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Characterization of the stability of polysaccharides during baking

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Zusammenfassung

Beta Glucan zählt zu den am häufigsten analysierten, sowie erforschten Ballaststoffen. Vor allem die verzweigte Form (1.3/1.6) des Beta Glucans findet wegen seiner stabilisatorischen Eigenschaften immer mehr Verwendung in der Backwarenindustrie und wirkt zudem gesundheitsfördernd. Ziel dieser Arbeit war es, eine gezielt herbeigeführte Veränderung des Molekulargewichtes von Beta Glucan zu verursachen und es in weiterer Folge mittels Size- Exclusion- Chromatographie (SEC) zu analysieren. Dafür wurde ein Szenario aus der Backwarenindustrie gewählt, wobei thermische sowie reologische Kräfte auf das Beta Glucan wirken. Die analytische Separation erfolgte durch die Verwendung einer *TSKgel PWxl* Säule von *Tosoh Bioscience GmbH*. Anschließend wurde das Eluat anhand eines Refractive Index Detektor (RID) gemessen.

Darüber hinaus wurden auch Analysen im Bezug auf die Wasserlöslichkeit von verzweigten Beta Glucan unter der Einwirkung von Scherkräften und Temperatur analysiert.

Die Resultate zeigten eine hohe Reproduzierbarkeit. Daraus lässt sich schließen, dass die SEC-Methode zur Bestimmung des Molekulargewichtes sehr verlässlich ist.

Die Veränderung des molekularen Gewichts von Beta Glucan korreliert sehr stark mit der Intensität des Bearbeitungsverfahrens (thermisch, reologisch). Um das molekulare Gewicht bestimmen zu können wurde eine Kalibrationskurve mit Polyethylenglycol erstellt. Kaum behandeltes Beta Glucan hatte ein Molekulargewicht von ungefähr 32.000 Da (Daltons), zweifach behandeltes Beta Glucan hingegen wies eine Bandbreite von 28.000-1000 Da auf.

Physikalische und thermische Kräfte unterstützen die Löslichkeit von verzweigten Beta Glucan im Wasser. Die Maximale Löslichkeit konnte nach einer Inkubation von 3 h erreicht werden.

Abstract

Recently beta glucan is one of the most common and well-studied dietary fibers, especially in terms of health benefits. The alteration of beta glucans molecular weight in baking processes was analyzed by size-exclusion chromatography (SEC).

A simulation with thermal and rheological conditions, typical in baking industries, was performed. For analytical separation a *TSKgel PWxl* column from *Tosoh Bioscience GmbH* was used and the reduction of the molecular weight in branched (1.3/1.6)-beta glucan was determined by using a refractive index detector (RID).

Furthermore measurements of the water-solubility of branched beta glucan induced by using high mechanical energy for stirring were conducted.

The results show that SEC is a reliable method for the analysis and molecular weight determination of beta glucan in food matrices. Physical and thermal treatments had an effect on the molecular weight. A correlation between the molecular weight and intensity of treatment could be observed. Compared to the calibration curve (PEG Standards) moderately treated beta glucan may have a molecular weight of approximately 32,000 Da while double treated beta glucan has a molecular weight of 28,000 down to 1,000 Da.

Physical forces (thermal and rheological) improve the water solubility of beta glucan. The maximum value of solubility was reached after 3 h of incubation.

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1. Polysaccharides

According to the polysaccharide definition of Brekke Peterson Munks: "*A polysaccharide is a long-chain carbohydrate made up of smaller carbohydrates called monosaccharides that's typically used by our bodies for energy or to help with cellular structure. Each monosaccharide is connected together via glycosidic bonds to form the polysaccharide.*

The typical polysaccharide is between 200 and 2500 monosaccharides long, and they can be either linear or branched carbon chains. Usually, the structure of polysaccharides is six-carbon repeating monosaccharides linked together by oxygen. The chemical formula is often $(C_6H_{10}O_5)_n$*, where the n is a number larger than 40. The specific formation of the polysaccharide is dependent upon its use."* (Munks, 2003)

The most common polysaccharides, which are clearly understood in their structures and functions, are starch, cellulose and glycogen. Starch is a polymer consisting of amylose, a linear polymer consisting of several thousand glucose units, which are linked with an α-1,4-glycosidic bond and amylopectin a branched polymer consisting of several hundreds of glucose units, which are linked by alpha 1,4 glycosidic bonds. Furthermore starch is used as storage in plants.

In animals and human body, especially in liver and muscles starch is stored as glycogen, a branched biopolymer, which consists of approximately 30000 glucose units linked by alpha 1,4 glycosidic bonds. Branches are linked to the chains from which they are branching off by alpha 1,6 glycosidic bonds between the first glucose of the new branch and a glucose on the stem chain. ((Jeremy M Berg, 2002)

Cellulose is an unbranched polymer, which consists of several glucose units, which are linked by beta 1,4 glycosidic bonds and plays an essential role in the structure of plants and other organism cell walls.

1.1. Digestible Polysaccharides

Digestible polysaccharides such as starch, dextrin and glycogen are broken down enzymatically by alpha amylase in animals and humans mouths and get depolymerized several steps in the small intestine to glucose which is absorbed from the cell walls of the intestine. Thereafter glucose is transported to different cells in the body and acts as an energy storage as well as carbon source for synthesis of lipids and proteins.

1.2. Dietary fibres

Non-digestible polysaccharides or dietary fibres are defined according to the American Association of Cereal Chemists (AACC) in the year 2000:"*Dietary fibre as the edible parts of plant that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin and associated plant substances.*" (Devinder Dhingra, 2011)

Furthermore during the year 2002 the National Academy of Sciences described dietary fibres as non-digestible carbohydrates, which belong to functional fibres. Those functional fibres are recognized for having beneficial effects in human bodies. Depending on the solubility in water dietary fibres can be classify into two main categories: Soluble and insoluble fibres.

1.3. Water insoluble dietary fibres

Cellulose, hemicellulose and lignin are water insoluble fibres and have less importance in the food industry because of their non-digestive properties in the human body. In agriculture, especially in breeding of ruminant animals the know-how about cellulose and hemicellulose plays an important role. Ruminant animals like cows have bacteria in their intestine, which are able to break down the beta 1,4 glycisidic bonds and recover about 70% of their forage. Furthermore insoluble fibres find importance in paper as well in methanol industry.

1.4.Water soluble dietary fibres

Water soluble dietary fibres like pectins, beta- glucans, gums (guar gums, microbial gums, xanthan, gellan) are useful tools in food industrial procedures and are added as a supplement to increase the water uptake rate and increase the viscosity (gelling) in products. Pectin consists of D-galacturonic acid units which are linked by alpha 1,4 glycosidically. Pectic substances are part of plant cell walls and act as an intracellular cementing substance in plants. In human gastrointestinal tract pectin decrease the rate of gastric emptying because of its swelling effects and extend the time of passing through the small intestine. Hypoglycemic properties can be explained by this fact. (Jenkins, 1978)

Gums are highly branched polysaccharides which are exudated by secretory plant cells. Gums mainly consist out of guar gum, an isolatad galactomannan from seeds of *Cyamopsis tetragonolobus* and gum arabic. Under enzymatically pretreatment guar gum gets soluble in water and can be used as a water-soluble fibre. Because of the high water uptake guar gum gets used to improve bowel functioning and relieve constipation. Gum arabic is an arabinogalactan polysaccharide complex with a glycoprotein. (Van Denffer D, 1976)

2. Beta glucan

Beta glucan is a non-starch polysaccharide from plants and fungi that aren't able to be converted to energy in human's body. Beta glucan is one of the most common and well-studied dietary fibre with regard to food supplements and health benefits because of its stability and easy handling. Based on the structure and in conjunction with the source, beta glucan can be classified into two groups. Due to that fact the functions of beta glucan in medical treatment differ from each other. Four significant areas of health benefits influenced by beta glucan can be proofed.

2.1. Medical and food industrial aspects

Heart health:

The FDA (United States Food and Drug Administrations) approved the health claim of soluble beta glucan in cereals for reducing the risk of heart disease. Studies have shown that a daily intake of more or less three grams over five weeks can reduce levels of low-density lipoprotein (LDL) cholesterol by 5-10%. (Jones, 2011)

Cancer:

Studies have shown that beta glucan (linked beta 1,3; 1,6 glycosidic) from fungi is able to suppress chemical and viral oncogenesis and prevent the growth of metastasis after surgery. The production of cytokines, which act as cell growth regulators in proliferation and differentiation for the defense mechanism in cells, gets supported by beta glucan.

Studies at the biotechnical Institute of Fukushima, Japan have shown that a daily ingestion of beta glucan in mice led to a higher proliferation of white blood cells, which are essential in cancer therapy. (Chihara, 1992)

Immunity:

The healing and immune-supporting properties of fungal beta glucan have been known for more than a century in eastern countries. Mushroom substances, especially polysaccharides which belong to the beta glucan groups support the immune complex by enhanching natural killer functions in B-; T-lymphocytes. Furthermore beta glucan is added as adjuvant in immunotherapy to induced the tumor killing-activity of monoclonal antibodies. (Akramiene D, 2007)

Infections:

Fungal beta glucan of yeast cells has shown a suppressing effect for getting a septic infection after a wound surgery. By using a guinea pig model studies have shown that by the presence of beta glucan in blood, bacteria were killed and due to that fact septic shock was reduced. (Onderdonk AB, 1992)

The use of beta glucan as an additive for food is common. Beta glucan have a high variability of molecular weight (9000-3000000 Da) and therefore it is a multi-functional tool. By using a high molecular weight β-glucan it is possible to create a pseudo plastic solutions with high viscosity. For creating soft gels beta glucan with low molecular weight is used. This phenomenon is achieved by chemical and

enzymatically hydrolysis as well as physical forces, which facilitate as well as the alteration of molecular size between 400000 and 2000000 Daltons. (Beer MU, 1997)

Beta glucan is suitable for many requirements in low fat products as ice-cream, cheese, sausages and soft- brined cheese. Furthermore it also finds application as an emulsifier and stabilizer in soups, sauces and beverages.

