system: Functional relevance to physiological states.* Progress in Retinal and Eye Research, 2018. DOI: 10.1016/j.preteyeres.2018.11.004.

Wang, Y., Zheng, Q., Li, W., Ma, Y., Yhao, X. and Zhang, C. *Measurment of Free Water in Foods by Secondary Derivative Thermogravimetry.* Journal of Food, 2018. **16** (1): pp. 438 – 443.

Ward, G., Hadar, Y., Bilkis, I., Konstantinovsky, L. and Dosorrety, C.G. *Initial steps of ferulic acid polymerization by lignin peroxidase.* Journal of Biological Chemistry, 2001. pp. 18734 – 18741.

Weiss, J., Mokoonlall, A., Sykora, L., Pfannstiel, J., Stefan, N. and Hinrichs, J. *A feasibility study on the application of a laccase-mediator system in stirred yoghurt at the pilot scale*. Food Hydrocolloids, 2016. **60**: pp. 119 – 127.

Wyss, H.M., Larsen, R.J. and Weitz, D.A. *Oscillatory Rheology, Measuring the Viscoelastic Behavior of Soft Materials.* G.I.T. Laboratory Journal, 2007. pp. 68 – 70.

Xu, F. *Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition.* Biochemistry, 1996a. pp. 7608 – 7614.

Xu, F., Shin, W., Brown, S., Wahleithner, J.A., Sundaram, U.M. and Solomon, E.I. *A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potentialsubstrate specificity and stability*. Biochemica et Biophysica Acta, 1996b. pp. 303 – 311.

Xu, F. *Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases.* Journal of Biological Chemistry, 1997. pp. 924 – 928.

Yamazaki, H.I. *Cuticular phenoloxidase from the silkworm, Bombyx mori: Properties, solubilization and purification. Insect.* Biochemistry, 1972. pp. 431 – 444.

Xu, Y., Gong, X., Xuan, S., Li, X., Qin, L. and Jiang, W. *Creep and recovery behaviors of magnetorheological plastomer and its magnetic-dependent properties.* Royal society of Chemistry, **2012. 8**: pp. 8483 – 8492.

Yapo, B. M., Robert, C., Etienne, I., Wathelet, B., and Paquot, M. *Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts.* Food Chemistry, 2007. **100** (4): pp. 1356 – 1364.

Yesilada, O., Birhanli, E., Ozmem, N. and Ercan, S. *Highly stable laccase from repeated-batch culture of Funalia trogii ATCC 200800.* Applied Biochemistry and Microbiology, 2014. **50** (1): pp. 55 – 61.

Yoon, W.B. and Gunasekaran, S. *Effect of Temperature and Concentration on Rheological Behaviour of Xanthan-Carob Mixed Gels.* Biotechnology and Bioprocess Engineering, 2007. **12**: pp. 295 – 301.

Yoshida, H. *LXIII. - Chemistry of lacquer (Urushi) Part I: Communication from the Chemical Society of Tokio.* Journal of the Chemical Society, 1883. pp. 472 – 486.

Zayas, J.F. *Water Holding Capacity of Proteins. In Functionality of Proteins in Food,* Springer, 1997. Berlin, Heidelberg, Germany. ISBN 978-3-642-63856-5.

Zhang, G. *Evaluating the viscoelastic properties of biologic tissues in a new way. Journal of Musculoskeletal Neuronal Interact, 2005. **5** (1): pp. 85 – 90.*

Zhang, X., Chen, T., Lim, J., Gu, F., Fang, F., Cheng, L., Campanella, O.H. and Hamaker, B.R. *Acid gelation of soluble laccase-crosslinked corn bran arabinoxylan and possible gel formation mechanism.* Food Hydrocolloids, 2019. **92**: 1 – 9.

Zorn, H., Langhoff, S., Scheibner, M., Nimtz, M. and Berger, R.G. *A peroxidase from Lepista irina cleaves β, β-carotene to flavor compounds.* Journal of Biological Chemistry, 2003. pp. 1049 – 1056.

Curriculum vitae

Personal Information

Full name: Sanaz Khalighi

Nationality: Iranian

Date of Birth: 03.06.1985 in Tehran, Iran

Academic Background

Since Jun. 2015	PhD student in Institute of Food Chemistry Leibniz University, Hannover, Germany **Thesis:** Rheological and Structural Properties of Gels Cross-linked with Fungal Laccases
Oct. 2009 – May 2012	M.Sc. of Food Science and Technology Tarbiat Modares University (TMU) Tehran, Iran. GPA: 17.24 out of 20. **Thesis:** Extraction and Investigation of Some Physical, Chemical and Rheological Properties of Iranian Carob Bean Gum
Oct. 2003 – Sep. 2007	B.Sc. of Food Science and Technology University of Science and Research Tehran, Iran. GPA: 16.38 out of 20
Oct. 1999 – Jun. 2003	High school Diploma, Tehran, Iran

List of Publications

This Thesis

1. **Khalighi,** S., Berger, R.G. Ersoy, F. **Cross-Linking of Fibrex Gel by Fungal Laccase: Gel Rheological and Structural Characteristics.** *Processes,* 2020. 8 (1), 16; https://doi.org/10.3390/pr8010016.

2. **Khalighi,** S., Berger, R.G. Ersoy, F. **Cross-Linking of Wheat Bran Arabinoxylan by Fungal Laccases Yields Firm Gels.** *Processes,* 2020. 8 (1), 36; https://doi.org/10.3390/pr8010036.

Posters

1. **Kalighi,** S., Abbasi, S. **Investigation of Some Physical, Chemical and Rheological Properties of Iranian Carob Bean Gum.** *Gums and Stabilizers for the Food Industry Conference,* Wageningen, Nederland. June 28th – July 1st 2011.

2. **Khalighi,** S., Abbasi, S. **Preparation and Chemical Modification of Edible Films Based on Farsi Gum (Zedu Gum).** *20th National Food Science and Industries Congress,* Sharif University, Tehran, Iran. 22 – 24 November 2011.

Oral Presentations (in Persian)

1. **Khalighi,** S., Abbasi, S. Azizi Tabrizi Zadeh, M.H. **The Effect of Some Parameters on Extraction and Some Physical, Chemical and Rheological Properties of Iranian Carob Bean Gum.** *20th National Food Science and Industries Congress,* Sharif University, Tehran, Iran. 22 – 24 November 2011.

Publications (in Persian)

1. **Khalighi**, S., Abbasi, S. **Effect of Different Dehulling Methods on Some Rheological Properties of Iranian Carob Bean Gum Dispersion**, *Journal of Food Science and Novel Technologies,* 2014. 1 (3): pp. 3 – 14.

2. **Khalighi**, S., Abbasi, S., Azizi Tabrizi Zadeh, M.H. **Effect of Different Dehulling Methods on Some Physical and Chemical Properties of Iranian Carob Bean Gum,** *Iranian Journal of Biosystems Engineering,* 2014. 44 (2): pp. 165 – 172.