2.2. Beta glucan in plants

In plants, especially in cereals like barley, wheat and oats beta glucan is mainly found in the cell walls, more precisely in the outer layer of the endosperm. Beta glucan in those cereals is linear, unbranched D-glucose polysaccharide chain which are linked by beta-1,3;1,4-glycosidic bonds (figure 1). According to the source, the macromolecular structure as well as the yield after isolation differs strongly. For extracting two common methods are used: Dry and wet milling. Commercially Oat (65%) and barley (53%) have shown the highest content of beta glucan. (Zielke C., 2017)

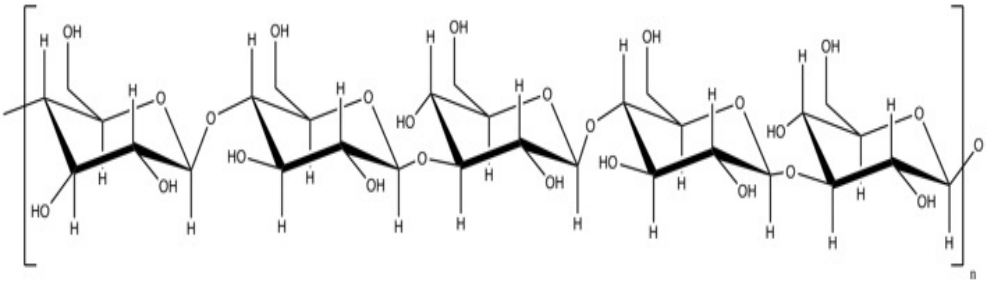


Figure 1 Structural chemical formula of 1,3/1,4 beta glucan

2.3. Beta glucan in fungi

In contrast to plants, the fungal structure of beta glucan differs in beta glycosidic binding. The backbone chain consists of D-glucose units which are linked by beta 1,3 glycosidic bonds and exhibit occasionally beta 1,6 gylcosidic linkages. Furthermore these branched chains are connected to each other to form a fibrillar 1,3 beta glucan (figure 2).

In mushrooms and yeasts beta glucans are major polymers in cell walls and act as a regulator for cell growth and morphogenesis. Highest contents of beta glucan was reported by *Cantharellus cibarius* (Chantarelle) with 24 g/100 g dry mass, *lentinula edodes* (Shiitake) and *pleurotus* species between 15-22 g/100 g dry mass.(Chang S.T., Buswell J.A., 2003)

The main interest in these branched glucans are the benfecial health effects. Studies have shown that beta glucans from fungal sources are more reactive in immunological and oncological pathways than from plants. This might be because of the beta 1,6 linkage.

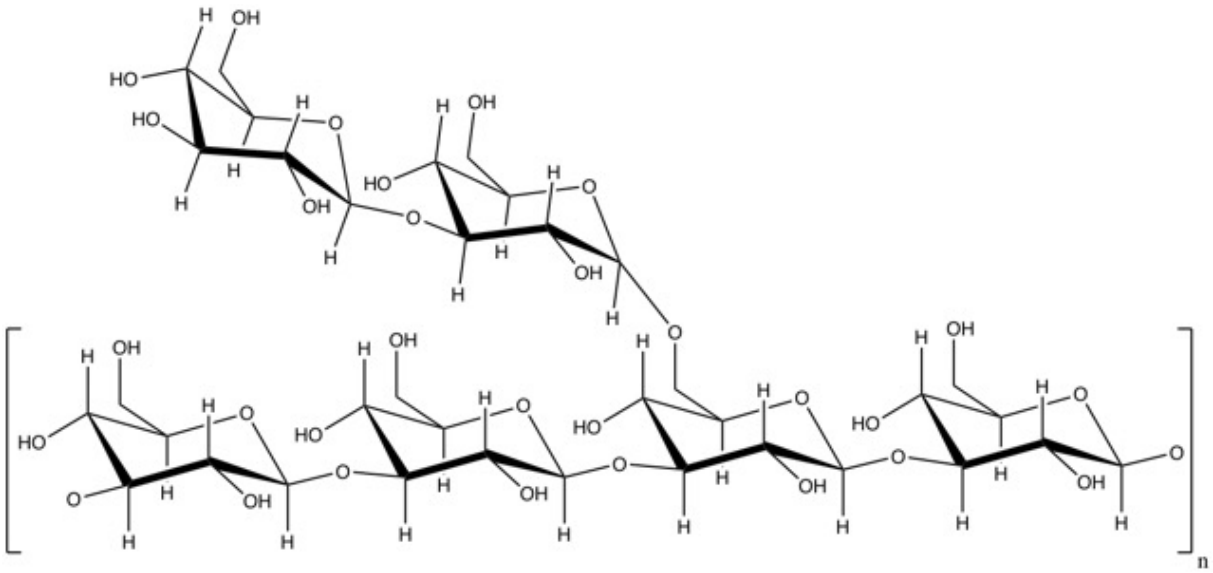


Figure 2 Structural chemical formula of 1,3/1,6 beta glucan

2.4. Sources of beta glucan

The list below shows summary of beta glucan content and structure difference in different sources

Table 1 Beta glucan sources categorized in their structure and taxonomy (Ilka Noss, 2012)

Glucan	Taxon	Linkages	MW (kDa)
Baker's yeast gl.	Fungi	β-(1,3), β-(1,6)	35-5000
Barley glucan	Plant	β-(1,3), β-(1,4)	23-137
Pullulan	Fungi	α-(1,4), α-(1,6)	200
Shizophyllan	Fungi	β-(1,3), β-(1,6)	76,8-450
Oat glucan	Plant	β-(1,3), β-(1,4)	1-300
Pleurotus ostreatus	Fungi	β-(1,3), β-(1,6)	30-300
Scleroglucan	Fungi	β-(1,3), β-(1,6)	1000-5000

3. HPLC (High Performance Liquid Chromatography)

High performance liquide chromatography is an analytical method in chemistry, which is used for disjunction of substances by their charge polarity or molecular size. Each HPLC consists of a mobile phase (eluent) and a stationary phase, mostly used silica- or kieselgel. Frequently used HPLC's are the normal phase (NP) and reversed phase (RP) chromatography methods. In NP the stationary phase is polar and the mobile phase is un-polar and in RP the stationary phase is un-polar and the mobile phase is polar.

Depending on the polarity of the analyte, the interaction between the stationary phase and the analyzed substance of various strenght and the analayte can be resolved by increasing the polarity of the eluent. Therefore the elutiontime, the time from column till the detecter is dependent to the polarity of the substance.

3.1. Size exclusion chromatography

Size Exclusion Chromatography (SEC) is a common HPLC method in which substances get separated based on their size and their molecular weight. Depending on the analyzed substance SEC can be classified in two different types. In Gel Permeation Chromatography (GPC), the stationary phase is hydrophobic packed and the mobile phase is non-aqueous, while in Gel Filtration Chromatography (GFC), a hydrophilic stationary phase and an aqueous mobile phase are used. GPC is used for measuring the molecular weight distribution of synthetic polymers and GFC is used for measuring the molecular weight distribution of molecules, which are soluble in water, such as polysaccharides and proteins. (Size Exclusion | https://www.separations.eu.tosohbioscience.com, 2017)

Other HPLC include for example normal-phase or reverse-phased chromatography. Depending on their molecular weight and the porous spherical beads containing pores of a specific size distribution in the stationary phase, the analytes retained in different degrees, resulting in a specific retention time for every compound. After elution the substances can be verified by using a detector.

G4000PWxl

TSKgel PWXL columns from *Tosoh Bioscience GmbH* are composed of spherical, hydrophilic polymethacrylate beads. The main application area for these columns is the analysis of water-soluble polymers, such as celluloses, acrylamides, glycols, dextrans, polyvinylalcohols, and oligosaccharides. (Size Exclusion | https://www.separations.eu.tosohbioscience.com, 2017)

The G4000PWxl column is ideally suited for analysis of water-soluble linear polymers with molecular weights up to 300,000 Da. The stationary phase consists of hydroxylated polymethacrylate beads, which makes it possible to use a maximum of 20% organic solvent.

According to the principle decription of a SEC – column by *Tosoh Bioscience GmbH*:*"Large biomolecules that cannot penetrate the pores of the packing material elute first from the column. They are said to be excluded from the packing; they flow with the mobile phase in the interparticle space of the packed column. The exclusion limit characterizes the upper limit of molecular weight (or size), beyond which molecules will elute at the same retention volume called the exclusion or void volume of the column. Many SEC columns are referred to by their exclusion limit.*

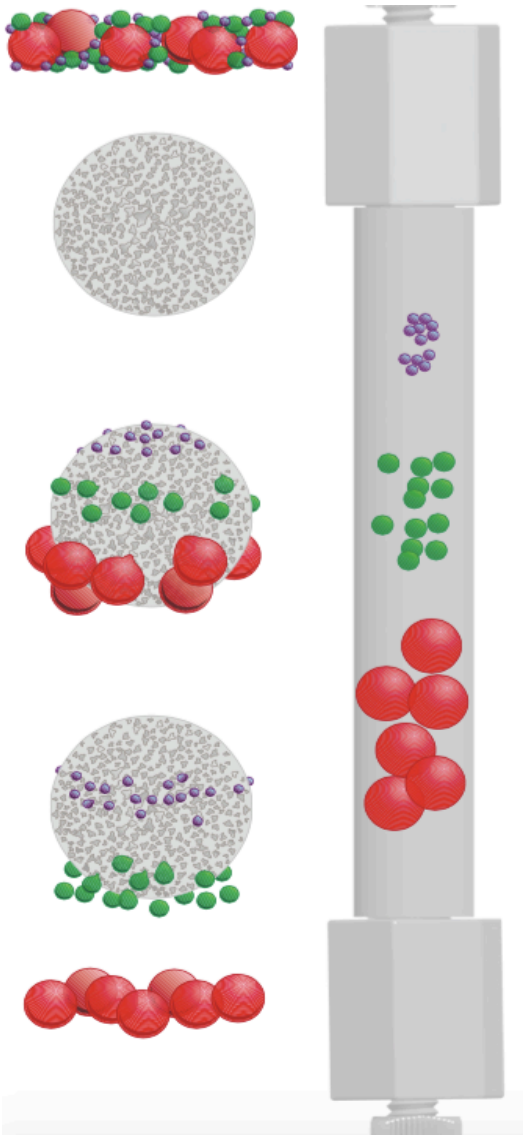


Figure 3 Principle of SEC column ((Size Exclusion | https://www.separations.eu.tosohbioscience.com, 2017)

Smaller molecules can partially or completely enter the porous particles. Because these smaller molecules have to flow through the interparticle space, as well as through the pore volume, they will elute from the column after the excluded sample components.

SEC is a very simple method for separating biomolecules, because it is not necessary to change the composition of the mobile phase during elution. However, the separation capacity of this method is limited." (Size Exclusion | https://www.separations.eu.tosohbioscience.com, 2017)

4. Materials and Methods

4.1. Materials

4.1.1. Instruments

All instruments and equipments which were used for sample preparing and sample analysis are listed in table 2.

Instrument	Manufacturer
Mettler Toledo AG135; AG245	Mettler Toledo GmbH, Vienna, Austria
Testo 104-IR	Testo GmbH, Vienna, Austria
Centrifuge 5804 R	Eppendorf AG, Hamburg, Germany
Centrifuge	Eppendorf AG, Hamburg, Germany
Vortex	Scientific Industries, New York, USA
DU© 800 photometer	Beckmann Coulter™ Inc., Vienna Austria
ThermoStat Plus	Eppendorf AG, Hamburg, Germany
Eppendorf Reference® pipettes	Eppendorf AG, Hamburg, Germany
LiChrolut vacuum chamber	Merck, Darmstadt, Germany
LiChrolut C18 SPE column elution bond	Merck, Darmstadt, Germany
Melag 206 Incubator	MTC, Hamburg, Germany
Oven	
Freeze dryer	
pH-meter	Mettler Toledo GmbH, Vienna, Austria
Kitchen mixer	Bosch, Vienna Austria
MR Hei-Standard	Heidolph Instruments GmbH, Schwabach

Table 2 List of used instruments and manufacturing companies

4.1.2. Chemicals

All chemicals, which were used for sample preparing and sample analysis are listed in table 3.

Standard substance	Manufactors
Sucrose	Sigma Aldrich Chemie GmbH, Vienna, Austria
Sodium bicarbonate	
Ammonium chloride	
beta-glucan	
Ethanol	
Methanol	
Dimethylsulfoxide (DMSO)	
Starch	
Xylose	
Hexane	
Sodium dihydrogen phosphate	
Potassium dihydrogen phosphate	
Sodium nitrate	
Phosphate buffer pH 6.5	
DMSO 20%	
PEG Standard	

Table 3 List of used chemicals and manufacturing companies

4.2. Methods

4.2.1. High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) was used for the determination of beta-glycan and other sugar compounds like glucose, sucrose. The separations in those HPLC's are achieved by different disjunction of sample substances between a stationary phase and a mobile phase. Size Exclusion Chromatography (SEC) a common HPLC method in which substances get separated based on their size and their molecular weight.

The HPLC-instrument by Agilent Technologies, Series 1100 (consisting of isocratic pump with solvent cabinet, manual injector (MI), a variable wavelength detector (VWD) with standard flow cell (10 mm path length, 14 µl volume, 20 bar maximum pressure), a thermostatic column compartment (TCC) and a refractive index detector (RID) was used. For data evaluation the software ChemStation32 for LC 3D© 1990-2003 by Agilent Technologies was used.

4.2.2. Baking Product Test (AACC Method)

For analyzing modification and degradation of beta-glucans under the impact of thermal and mechanical conditions a repeatable standard has to be found. Therefore an International Standard of the "American Association of Cereal Chemists" (AACC) for measuring the baking quality of cookie flour was used and the recipe was modified. For extraction of the beta- glucans out of the well baked cookie different kinds of extraction steps were used. Altogether the process can be separated in three steps: 1. Removing of fatty compounds, 2. Removing of Proteins and 3. Purification of beta- glucans and carbohydrates.

4.2.3. Solid-phase extraction (SPE)

Complex samples can lead to many problems during chromatographic analysis because of their compositions. These problems include quantification errors, baseline interferences, blocking of columns and resolution issues (Arsenault, 2003). To minimize such problems solid-phase extraction (SPE) is a common method for sample pre-treatment. The main targets are to clean-up compounds and matrix of the sample, to concentrate the analyzing substances and to change solvents (Simpson N., 2000). The principle of solid-phase extraction is to absorb the analyzed substances by proper sorbent, when they are passed through a glass or plastic cartridge (Nollet L.M.L, 2000). Alltogether the process can be separated in five steps: 1. sorbent conditioning, 2. sample loading, 3. sorbent washing, 4. elution of the analyte with a selective organic solvent (Simpson N., 2000). The main steps are shown in figure 4.

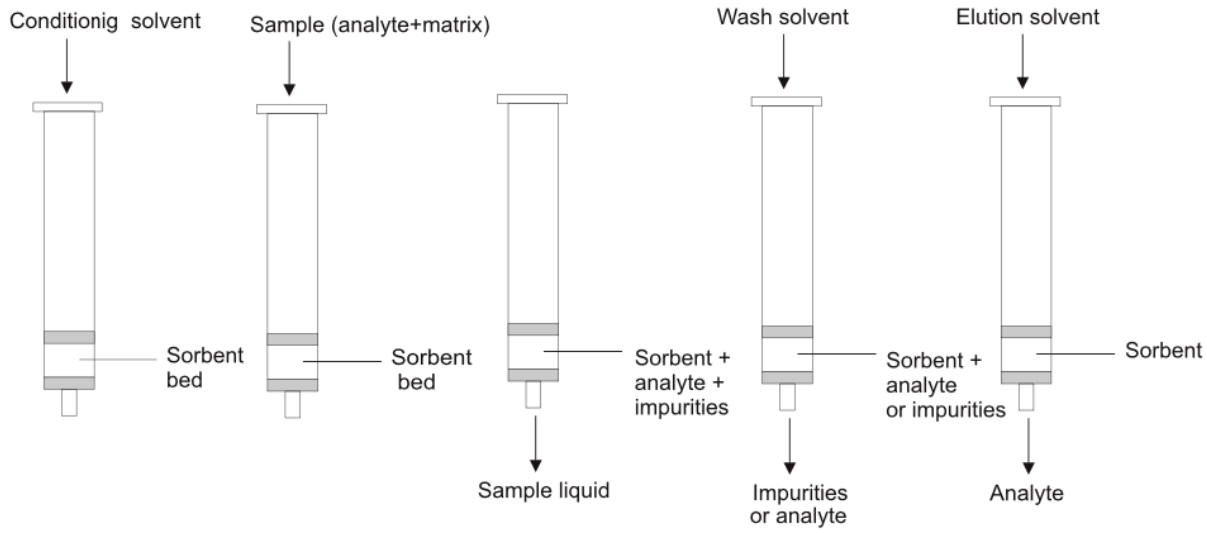


Figure 4 Steps of the solid-phase extraction procedure (Modfied from (Żwir-Ferenc, Bizuik, 2006))

The most common retention mechanisms include van der Waals, electrostatic, hydrogen, non-polar and polar interactions (Żwir-Ferenc, Bizuik, 2006). For ion-pair interactions, a polar group and a long aliphatic chain are used. For non-polar (reverse phase) interactions an octadecyl modified silica and a C18 sorbent phase are used. During the experiment a C18-SPE elution cartridge by varian were used for purification of the polymer samples and to remove all protein compounds of them.

Sample preparation

All ingredients, which were used for the dough, are listed in the table below. Every ingredient was pre-weighed (Table 5).

Ingredient	Formula (g)
Flour (Farina)	39
Beta-glucan (1,3-1,6) from fungi	1
Sucrose	24
Not Fat Dry Milk	1.2
$NaHCO_3$	0.72
NH_4Cl	0.20
Shortening (rape seed oil)	12
NaCl	0.18
Water	6.4
Total amount	84.6

Table 4 Ingredients for preparation of six cookies

Mixing procedure

1. Dry ingredients (sucrose non-fat dry milk, etc.) were put together until they were well mixed. These ingredients were put together with shortening (rape seed oil) by using a mixer, using a paddle attachment, on low speed for one minute. Thereafter the mass in the bowl were scraped and paddled at medium speed for 1minute. Afterwards it was scraped again on high speed for 30 seconds and this step was repeated twice.

2. The creamed mass was scraped into a cookie dough mixing bowl and water with the pre-dissolved ammonium chloride and sodium bicarbonate was added. This mass was mixed for three minutes (stopping mixer and scraping after first few seconds if the shortening is stuck on the side of the bowl) and for scraping the sticking rest a small spatula was used.

3. At least 39 g flour was added to the bowl and mixed for 25 seconds. For getting a better homogeneity the dough it was scraped from the edges of the bowl and were put under the pins of the mixer. This step was repeated three times.

4. The dough was taken out of bowl and was formed to a single dough mass, which was separated into six portions with the same weight of 14.1 g

5. Two portions were rolled out by a rolling pin on an aluminum cookie sheet and were placed in an oven for ten minutes and 180 °C. The rest of the portions were frozen.

6. For references the same procedure and the same steps were done, but instead of taking β-glucans 1 g wheat flour was taken. (AACC International Approved Methods - AACC Method 10-54.01. Baking Quality of Cookie Flour-Micro Wire-Cut Formulation, 2017)

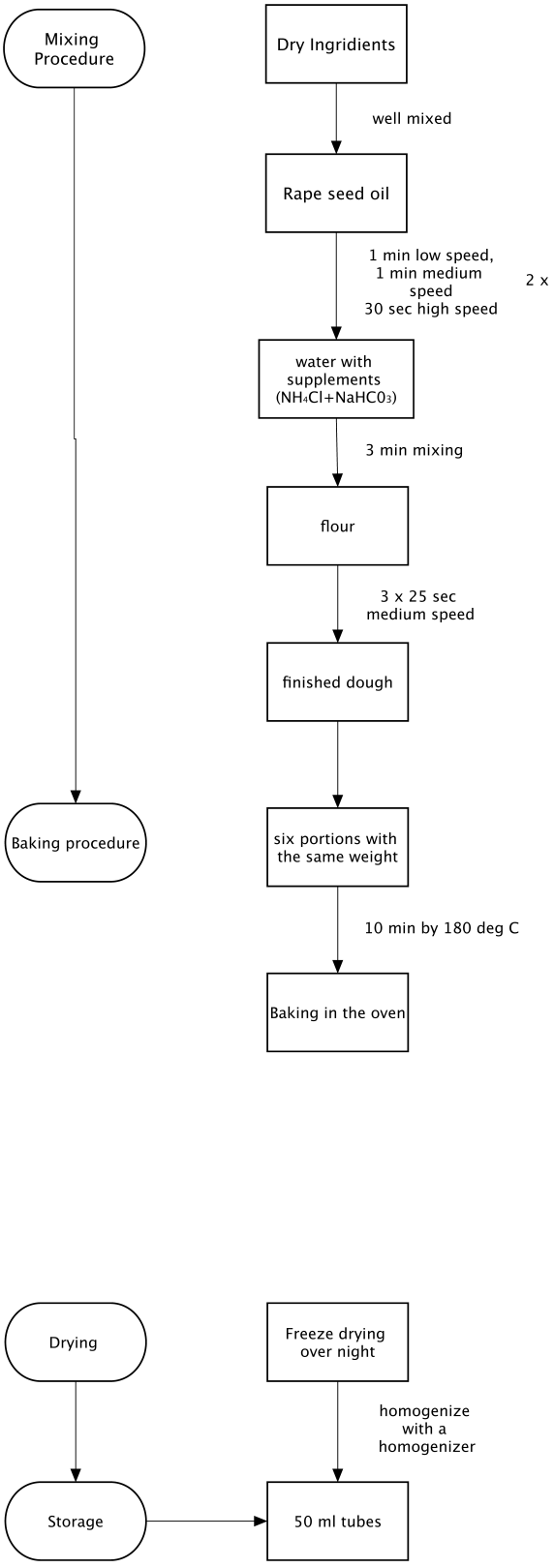


Figure 5 Flowchart of the mixing procedure of an AACC method „Baking quality of cookie flour micro“ and the following drying steps.

Sample Preparation

To dehydrate the baked cookie portions the samples were freeze-dried over night. Thereafter all portions had a loss of 1.7gramm and a total weight of 12.4 g. For the extraction of the carbohydrates of the cookie sample several process steps were done. First of all the dried cookie sample was crushed by a homogenizer for getting a much higher surface of the sample. 2. For separation of the hydrophobic (fatty compounds) and the hydrophilic parts a phase separation was done. The samples were filled up with 20 ml of hexane, were shaked for 30 min and were centrifuged for 10 min by 4500 rpm at room temperature. Thereafter the supernatant was discarded. This step was repeated for three times in a row. The rest of the hexane was removed with nitrogen. A phosphate buffer with a pH of 6.5 was prepared and the sample was redissolved in 30 ml buffer. For denaturation of the proteins the dissolved sample was transferred to a shaking flask and was cooked while stirring for 10 min in a water bath. After cooling down the cooking process the sample was centrifuged for 20 min by 4500 rpm at room temperature and the supernatant was used. To purify the supernatant of the sample it was portioned in 1.5 ml Eppendorf tubes and were centrifuged for 5 min at 16000 rpm at room temperature. The supernatant was transferred to another by Eppendorf tube and this step was repeated three times. At last the supernatant was collected and was purified by using the SPE C18-elution method to get rid of the remained proteins.

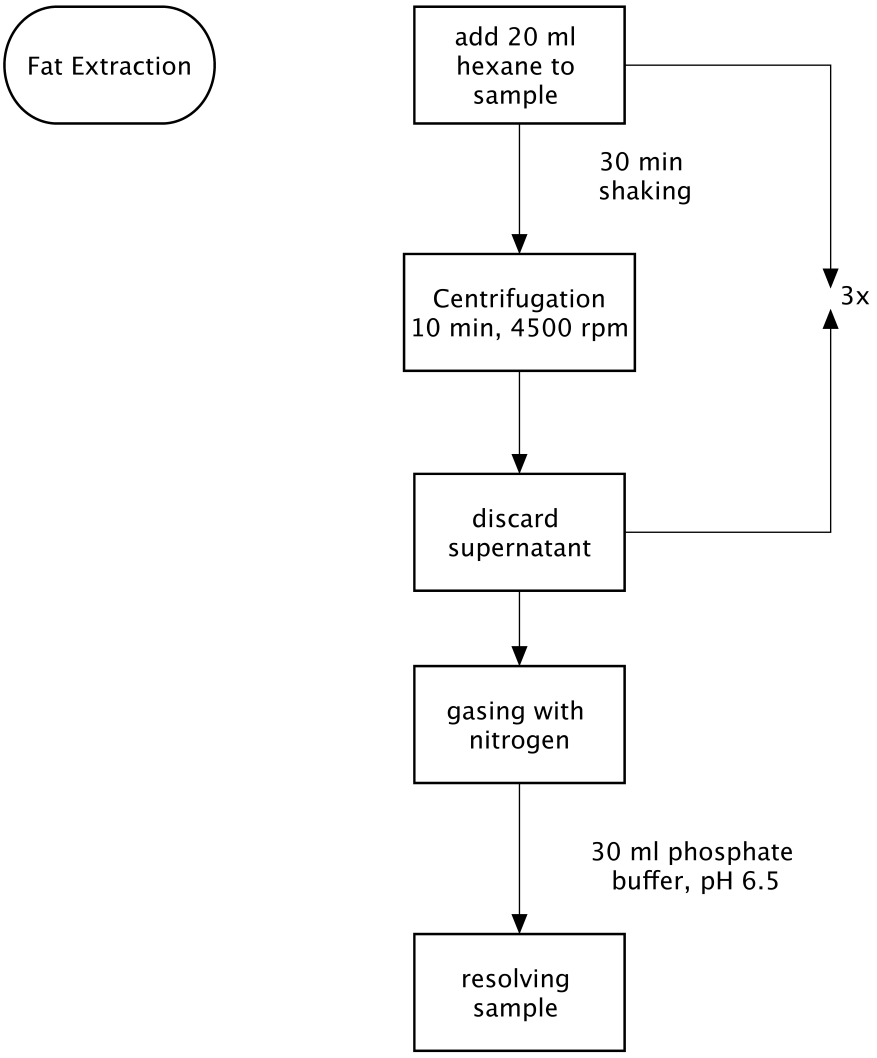


Figure 6: Fat extraction of the homogenized cookies with the two-phase principle

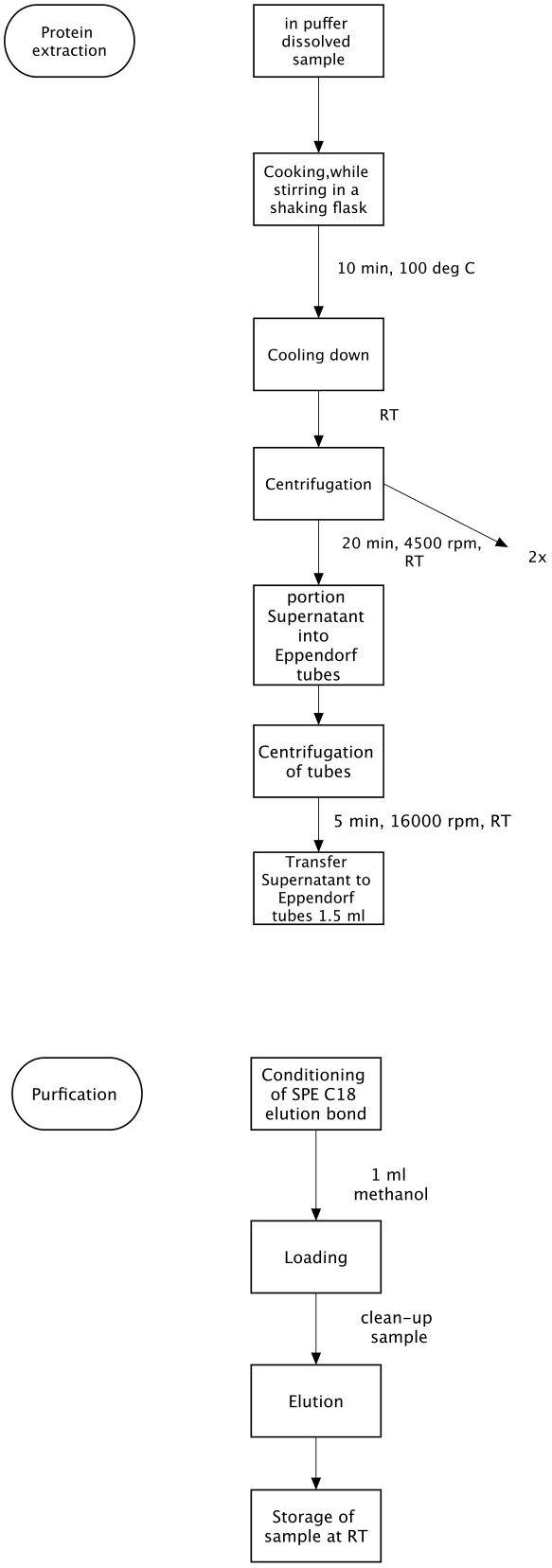


Figure 7; Steps of the protein extraction procedure and the steps of carbohydrate purification with SPE C18 elution columns

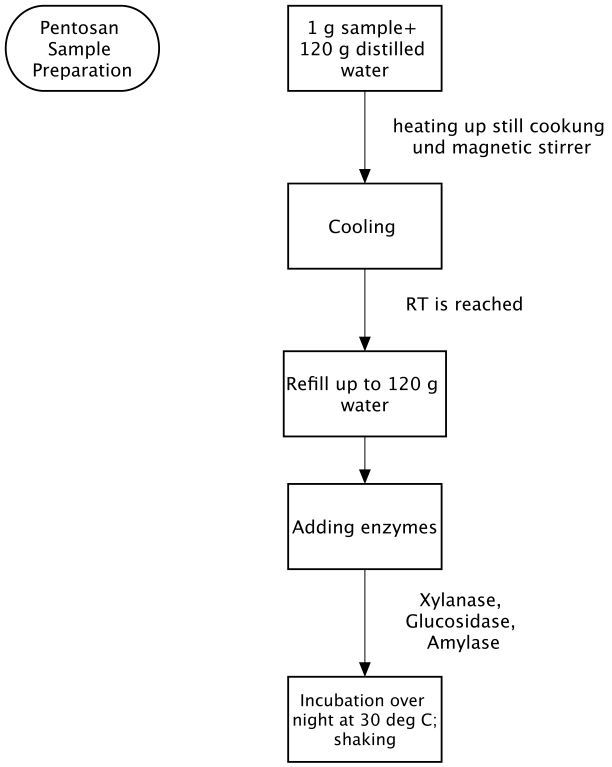
4.2.4. Pentosane Assay

The total amount of beta-gluc can be measured by using a colorimetric assay. This assay is a modification of the Tollens method, which is typically used for xylose measurements. The reactive orcinol/iron solution is a mixture of glacial acetic acid, concentrated hydrochloric acid, phloroglucinol solutions (20%) and glucose. The Tollens/orcin method makes use of the conversion of pentosane to furfural by hydrochloric acid. Thereafter furfural is distilled at a controlled rate and determined colorimetrically at a wavelength of 552 nm and 510 nm (Fraser,J.R.,Branden-Bravo,M. &Holmes,O. C., 1956)

Sample preparations

1 g of flour was weighted into a conical flask (250 ml) and 120 ml of distilled water was added. Thereafter the mixture is heated on a heating plate until it starts to cook and was slowly stirred by a magnetic stirrer. Under this conditions a perfect dispersion between water and flour can get reached. Thereafter the dispersion get cooled down and was refilled with distilled water, to reach the value of 120 ml. For degradation of xylose, glucose and amylose, different kinds of enzymes with different enzyme activities, which are shown in the figure below, were composed together and were added to the prepared samples. In the control sample the whole mixture was incubated over night at 30 °C under slow shaking conditions. 1 ml of the reactive solution, which is described in the pentosane assay, was mixed with 0.2 ml of the sample (for control sample purified water was used) and was incubated for 25 minutes in a cooking water bath. Furthermore the sample was cooled down by covering in ice and the absorbance of the resulting solution was measured at 510 nm and 552 nm. The percentage of the total amount of pentosanes in flour was calculated by substitution of both wavelength values and was compared to a standard curve. All samples were measured as a triplet. For negative standard distilled water was used and for positive standard D-(+)-xylose was used. Both standards were placed into the water bath next to the flour samples and were also measured at the same wavelengths. For the calibration curve 1 mg/ml D- (+) xylose were weighed and dissolved in water. Out of the stock solution a five-point calibration curve between a range of 25 µg/ml and 500 µg/ml were established.

Enzyme	Units/gramm	Manufacturer	Amount in mg per 1gramm of sample
Xylanase	2600	Sigma Aldrich Chemie GmbH, Vienna, Austria	500
Glucosidase	193	Sigma Aldrich Chemie GmbH, Vienna, Austria	0.3
Amylase	99	Sigma Aldrich Chemie GmbH, Vienna, Austria	8



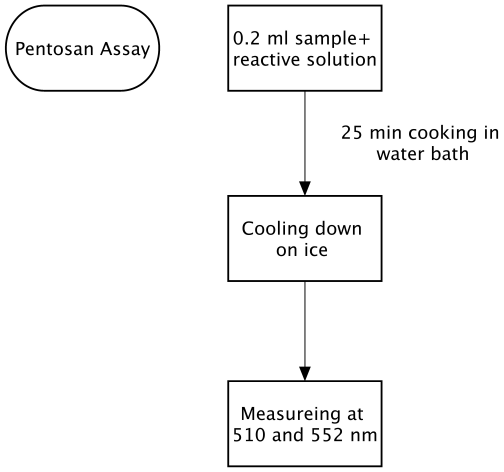


Figure 8 Steps of the preparation of a pentosane assay (Modified from Fraser et al., 1956)

4.2.5. Solubility of carbohydrates

The solubility of samples plays an essential role in the analytics procedure due to the fact that each sample have their own structure and affinity to polar or un-polar solutions. A large number of carbohydrates are hydrophilic because of their reactive OH groups and are soluble in polar fluids because of the interactions between hydrogen bonds and van-der-Waals forces. But in the case of beta glucan both options are possible because beta glucan have water soluble and water-insoluble parts. For the thesis the water soluble part was the point of interest and the aim was to find out how temperature and rheology and the use of different solutions influence the solubility of beta glucan under a defined time period.

Sample preparations

15 mg of a carbohydrate sample was added to 1 ml solution (water, 30% DMSO, 6.5pH phosphate buffer) in a 1.5 ml Eppendorf tube. Each sample has to be prepared 6 times .The thermo-mixer was heated up to 95 °C and a rheology of 450 rpm was chosen. Samples were incubated for 24 hours and after 1, 2, 3, 4, 24 hours one sample was taken out. After collection all samples they were centrifuged for 5min by 16000 rpm at room temperature. Depending on the sample concentration a right dilution factor was found to avoid an overload of the column.

5. Results and Discussion

5.1. Alteration of beta glucan's structure by inducing mechanical and thermal forces

Before starting the HPLC analysis all cookie samples were hydrolyzed enzymatically with alpha- amylase for two hours at room temperature, for minimizing carbohydrate side products. Thereby alpha- glycosidic bonds were broken up and sucrose should be the end product, which should elute by a time of 28 minutes. Due to that fact that beta glucan is linked beta glycosidic, alpha amylase isn't able to interact with beta glucan. Depending on the molecular size beta glucan elutes between 14 and 24 minutes and alpha amylase elutes by 25 minutes. Furthermore all raw material were analyzed and eluted through G4000Pwxl column.

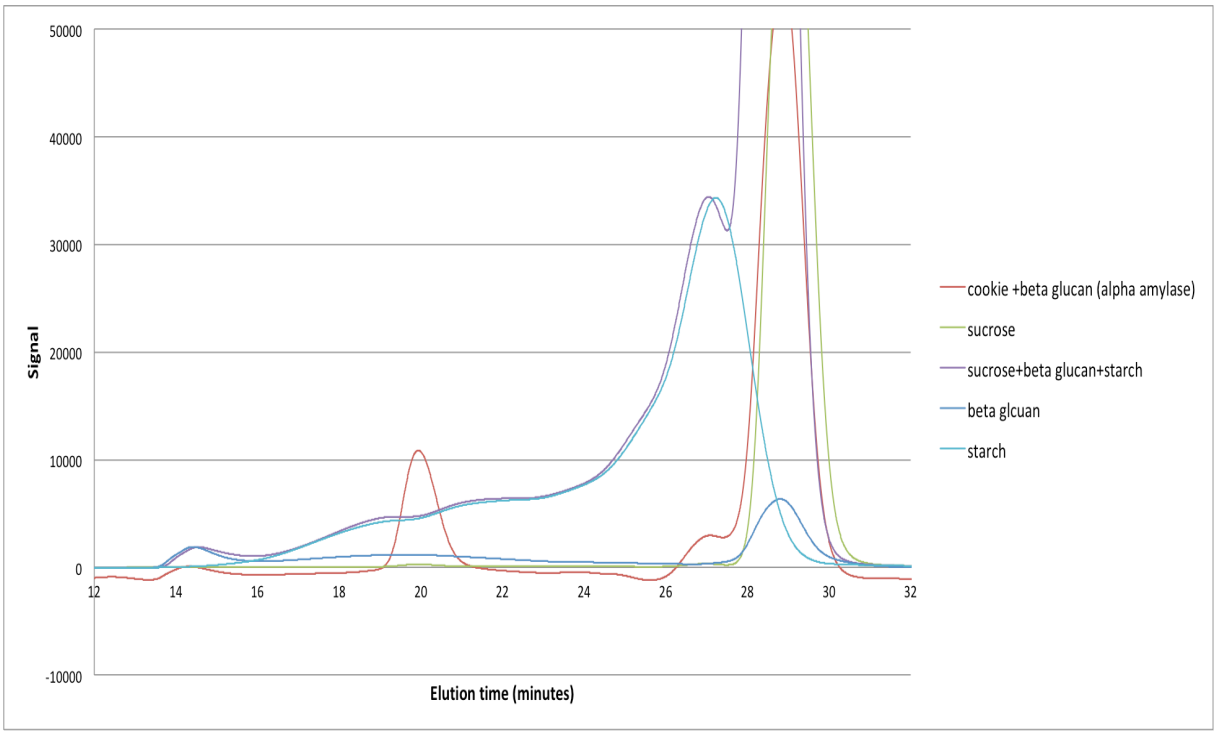


Figure 9 Chromatogram of sucrose (green), beta glucan (dark blue), sucrose+beta glucan+starch (violet), starch (light blue), cookie (alpha amylase) (red) for identifing raw materials

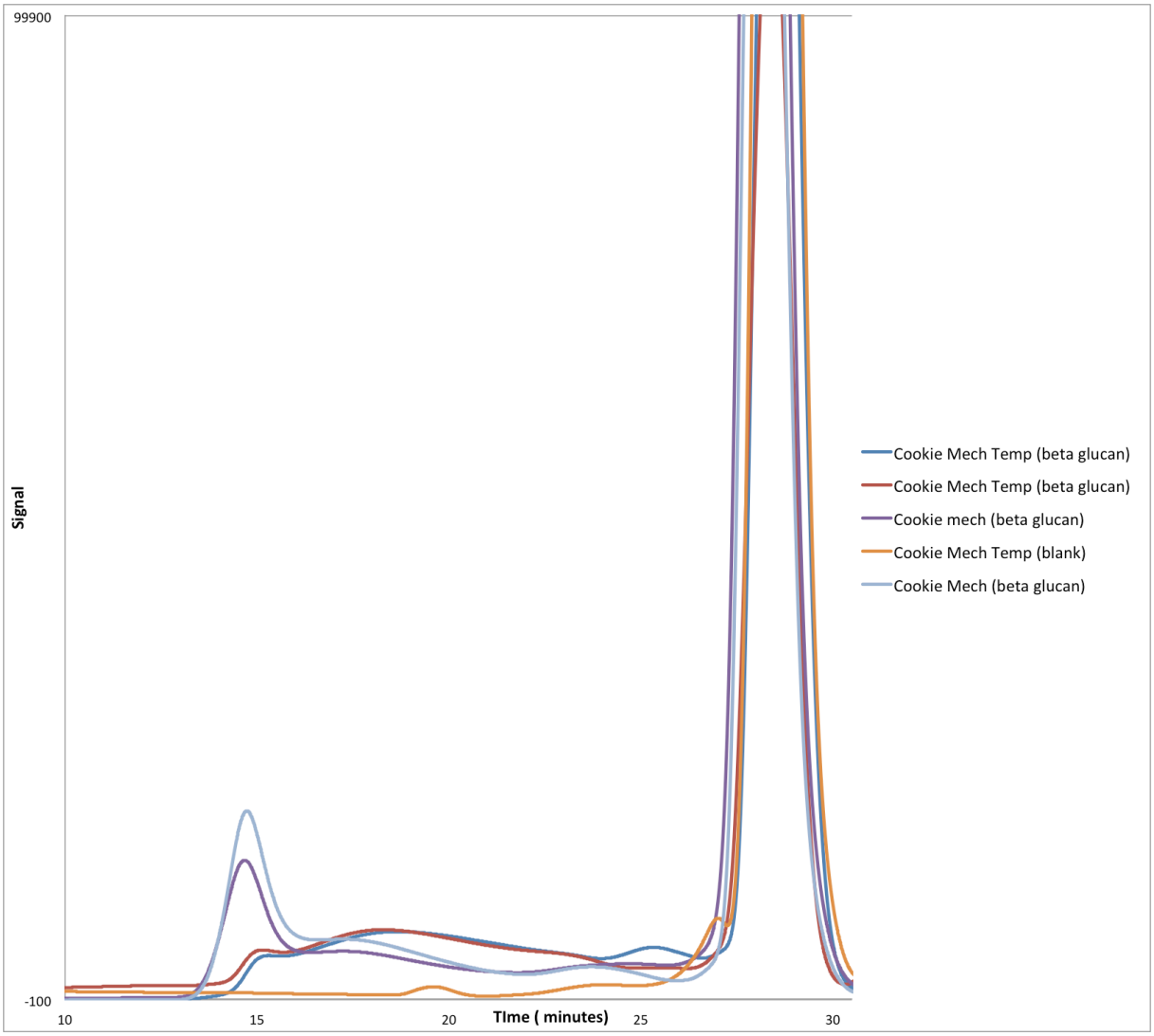


Figure 10 Chromatogram of cookie samples influenced by rheological and thermal actions

In figure 9 all cookie sample exhibited a sucrose peak at around 26 to 28 minutes. The blank that is colored yellow showed the highest peak. Rheological treated cookie samples (light-blue; violet), had peaks by 15 minutes and belong to the right side a broad distribution between 15 to 20 minutes. Cookie samples that were treated rheological and thermal (red and dark-blue) exhibited a higher broad peak between 15 to 25 minutes. Therefore we noticed that a thermal and rheological treatment reduced the molecular weight of beta glucan in cookies compared to the single-treated cookies. We can assumed that thermal treatment induces the breakdown of beta glucan chains.

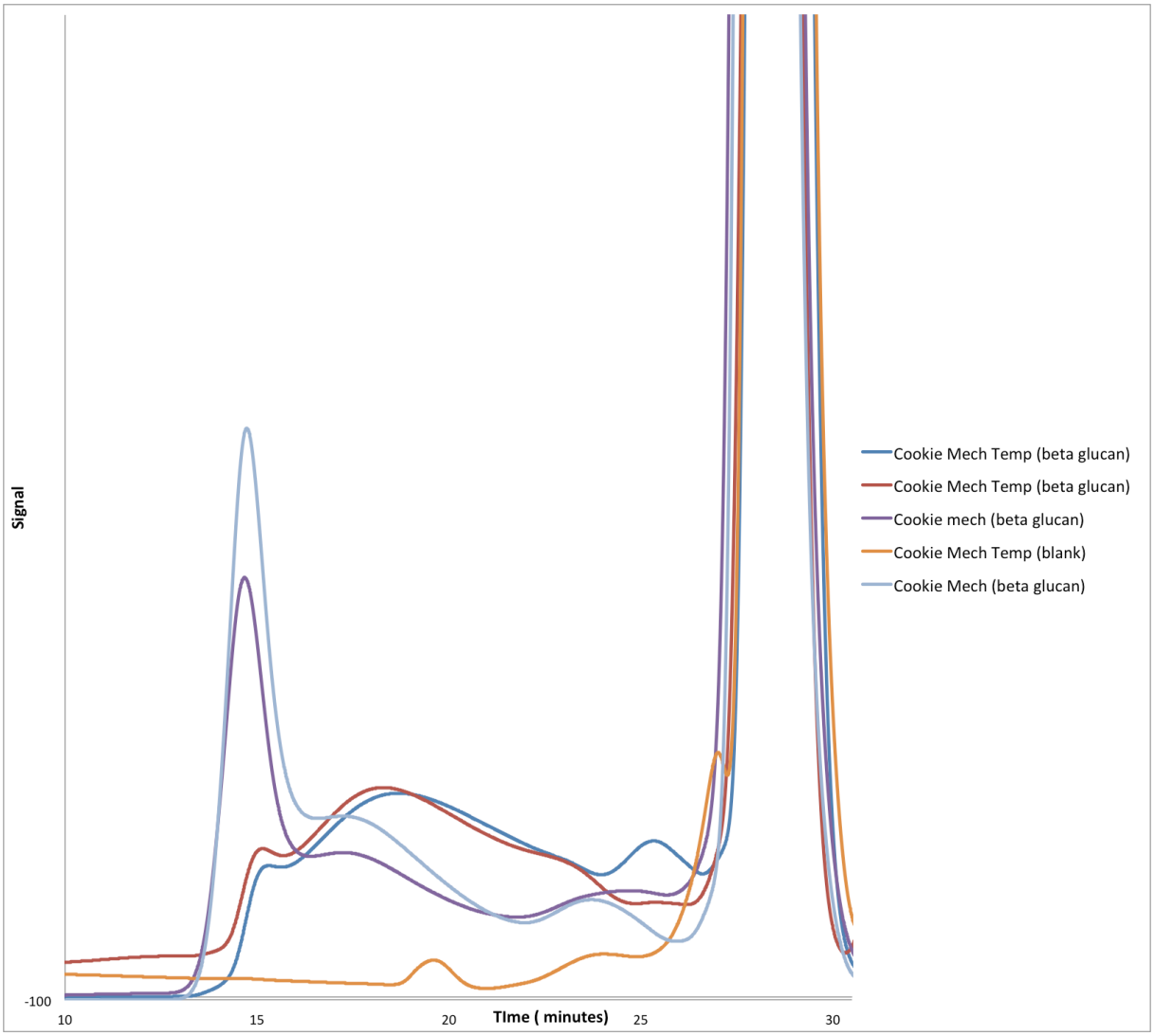


Figure 11 Zoomed Chromatogram of cookie samples influenced by rheological and thermal actions

Figure 10 showed figure 9 in a higher resolution (300%). Thereby smaller peaks, especially peaks between 14 to 25 minutes can be seen in detail. Beta glucan was occurred in all cookie samples. Mechanically treated sample exhibited an other distribution of beta glucan as samples which were treated mechanically and thermal. The elution time for beta glucan in single treated samples were around 14.5 minutes and shown a high peak with a shoulder. In case of the double-treated samples the time of elution started at 15 minutes and showed the highest signal peak at 18 minutes. All samples exhibted an alpha amylase peak at 25 minutes. This figure further confirms our previously mentioned theory.

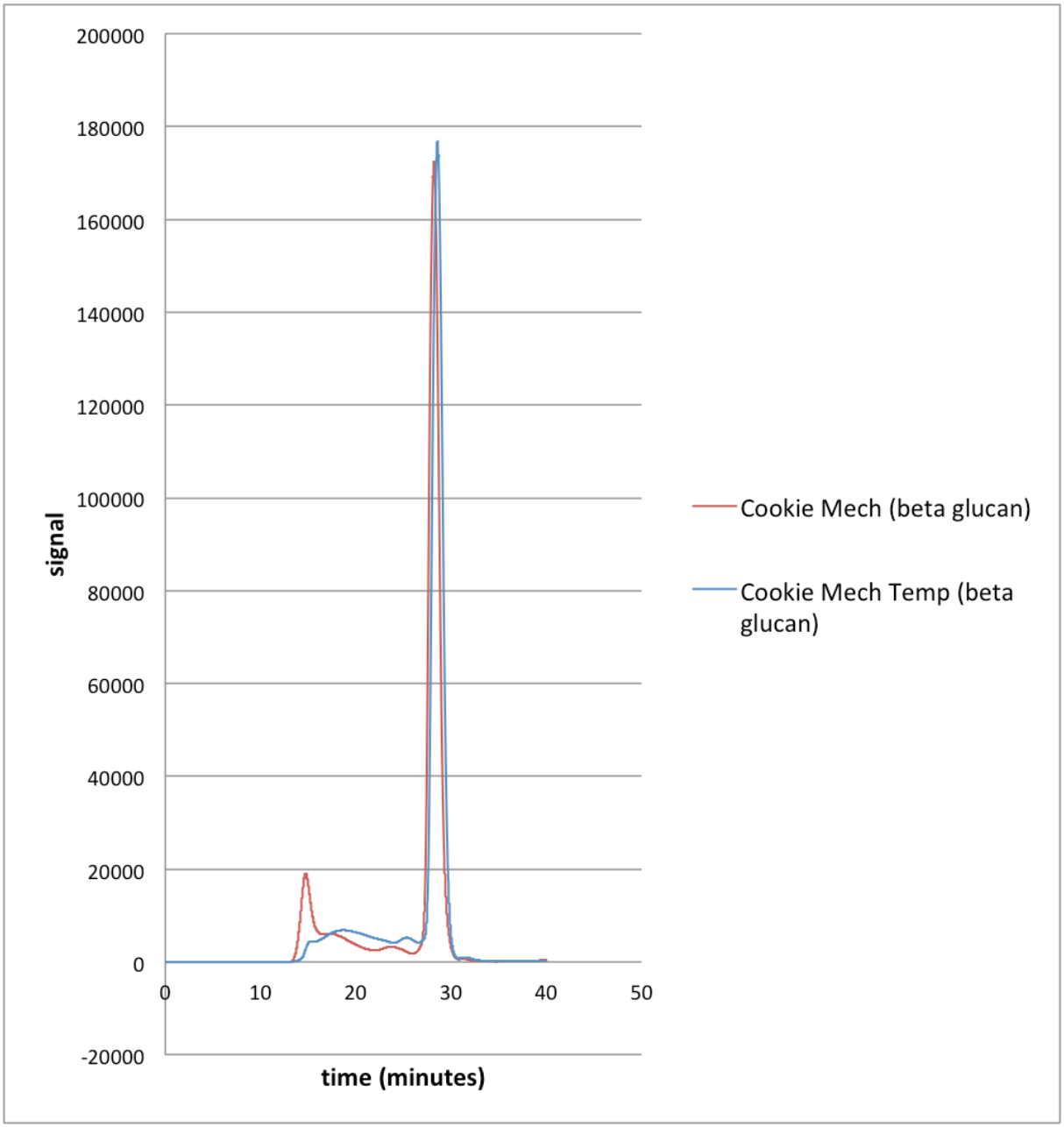


Figure 12 Comparison of two Cookie samples influenced by rheological steps and influenced by the combination of rheological and thermal steps

Figure 12 showed the significant differences between two treated samples more clearly. Cookie Mech (beta glucan) displayed higher yield by earlier elution time than sample Cookie Mech Temp (beta glucan).

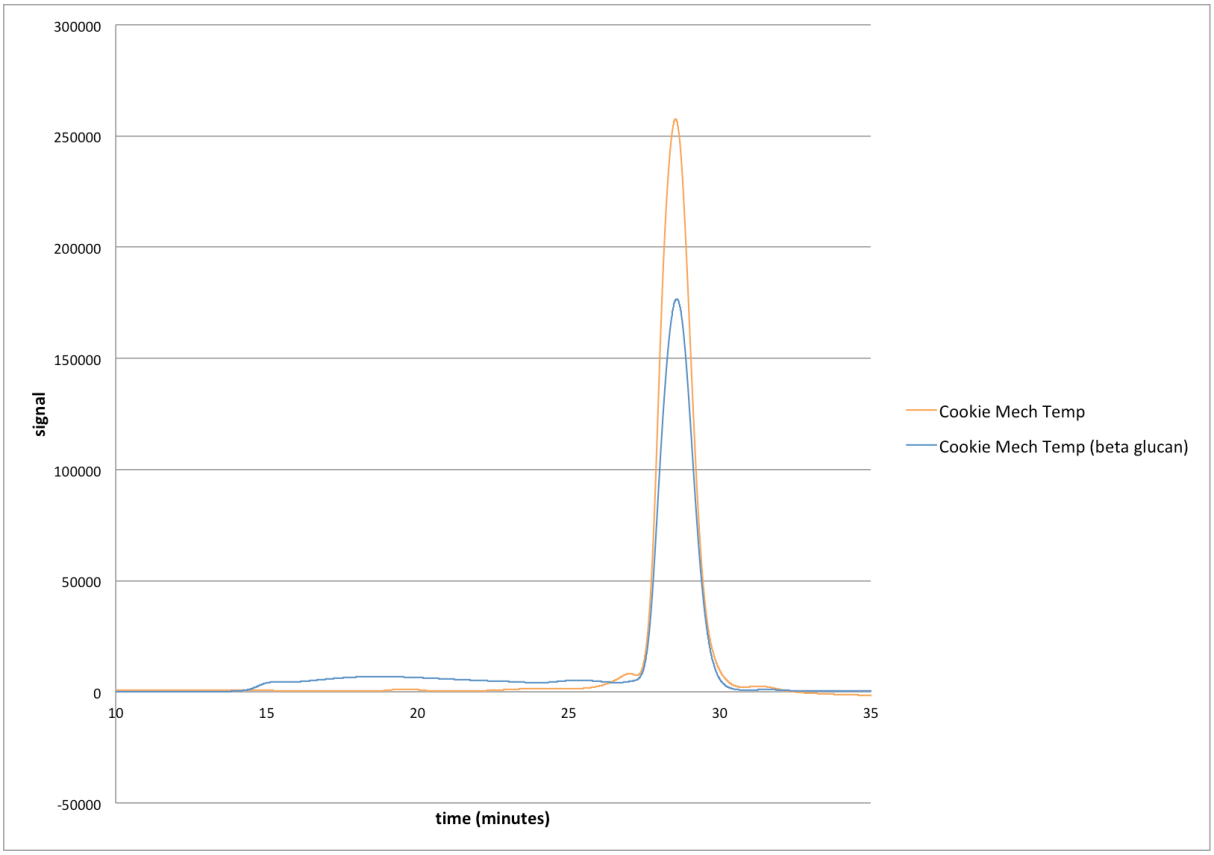


Figure 13 Chromatogram of cookie samples with and without beta- glucan

In chromatogram 13, the yellow colored blank (without beta glucan) was matched with a beta glucan sample which is marked blue. As it was aspected, the orange chromatogram (blank) didn't show any increasing values before 27 minutes and the peak for glucose at 28 minutes was quite high. Compared to the beta glucan sample, the blue chromatogram showed a smaller sucrose peak at 28 minutes and a broad distribution of beta glucan between 15 and 26 minutes. Figure 13 affirms the fact that addition of beta glucan to the cookie recipe did not affect the position of the sucrose peak in the chromatogram. Also there is no interaction between the cookie constituantes and beta glucan in the chromatogram.

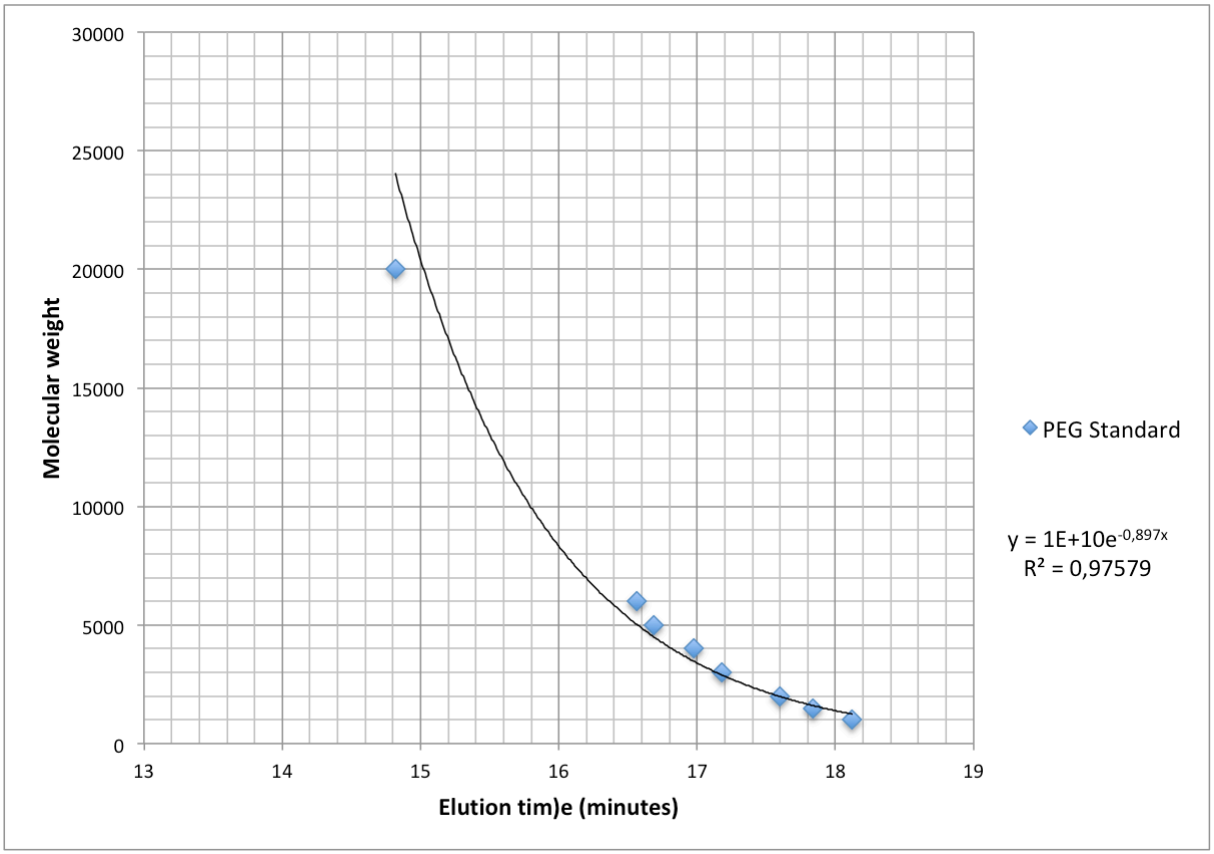


Figure 14 Exponential calibration curve of a Polyethylengylcol (PEG) standard between a molecular weight of 1000 and 20000.

Figure 14 is the calibration curve of the column G4000Pwxl from Tosoh Bioscience GmbH using PEG standards with different molecular weights from 1000 to 20000 Daltons. By extending the calibration curve exponentially the molecular weight by the elution time of 14.5 minutes would be around 30000 to 35000 Daltons.

According to this calibration curve the range of beta glucan in rheologically treated cookies is around 32000 Daltons, while the range of beta glucan in double-treated samples is around 28000 to 1000 Daltons.

5.2. Solubility of beta glucan in distilled water

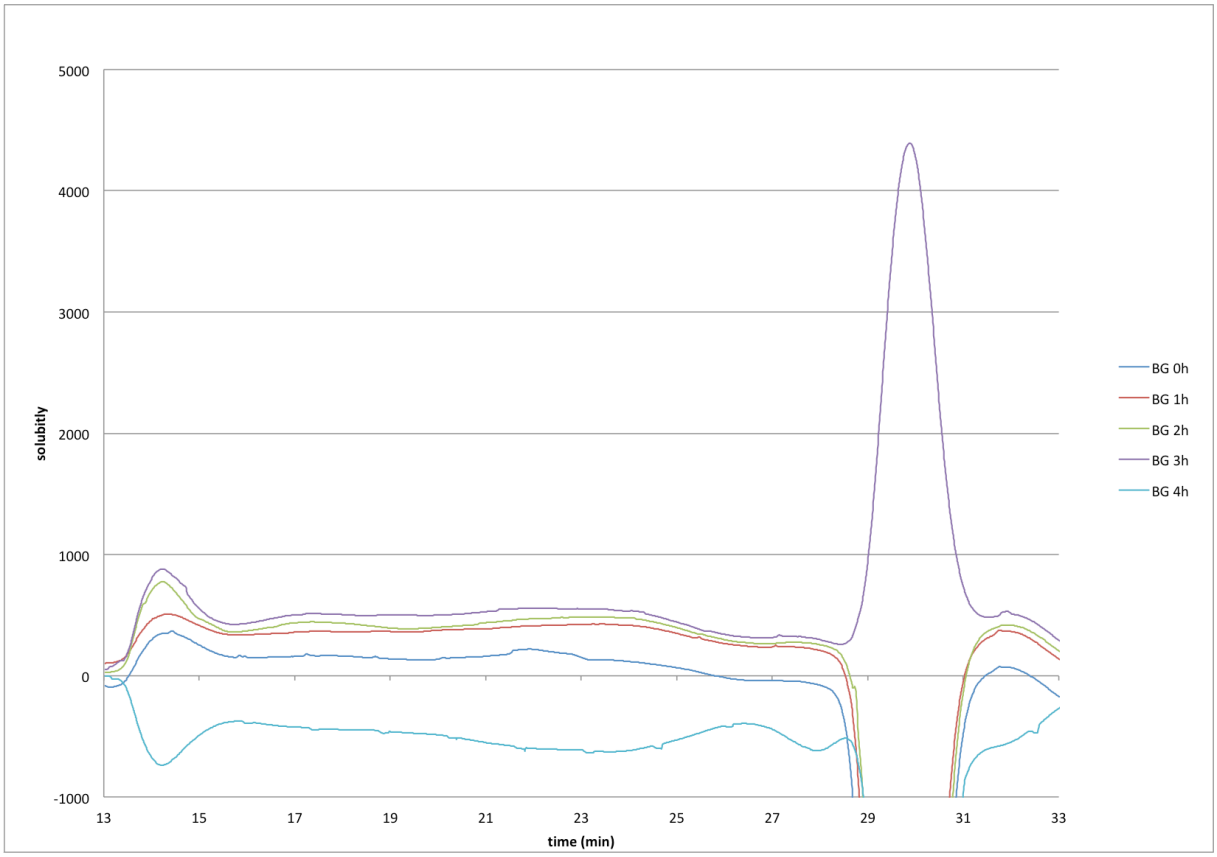


Figure 15 Solubility of water-soluble beta glucan by 99°C

According to figure 15 the solubility of beta glucan increases with time of thermal treatment.

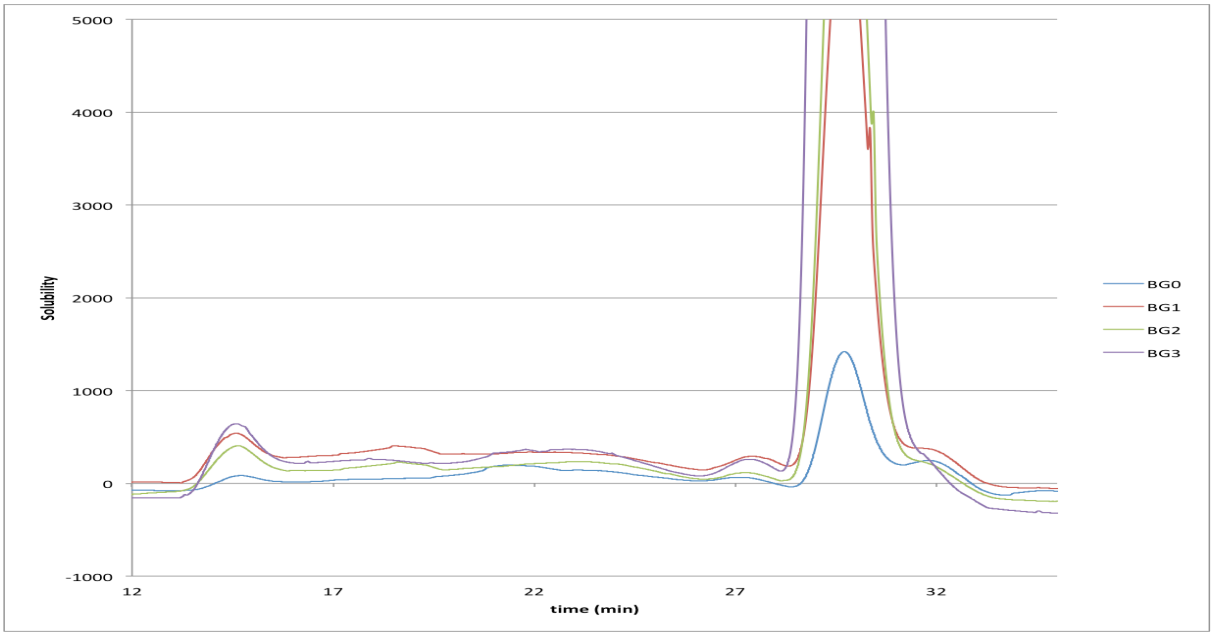


Figure 16 Solubility of water-soluble beta glucan by 150rpm at room temperature

According to figure 16 the solubility of beta glucan increases with time of rheological treatment.

6. Conclusion

The size exclusion chromatography is a relieable method for the analysis and molecular weight measurement of beta glucan in food matrices. No interactions between beta glucan and cookies were observed. Physical and thermal treatments were applied to cookies containing beta glucan. Our results indicate that double treated cookies contained lower molecular weight of beta glucan compared to single treated cookies. Depending on the number of treatments beta glucan with higher molecular weight elutes at 14.5 minutes, while beta glucan with smaller molecular weight elutes later on. Compared to the calibration curve single treated beta glucan may have a molecular weight of approximately 32000 Daltons. On the other hand beta glucan that was double treated may have a molecular weight of 28000 to 1000 Daltons. This is a significant reduction in MW of beta glucan chains that may have resulted from the thermal induction of cleavage glycosidic bonds.

In experiment two the solubility of beta glucan induced by rheological or thermal forces, the time of incubation was an essential factor. Both pysical forces supported the solubility in water. After three hours of incubation the solubility of beta glucan reached the maximum and no further elevation was observed.

7. Literature

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